Conservation genetics, Speciation and Biogeography in African Dragonflies

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades
Doktorin der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation

von

Dipl.-Biol. Sandra Damm (geb. Giere)
geboren am 7. April 1974 in Bremen

2009

Referentin: PD Dr. Heike Hadrys
Institut für Tierökologie und Zellbiologie
Stiftung Tierärztliche Hochschule Hannover

Korreferentin: Prof. Dr. Elke Zimmermann
Institut für Zoologie
Stiftung Tierärztliche Hochschule Hannover

Tag der Promotion: 27.07.2009

„Und auf das Zusammenwirken der Kräfte, den Einfluß der unbelebten Schöpfung auf die belebte Tier- und Pflanzenwelt, auf diese Harmonie sollen stets meine Augen gerichtet sein!“

Alexander von Humboldt (1799)

Contents

Zusammenfassung

Artenschutzgenetik, Artbildungsprozesse und Biogeographie afrikanischer Libellen

Die Erhaltung der Biodiversität ist eines der wichtigsten Ziele im Naturschutz. Die Einbeziehung verschiedener Forschungsdisziplinen ermöglicht die Betrachtung ihrer Entstehung auf unterschiedlichen Ebenen – von Populationen bis hin zu Arten und deren biogeografischer Geschichte. Im modernen Artenschutz werden dafür zunehmend auch molekulargenetische Methoden in die Untersuchungen mit einbezogen, da sich mit ihrer Hilfe wichtige Informationen über den Entstehungsprozess der biologischen Vielfalt herleiten lassen. Mit der vorliegenden Arbeit werden Studien auf den Gebieten der Populationsgenetik, Artbildung und Phylogeographie an afrikanischen Libellen, insbesondere in der Gattung *Trithemis* vorgestellt.

Hierfür wurden zunächst neue Marker-Systeme und Methoden entwickelt und getestet. Mikrosatelliten sind auf Populationsebene eines der besten Marker-Systeme. Daher wurde im Rahmen dieser Arbeit ein Protokoll zur Isolierung von Mikrosatelliten entwickelt und im Anschluss an vier verschiedenen Libellenarten erfolgreich angewandt, um Fragen bezüglich ihres Fortpflanzungsverhaltens (*Anax imperator* und *A. parthenope*), ihrer Parthenogenese (*Ischnura hastata*), ihrer genetischen Diversität (*Orthetrum coerulescens*) und ihrer Populationsstruktur (*Trithemis arteriosa*) zu untersuchen.

Für umfassende populationsgenetische und phylogenetische Studien wurden außerdem neue Sequenzmarker ausgewählt (ND1, COI, 16S, ITS I - II sowie eine Mikrosatelliten-flankierende Region) und auf ihre Aussagekraft für das Erkennen von Populationsstrukturen sowie die Auflösung von Verwandtschaftsverhältnissen untersucht. Desweiteren wurden zwei unterschiedliche Methoden auf ihre Anwendbarkeit hinsichtlich einer gesicherten Identifizierung und Entdeckung neuer Arten überprüft: zum einen das auf Merkmalen basierende Barcoding (CAOS-barcoding) und zum anderen der sogenannte Taxonomische Zirkel, durch dessen analytischen Überprüfungsprozess die Hypothese einer Artentdeckung bestätigt oder verworfen werden kann.

Der geografische Schwerpunkt dieser Arbeit liegt auf Namibia, einem der trockensten Länder des afrikanischen Kontinents. Libellen sind aufgrund ihres komplexen aquatisch-terrestrischen Lebenszyklus an Gewässer gebunden und werden daher in Gebieten mit wüstenähnlichem Klima nicht unbedingt erwartet. Dennoch konnten einige Arten

Überlebensstrategien entwickeln, die es ihnen ermöglichen, sich auch an sehr trockene Gebiete anzupassen. Um zu untersuchen, wie sich das (Über-)Leben eines wasser-assoziierten Organismus in der Wüste auf dessen genetische Diversität und Verhalten auswirkt, wurde eine populationsgenetische Studie an der Segellibelle *Trithemis arteriosa* durchgeführt. Mit Hilfe des neu entwickelten Mikrosatelliten-Systems, zwei nicht-kodierenden nukleären und einem mitochondrialen Sequenzmarker (ITS I - II; TartR04; ND1) wurden zwölf Standorte in Namibia und Kenia untersucht. Die Ergebnisse der nukleären Marker zeigten hohe genetische Diversitäten und Genfluss zwischen allen untersuchten Standorten an. Die Analyse des mitochondrialen Markers ließ jedoch eine Strukturierung der Populationen mit fast ausschließlich privaten Haplotypen erkennen. Die sich widersprechenden Ergebnisse weisen auf eine geschlechterspezifische Ausbreitung hin. Während die Weibchen standorttreu sind und dabei Energie für Fortpflanzung und Eiablage sparen, zeigen die Männchen hohe Migrationsraten in Abhängigkeit von der Gewässerstabilität. Diese Studie liefert erstmalig Einblicke in die Verhaltens- und Ausbreitungsstrategien eines in der Wüste lebenden und an Gewässer gebundenen Insekts.

Die populationsgenetische Studie an *Trithemis stictica*, einer Libellenart mit hohen Habitatansprüchen, lässt ein anderes Biodiversitätsmuster erkennen. Aufgrund ihres stenöken Verhaltens konnte diese Art nur zwei regional begrenzte Populationen in Namibia etablieren. Zusätzlich zu den Standorten in Namibia wurden Proben aus dem gesamten Verbreitungsgebiet der Art im südlichen Afrika miteinbezogen und mit Hilfe von vier Sequenzmarkern (ND1, COI, 16S und ITS I - II) genetisch untersucht. Die Analysen aller vier Marker zeigen übereinstimmend eine klare genetische Aufspaltung der Individuen in drei Gruppen. Die Überprüfung und anschließende Bestätigung der Entdeckung zweier neuer Libellen-Arten (*T. morrisoni* und *T. palustris*) erfolgte durch eine vergleichende Analyse der Teildisziplinen Morphologie, Ökologie, Geografie und Genetik mit Hilfe des Taxonomischen Zirkels. Morphologisch konnten Unterschiede zwischen *T. stictica* und den neuen Arten *T. morrisoni* und *T. palustris* aufgedeckt werden. *T. stictica* ist im südlichen Afrika weit verbreitet, wohingegen die beiden neuen Arten regional begrenzt an den Flussläufen des Okavango und Sambesi vorkommen, wo sie unterschiedliche ökologische Nischen besetzen. Mit Hilfe des neu entwickelten, auf Merkmalen basierenden CAOS-Barcodings wurde eine Merkmals-Matrix erstellt, welche eine sichere Identifizierung zweier neuer Arten bestätigt. Da sich diese jedoch morphologisch nicht voneinander unterscheiden lassen, handelt es sich hierbei um die beiden ersten kryptischen Libellen-Arten. Die Zuordnung eines bestimmten Speziationsmodelles ist schwierig. Allerdings scheint ein Habitat-Shift, also die Anpassung an

unterschiedliche ökologische Nischen, als Hauptursache der Aufspaltung der Arten am wahrscheinlichsten zu sein. Diese erfolgte vor ungefähr 0,7 - 2,4 Millionen Jahren, induziert durch die einschneidenden Umweltveränderungen Afrikas in jener Zeit (Regenwald-fragmentierung und Wüsten-Entstehung).

Der Einfluss dieser Umweltveränderungen in Afrika auf historische Artbildungsprozesse wurde in einer weiteren Studie an der besonders artenreichen Gattung *Trithemis* untersucht. Molekulargenetische Analysen dreier Sequenzmarker (ND1, 16S und ITS I - II) wurden mit ökologischen, geographischen und morphologischen Daten verglichen, um daraus Rückschlüsse auf die phylogeographische Historie der Gattung zu ziehen. Arten der Gattung *Trithemis* kommen an fast allen Gewässertypen Afrikas vor und zeigen hierbei eine große Bandbreite verschiedener Ausbreitungsmöglichkeiten und ökologischer Ansprüche. Morphologisch lässt sich die Gattung in zwei Gruppen aufteilen, und zwar in rote und blaue bzw. dunkle Arten. Durch die Anwendung der molekularen Uhr wird eine Entstehung der Gattung vor ca. 6 - 9 Millionen Jahren angenommen. Die Ergebnisse zeigen, dass durch drastische klimatische Veränderungen die Artbildung hauptsächlich allopatrisch stattgefunden und an Trockenheit angepasste Arten bevorteilt hat. Im Verlauf des Pliozäns kam es zu einer sehr schnellen Radiation resultierend unter anderem in der Bildung dreier Kladen blau/dunkler Arten mit einer gruppenspezifischen Habitat-Anpassung an (i) Gewässer im Flachland, (ii) Gewässer in Gebirgsregionen und (iii) in sumpfigen Gebieten. Die roten Arten sind demgegenüber besonders gut an das vorherrschende trockene Klima angepasst und heute wie damals über den ganzen afrikanischen Kontinent hinweg verbreitet.

Schlüsselwörter: Artenschutzgenetik, Artbildung, Biogeographie, Libellen

Summary

Conservation genetics, Speciation and Biogeography in African Dragonflies

Conservation biology aims to study the biological diversity and to protect species, their habitats and ecosystems. It integrates a variety of disciplines providing different kinds of information from populations to species and their biogeography. The addition of molecular techniques is an important new component which effectively contributes to all disciplines to allow a better understanding of the processes of diversification in nature. The thesis covers a wide range of aspects from population genetics to speciation processes and phylogeographic analyses in African odonates (dragonflies and damselflies) with focus on the genus *Trithemis*.

In the context of the different aims of this thesis new marker systems and methods were developed. For conservation genetic studies microsatellites are the state-of-the-art method. Therefore a protocol for the isolation of microsatellite systems was developed and successfully applied on four different odonate species to address different questions concerning mating strategies (*Anax imperator* and *A. parthenope*), parthenogenesis (*Ischnura hastata*), conservation (*Orthetrum coerulescens*) and population genetic structures (*Trithemis arteriosa*).

For comprehensive population genetic and phylogenetic analyses new sequence markers (ND1, COI, 16S, ITS I - II and a microsatellite flanking region) were chosen and analysed concerning their ability to identify population structures and to resolve phylogenetic relationships. Furthermore two different approaches were tested in regard to their suitability for unambiguously identifying and discovering new species: on the one hand the newly developed character-based barcoding (CAOS barcoding) which gives the possibility to integrate traditional with genetic diagnostic characters and on the other hand the taxonomic circle, an analytical approach to test first discovery hypotheses.

The geographical focus of this thesis is Namibia which is one of the most arid countries in Africa. Odonates as freshwater-associated organism with a complex life cycle composed of an aquatic larval and a terrestrial adult stage would not be expected to inhabit desert regions. Nevertheless many species have evolved survival strategies for arid conditions. To examine the genetic and behavioural consequences of a freshwater-associated organism living in desert regions the genetic diversity, population structure and dispersal behaviour of the dragonfly species *Trithemis arteriosa* was studied. Twelve populations from Namibia and

Kenya were analysed using nine microsatellite loci, two non-coding nuclear fragments (ITS I - II; microsatellite flanking region TartR04) and the mtDNA fragment ND1. The nuclear markers revealed a high allelic and haplotype diversity in all populations with high levels of gene flow. In contrast, ND1 sequence analyses showed sub-structuring and exhibited, except of two main haplotypes, only private haplotypes. The conflicting patterns of nuclear markers versus a mitochondrial sequence marker can be explained by a male-biased dispersal. Females might be philopatric to save energy for mating and oviposition, while males disperse dependent on the environmental stability of the habitat. This study gives first direct insights into the dispersal behaviour of a desert inhabiting, strongly water dependent flying insect.

A different pattern of biodiversity was observed by analysing the population genetic structure of a species with high habitat specificities. *Trithemis stictica* occurred only at two regions in Namibia. Samples from its whole distributional range in Southern Africa were included and analysed with four different sequence markers (ND1, COI, 16S and ITS I - II). Genetic results surprisingly unravelled three highly distinct but morphological cryptic clades. A corroborative approach applying the taxonomic circle by combining molecular data with ecological, morphological and geographical information supported the hypothesis of two new species. *T. stictica* is distributed throughout sub-Saharan Africa and the two new species coexist in the same geographical range, the Okavango and Zambezi floodplains, where they occupy different habitats. All characters of the different analysed disciplines were incorporated in an elaborated character-based barcoding matrix which allows a better identification of the two new species. Significant morphological differences were found between *T. stictica* and the two new species, *T. morrisoni* and *T. palustris*, while between the latter two no such differences were observed. All evidence confirmed the hypothesis of the discovery of the first cryptic odonate species. Molecular clock analyses date back the time of their divergence approximately 0.7 - 2.4 million years ago. Environmental changes during this time period with increasing aridity and habitat fragmentation might have forced the divergence of the two species. Assigning a specific mode of speciation is difficult, but a historical habitat shift might be a promising explanation for their divergence since both species occur in different ecological niches.

In a comparative phylogenetic analysis of the species-rich genus *Trithemis* we aimed to study the influence of historical environmental changes on speciation events. We combined molecular analyses of three target genes (ND1, 16S and ITS I - II) with ecological, geographical and morphological data to reconstruct the biogeographical history of the genus. The species occupy most freshwater habitats on the African continent, from deserts to forests,

from cool permanent streams to warm temporary pools. They differ in their dispersal capacity and ecological requirements and can be divided into two colour groups (red and blue/dark species). Molecular clock analyses estimate the time of the genus origin 6 - 9 million years ago. At this time the drastic climatic fluctuations with increasing aridification and forest fragmentation forced speciation mainly in form of allopatry and favoured dry-adapted open-land species. During a rapid radiation in the Pliocene three distinct clades of dark species evolved different habitat adaptations by colonizing (i) lowland streams, (ii) highland streams and (iii) swampy habitats to deal with the changing environmental conditions. The red-coloured species developed special adaptations to the arid climate and were therefore able to expand their ranges. Today the group of red species harbours the most widespread species of this genus.

Keywords: Conservation genetics, Speciation, Biogeography, Odonata

1. General Introduction

This thesis covers a variety of different aspects in conservation biology ranging from population genetics to speciation processes and phylogeography in African dragonflies. By using dragonflies as a model system the presented studies analyse ecological, evolutionary as well as biogeographical questions to give insights into behavioural traits and speciation processes of African insects. In this context new marker systems are developed and applied at three different levels (population, species and genus) with a focus on the species-rich genus *Trithemis*. Due to the different goals of the presented studies, I will initially provide an overview of modern conservation biology and biodiversity research and its important different disciplines population genetics, species diversification and phylogenetics.

1.1. Conserving the biodiversity of life

The Earth's biodiversity is of inestimable value for all living organisms. The benefit for humans from nature's diversity covers a vast range of aspects from inspiration to scientific and economic interests (e.g. Avise *et al.* 2008; Wake & Vredenburg 2008). However, species extinction rates are rising. E.O. Wilson (1993) estimates a loss of the world´s remaining species at 0.25% per year. The effects of global warming and growing human impact are accelerating the extinction rate and its current magnitude is comparable to the five great mass extinctions revealed in geological records. Therefore the loss of today's diversity is also called the "sixth mass extinction" (Wake & Vredenburg 2008). Many species, especially insects or rainforest species, are not even discovered yet (Dunn 2005; Samways 2007). The increasing understanding of the importance and value of biodiversity has led to crisis disciplines like conservation biology. Conservation biology has the aim to study and protect biodiversity with its species, habitats and ecosystems by integrating different scientific fields from classical ecology to geography and genetics. The expansion of genomic technologies in conservation biology greatly improves decision-making (e.g. DeSalle & Amato 2004; Schwartz *et al.* 2007). In combination with traditional ecological approaches the newly developed high-throughput methods allow a fast assessment and analysis of complex study systems.

For the evaluation of biodiversity several programs were organised such as the "World Atlas of Biodiversity" of the World Conservation Monitoring Centre (see www.unep-

wcmc.org). Also monitoring projects like the African-wide BIOTA network (Biodiversity Transect Analyses in Africa, BMBF) have become more important. For such approaches a rapid assessment and identification of species is the most important precondition (Schwartz *et al.* 2007). One promising method is DNA-barcoding which uses a standardised DNA region for taxon assignment and can accelerate and simplify species identification (Hebert *et al.* 2003). The international initiative of the Consortium for the Barcode of Life (CBOL) established a worldwide database (BOLD) with sequences of the proposed standard mitochondrial gene cytochrome c oxidase 1 (COI) for animals and included 37,000 species records by the end of 2008 (http://barcoding.si.edu). However, the barcoding initiative based on sequences alone has limitations and problems in undescribed or cryptic species as well as in species groups which show only low variability in COI (DeSalle *et al.* 2005; Hickerson *et al.* 2006; Rubinoff 2006).

For the definition of conservation units different approaches are suggested. One method is to assign individuals to molecular operational taxonomic units (MOTU`s) according to their genetic similarity without designation of its taxonomic rank (Blaxter *et al.* 2005). This method enables the inclusion of groups with taxonomic uncertainties. Another way to identify conservation or evolutionary significant units is combining ecological and genetic aspects and thereby defining a population, species or region of high conservation value (Moritz 1994; Vogler & DeSalle 1994). By analysing species composition, genetic diversities and interactions between populations the status of a population will be assessed. A special example of regions with high conservation value are the so-called biodiversity hotspots. Here the level of biodiversity is above average by also harbouring many endemic species. These regions like e.g. the Eastern Arc Mountains of Tanzania exist worldwide and are of highest conservation interest (Burgess *et al.* 2007).

1.2. Evolution of diversity

For the application of appropriate conservation strategies it is not only important to identify and assess diversity, but also to understand the patterns and processes underlying species diversification (Bowen 1999). Here the different levels from population to species and phylogeography provide crucial information about the evolution of diversity. The use of genetic tools allows the expansion of traditional approaches for a deeper understanding of the complexity of the underlying processes.

Population level

Population genetics enable the quantification of important factors such as effective population size, inbreeding, migration and gene flow (Hartl 2000; DeSalle & Amato 2004). This provides specific and comparable quantifications of processes that affect endangered populations. Additionally it adds an important new level to biodiversity research, the genetic diversity (Avise *et al.* 2008). Preservation of genetic diversity is the fundamental level for conserving the diversity of life. High genetic diversity gives a population the ability to adapt to changes in their environment and to avoid inbreeding depression (Hartl 2000; Frankham *et al.* 2002). Since "isolation by distance" and reduced gene flow can promote speciation, a basic step for understanding diversification is also to analyse the intraspecific dispersal abilities and the population structure (Wright 1943).

The population genetic parameters can be analysed with a variety of modern techniques like microsatellites or sequence markers. The varying mutation rates of the different marker systems provide the possibility of analyses at different geographical scales. Today, due to high variabilities between individuals and populations, microsatellites are the state-of-the-art method not only in population genetics but also in analyses of paternity, mating systems and sexual selection (Goldstein & Schlötterer 1999). For conservation concerns the application of these sensitive markers facilitates the rapid detection of environmental changes and could also be used in long-term monitoring of important population sites (Ridley 1996; Hartl 2000).

Species level

Situated at the interface to population genetics are problems of defining species boundaries, subspecies and cryptic species (Bickford *et al.* 2007). A species is the basic taxonomic unit of biological classification and its definition has long been discussed (reviewed in De Queiroz 2007). Different concepts were proposed of which the "biological species concept" is the most widely accepted (Ridley 1996). According to this, a species is defined as a group of organisms capable of interbreeding and producing fertile offspring. Other species concepts focus on morphological similarities (morphological species concept), genetic or phylogenetic similarities (genetic or evolutionary species concept) or the ability of individuals to recognise each other as possible mating partners (recognition species concept)(De Queiroz 2007).

While delineating and identifying species is crucial for the assessment of biodiversity, understanding the mechanisms and forces which promote speciation are of additional importance for conservation. The three main modes of speciation are allopatric, parapatric and

sympatric speciation (Coyne & Orr 2004). Allopatric speciation is defined by reproductive isolation through geographical barriers (for overview see Gavrilets 2003). Parapatric speciation is a speciation mode where geographical variation ultimately leads to the splitting of a subdivided population into reproductively isolated units (Gavrilets et al. 2000). The most controversially discussed mode of speciation is sympatric speciation (Bolnick & Fitzpatrick 2007). Driven by various internal traits, selection occurs within or between populations with a broad geographical overlap. Although mating is generally possible, gene flow is interrupted. In contrast to allopatric speciation, verifying the two other modes of speciation is often difficult in empirical case studies (Fitzpatrick *et al.* 2008). Regardless of the mode of speciation, revealing the underlying mechanisms is of great importance for understanding the development of diversity. Despite intensive research in this complex area, many mechanisms still remain unclear (Bolnick & Fitzpatrick 2007).

Furthermore, many aspects concerning the evolutionary processes underlying cryptic speciation are still unresolved (Bickford et al. 2007). Cryptic species are genetically distinct species which were erroneously classified under one species name because of their high morphological similarity (Bickford *et al.* 2007). Uncovering and incorporating cryptic species in the global biodiversity assessment is of particular importance for conservation. Even though cryptic species have previously been discovered, the establishment of DNA barcoding increases the recognition of "new", formerly undetected species enormously (e.g. Hebert *et al.* 2004; Hajibabaei *et al.* 2006). This also leads to discussions about species definitions and delineations and highlights the importance of further integrative research at the species level (DeSalle 2006; Vogler 2006; Waugh 2007).

Phylogenetic level

The aims of phylogenetic research are to reconstruct the evolutionary history and to study the patterns of relationships among organisms (e.g. Mayr 1963; Wägele 2001). Understanding how species evolve and adapt to changing environmental situations is of great importance for future conservation management (Dobzhansky 1973; Avise & Ayala 2007). Historic events are often not obvious and only fossils remain as relicts of the past. However fossils linking different groups of organisms are often missing or have not yet been discovered. Since the introduction of molecular methods, analysing the relationships among different species, families or even phyla has become much easier. By combining palaeontology with molecular analyses phylogenetic trees can be calibrated and substitution rates for prominent genes can be estimated (e.g. Donoghue & Benton 2007; Whitfield & Lockhart 2007). Comprehensive

biogeographical analyses which combine the geographical history of islands and continents with genealogy and distributional data help to reconstruct past speciation events and to understand the processes of evolution in general (Cox & Moore 2005).

Another important aspect of phylogenetic research is taxonomy. Our knowledge of the systematic system is traditionally based on morphological characters. Incorporating molecular data has recently led to several revisions, especially in groups of high morphological resemblance (Monaghan *et al.* 2005; Vogler & Monaghan 2007). Taxonomic research based on DNA could therefore help to discover and delineate species which is crucial for assessing biodiversity and conservation management.

1.3. Conservation, Speciation and Biogeography in African Dragonflies

Dragonflies as a model system

Odonates are considered to be the earliest flying insects with an age of 250-200 million years (Grimaldi & Engel 2005). They constitute approximately 6,000 described species and have a worldwide distribution (Kalkman et al. 2008). The insect order is divided into two main suborders, Zygoptera and Anisoptera, known as damselflies and dragonflies, and a third suborder, the Anisozygoptera, which harbours only two relict species (Askew 1988).

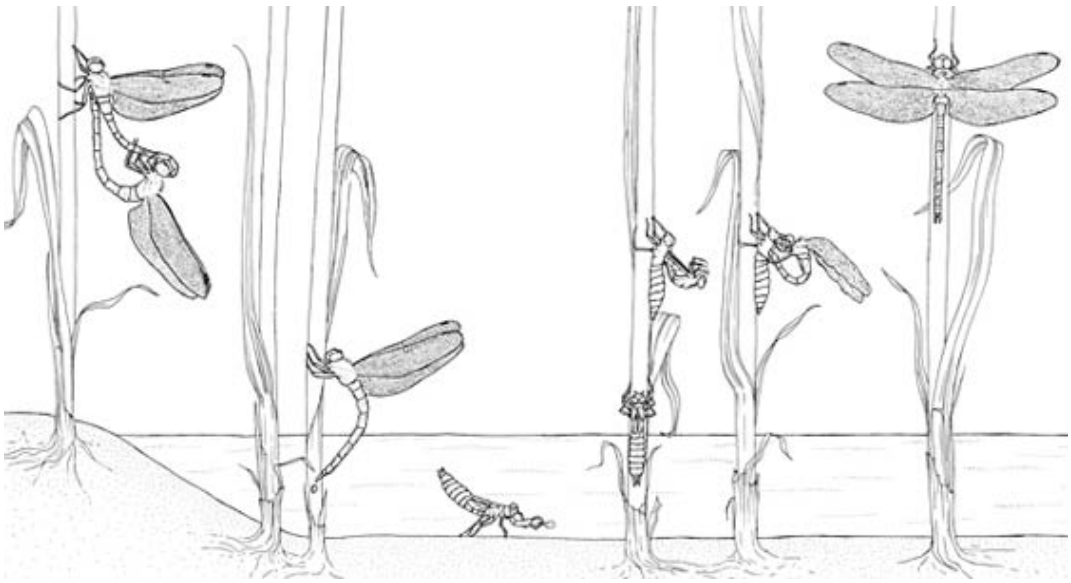

Figure 1 The complex life cycle of odonates from mating to oviposition, larval stage and emergence.

Odonates are associated with freshwater habitats by their complex life cycle composed of an aquatic larval and terrestrial adult stage (Figure 1). Both larvae and adults show a more-or-less strong selection in habitat choice concerning e.g. the substrate, water quality and flow as well as structural characteristics of the surrounding vegetation.

While odonate species in general are highly mobile organisms, their different ecological requirements are often linked with their dispersal capacities (Corbet 1999). The range from extremely good to poor dispersers offers insights into different degrees of vicariance and dispersal. Altogether, their habitat sensitivity makes them good indicator organisms for evaluating environmental changes in the long term (biogeography) and in the short term (conservation) of all kinds of freshwater systems (e.g. Samways 1993; Corbet 1999; Clausnitzer 2003; Samways 2007; Cordoba-Aguilar 2008).

The genital morphology of odonates is unique in the animal kingdom. The females have sperm-storage organs and the males primary (sperm production) and secondary (sperm transfer) genitalia. With these peculiar morphologies, odonates evolved a very special mating system and a variety of different reproductive strategies. The pioneering studies of Waage (1979; 1984) and Parker (1970) demonstrated the mechanisms of sperm displacement for the first time. Since then, studies analysing the evolution of the reproductive system in the context of sexual selection, sperm competition and female choice have changed our understanding of mating systems in general (e.g. Fincke & Hadrys 2001; Cordoba-Aguilar *et al.* 2003; Cordero Rivera *et al.* 2004). Reproduction is the basic unit of evolution. In odonates, habitat as well as sexual selection are involved in reproductive behaviour and mate recognition and could therefore promote speciation (McPeek & Gavrilets 2006; Svensson *et al.* 2006). With the introduction of molecular methods, paternity studies in odonates can give additional insights into mating strategies and provide, through the combination of behaviour, population genetics and speciation processes, crucial information for conservation and evolution (e.g. Hadrys *et al.* 1993). The combination of their unique reproductive system and complex life cycle makes odonates excellent model organisms for many evolutionary questions concerning speciation processes and phylogenetic questions.

Figure 2 From mating to emergence
Left, a copulation wheel of *Orthetrum chrysostigma*, a widespread species in Africa. In the middle the aquatic larval stage and right an exuvia, both examples show species of the family Aeshnidea which harbor the largest dragonfly species.

Africa, Namibia and its Odonates

The African continent forms a large continuous landmass which is, in comparison to other continents, virtually uninterrupted by mountain chains or large waterbodies (Griffiths 1993). The most significant barrier is the Sahara, separating the Afrotropics from the Palearctic. Africa has only been moderately affected by tectonic changes in the past, but the climate is characterised by extreme variability from the mid-Tertiary onwards (Morley 2000). The closure of the Tethys Sea (20-10 Mya) and the central African uplift resulted in an increasing aridity with the development of the Sahara and a savannah dominated landscape. The formerly uninterrupted rainforest belt in the equatorial region got fragmented. Today it comprises the East African coastal rainforests and the West and Central African rainforests (Guinea-Congolian).

The geographical focus of the studies presented here is Namibia and the floodplains of its surrounding countries, the Upper Zambezi and the Okavango river systems. Namibia is the most arid country of the Afrotropical region (i.e. south of the Sahara). It possesses two deserts, the Namib Desert at the Atlantic west coast and the Kalahari Desert shared with Botswana in the east (Mendelsohn *et al.* 2002). Most of the landscape is characterised by desert, semi-desert and savannah. The only perennial rivers are located along the northern and southern borders of the country. Natural permanent surface water in the interior parts of Namibia only occurs at widely separated springs around mountains and in the ephemeral river courses. Water is therefore one of the most relevant and limited resources in Namibia.

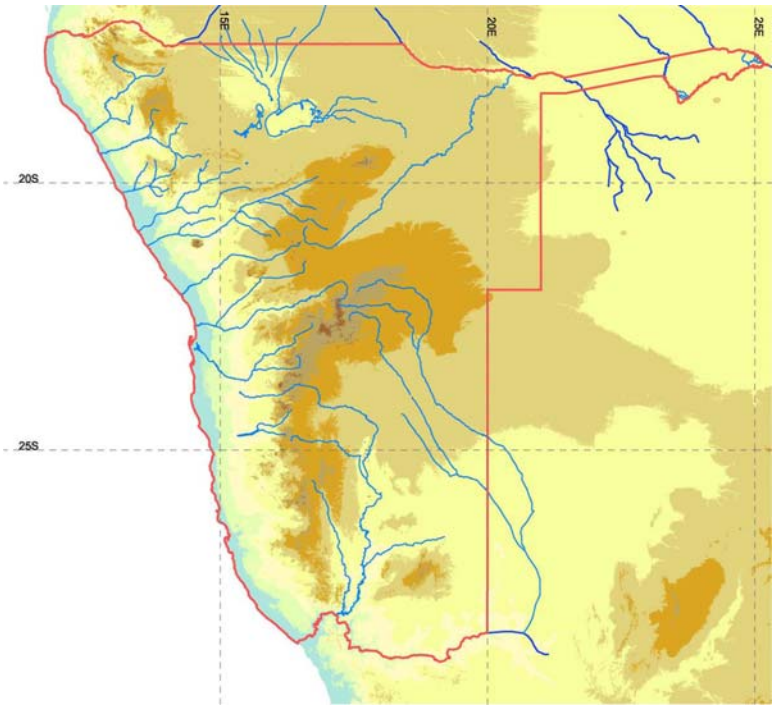

Figure 3 Map of Namibia showing the major ephemeral rivers and the geological relief from central to south Namibia.

In comparison to other tropical regions, the Afrotropical odonate fauna is relatively poor with approximately 850 described species. Its composition is similar to that of the Holarctic, with few families and a large proportion of Coenagrionidae and Libellulidae (Dijkstra 2003). This may be explained by the unstable climatic history of the continent, which favoured species capable of colonising recent or temporary habitats. As a consequence of changing climate and rainforest reduction, the 'old' African fauna is now generally rare and restricted to stable, but isolated areas (Kalkman *et al.* 2008). On the other hand, the recently distributed species inhabit all kinds of different habitats in forests and savannahs with a remarkable speciation in a few genera (e.g. *Pseudagrion, Orthetrum* and *Trithemis*). The highest odonate diversity, as well as the greatest number of regional restricted species, is found in the Guineo-Congolian forest, which stretches from Senegal to western Kenya (Dijkstra & Clausnitzer 2006). The highest amount of endemism is found in coastal East Africa, with the Eastern Arc Mountains, the Ethiopian highlands and South Africa as well as on Madagascar (Kalkman *et al.* 2008).

Odonates as freshwater-associated organisms would be expected to be absent or poorly represented in desert environments. Nevertheless, deserts do contain wetlands which are colonised by a number of aquatic animal groups, including dragonflies and damselflies (Suhling *et al.* 2003). Springs in mountainous regions provide permanent water bodies and episodic rainfall may establish ephemeral (or temporary) rivers or ponds. Additional water resources occur along the course of the normally dry ephemeral rivers at rare places where

groundwater surfaces, dependent on geology or topography (Suhling *et al.* 2006). Odonates are excellent flyers which enables them to cover long distances and colonise even the most isolated habitats (Corbet 1999). Although there are a few desert endemic odonates the majority of species inhabiting deserts or dry savannah regions are widespread in Africa. In Namibia 126 different odonate species are described (Suhling & Martens 2007).

The genus *Trithemis*

Besides three more technical related studies in other odonate species, this thesis mainly focuses on the dragonfly genus *Trithemis* (Odonata, Libellulidea). *Trithemis* provides an excellent example of a very successful genus on the African continent that dominates modern odonate communities. Harbouring 40 recognised species, it is one of the most speciose odonate genera in Africa with a continent wide distribution, including two endemic Madagascan and five Asian species (Pinhey 1970; Dijkstra 2007). It occupies most freshwater habitats, from deserts to forests, and from cool permanent streams to warm temporary pools. The species differ in their dispersal capacity and show wide ranges of habitat preferences from generalists to specialists. Morphologically the genus can be divided in two colour-groups (see Figure 4). Species from warmer (i.e. exposed, stagnant, lowland) habitats are mostly red-coloured, while those from cooler (shaded, flowing, highland) habitats are generally blue or blackish.

Trithemis festiva *Trithemis hecate* *Trithemis annulata* *Trithemis arteriosa*

Figure 4 Four different *Trithemis* species representing the two different colour groups in this genus.

2. The aims of the thesis

The thesis aims to address a variety of questions concerning (i) population and conservation genetics, (ii) speciation, (iii) phylogeny and phylogeography in African dragonflies. Despite of their high suitability as model organisms studies on odonates are still underrepresented. Especially knowledge about biodiversity patterns in afrotropical regions is still limited and mostly concentrates on vertebrates. By using the highly successful genus *Trithemis* the presented studies add new approaches to conservation genetics and biodiversity research by applying novel techniques and markers. In the following I will briefly introduce the topics and summarise and discuss the main results of the publications and manuscripts upon which this cumulative dissertation is based (see 6.1- 6.9).

2.1. New markers – new approaches

An important aim of this thesis is to develop marker systems and approaches in conservation genetics which allow efficiently analysing population structure, phylogenetic relationships and identifying and unambiguously discover new species.

Microsatellite systems

Microsatellites are state-of-the-art technique in conservation genetics. Due to their high variability microsatellites provides a powerful tool to analyse mating systems, paternity issues and population genetic patterns. Most microsatellite primers are species specific or only applicable in closely related species because of the high variability of the microsatellite flanking regions. Therefore a protocol was developed and established for the isolation of microsatellites. Four odonate species were chosen to analyse different aspects of paternity, mating systems and population genetics.

In order to study and compare the mating system of the two closely related sister species *Anax imperator* and *Anax parthenope* (Aeshnidae) a microsatellite system was developed for both species (Hadrys *et al.* 2007a). Despite of their close relationship they developed different traits of sperm competition and are therefore an interesting model system to study sperm precedence mechanisms in the context of female choice (see also 6.1).

In strong contrast the damselfly *Ischnura hastata* (Coenagrionidae) is the only known odonate species which exhibits parthenogenesis. In North and South America, the Caribbean and Galapagos Islands the species have normal bisexual populations, but at the Azores Islands only female populations were found. With help of a microsatellite system the genetic

diversities within and among bisexual and parthenogenetic populations as well as the origin and type of parthenogenesis can be analysed (Carballa *et al.* 2007).

For the endangered European species *Orthetrum coerulescens* a panel of microsatellites was developed to analyse and monitor the effects of environmental changes and human impact on this species (Hadrys *et al.* 2007b). With its very special habitat requirements, occurring only at small riverine habitats, it is already a red-list species (see also 6.3).

Finally, for population genetic analysis in an African-wide distributed dragonfly species a microsatellite system for *Trithemis arteriosa* was developed (Giere & Hadrys 2006). As indicator species for perennial water bodies, the application of microsatellites in *T. arteriosa* could add crucial information for targeting the protection of dragonfly habitats in Africa (see also 6.4 & 6.5).

Population genetic marker

Because microsatellite analyses are highly dependent on sample sizes the application of additional marker systems might be a good solution to independently revise the results. The aim is therefore to test other marker sets for their applicability in population genetics, covering both the nuclear and the mitochondrial genome. Two different non-coding nuclear sequence markers are applied, the ribosomal ITSI and II regions and a microsatellite flanking region. While ITS was used for population genetics in other species groups before, in this thesis the suitability of a microsatellite flanking region as a sequence marker was tested for the first time. In addition two different mitochondrial markers were chosen, the NADH dehydrogenase subunit 1 (ND1) and the cytochrome c oxidase I (COI). The comparison of mtDNA and nuclear markers allows comprehensive analyses on maternally as well as bi-parentally inherited markers at different levels of sensitivity (see 6.5 & 6.6).

Approaches for species discovery and identification

Since the use of molecular genetic methods in population analyses and taxonomic research the number of new, formerly undetected species highly increases. The discovery of new species based solely on DNA, like in the traditional DNA barcoding approach, is mostly insufficient and often ill-suited. The need for an analytical discovery process increases in cases where traditional taxonomy fails to identify species. In this thesis two different approaches are tested to unambiguously discover new species (see 6.6).

While DNA barcoding as suggested by Hebert *et al.* (2003) only relies on genetic distances between species, the newly established method by Rach *et al.* (2008), the character-based DNA barcoding, is based on diagnostic characters in a molecular dataset. It therefore allows the incorporation of classical taxonomic characters. In this thesis the new method is applied for the first time, incorporating also characters of different sources (morphology, geography, ecology and genetic data) to test its applicability in the discovery of new species.
An analytical approach for identifying new species and verifying discovery hypotheses is the "taxonomic circle" (DeSalle *et al.* 2005) The taxonomic circle describes the interaction of different datasets (morphology, reproductive isolation, geography, ecology, genetics). A species status can only be confirmed if at least two disciplines support the species hypothesis and could therefore be based on different species concepts. In this thesis the taxonomic circle was applied to prove the discovery of cryptic species.

2.2. Population genetic structure and diversity in a desert-inhabiting dragonfly

Water dependent species inhabiting desert regions seem to be a general contradiction. Nevertheless many species have evolved strategies to survive under arid conditions. Desert inhabiting odonates are mostly opportunistic in their habitat preferences and are therefore able to colonise nearly every freshwater habitat. This study aims to analyse the behavioural and genetic consequences of a water-associated insect species in desert regions. Therefore the population genetic structures and genetic diversities of the African-wide distributed dragonfly species *Trithemis arteriosa* of eight Namibian population sites were examined. In addition four sites from Kenya were included in the analyses to compare the genetic patterns of an arid and a tropical region. Inhabiting only open perennial water habitats with emergent vegetation the species provides a good model system to gain first insights into the consequences and adaptive value of a strongly water-associated insect in desert regions (see 6.5).

2.3. Cryptic speciation in the genus *Trithemis*

Trithemis stictica was chosen as model organism to perform a population genetic study of a species with highly specialised habitat requirements. In Namibia this species was found in only two regions, the Naukluft Mountains and the Okavango and Zambezi Rivers (shared with its adjacent countries Botswana and Zambia). To cover the whole distributional range of

T. stictica samples from South Africa, Kenya, Tanzania, Botswana, Zambia and Ethiopia were included. First results of the sequence marker ND1 surprisingly revealed three distinct genetic groups. Although all individuals were previously identified as *T. stictica*, the three groups clearly differ genetically at the species-level.

Discovery of the first cryptic odonate species

Based on the above described results the aim of the first study in this species complex is to prove the hypothesis of two new cryptic species in the genus *Trithemis* (see 6.6). Therefore a comprehensive morphological analysis was done to find phenotypic differences and potential reproductive barriers between the three clades. In addition the genetic distances between closely related *Trithemis* species were evaluated by including already described species of this genus. In an integrative approach using morphological, ecological, geographical and genetic data the above described taxonomic circle was applied. Furthermore a character-based barcode matrix was established by incorporating characters of the different analysed disciplines to test the applicability of such a comprehensive barcode to discover and delineate species (Damm *et al.* 2009b).

Species description

According to the results of the first study all evidence supports the discovery of two new and cryptic *Trithemis* species. For the introduction of new species to the scientific community a species description is required which delimits the new entity from described species of a given genus. The second study (see 6.7) in this species complex aims to describe holotypes of each sex of the two new species, *T. morrisoni* and *T. palustris*, and discuss the morphological variations. To point out the differences between *T. morrisoni, T. palustris* and *T. stictica*, a re-description of *T. stictica* was performed. By considering the relevant published information about varieties, sub-species and species the differences between the three species are discussed (Damm & Hadrys 2009c).

Speciation processes

The third study (see 6.8) in this species complex aims to examine the reasons for the divergences of the three *Trithemis* species resulting in two cryptic and regionally sympatric species. The underlying speciation processes were analysed by studying genetic diversities, population genetic parameters between the analysed population sites of each species, their morphological variation and ecological niche separation. The time of divergence was

estimated via molecular clock analyses and different modes of speciation are discussed in the biogeographical context (Damm & Hadrys 2009a). Furthermore this case study of diversification allows to investigate two different speciation processes in closely related sister species and the first discovery of a cryptic speciation process in odonates.

2.4. Phylogeographic analyses of the genus *Trithemis*

Only little is known about how the severe climatic changes in Africa's history with an enormous decrease in water resources affected macro-invertebrates. With their aquatic and terrestrial life stages, odonates are interesting model systems for studying the effects of a changing environment and increasing aridity during the Pliocene and Pleistocene.

This study (see 6.9) aims to analyse the phylogenetic relationships within the genus *Trithemis* in the context of the historic climatic shifts in Africa. With its successful radiation and widespread distribution, it provides an excellent study system concerning these questions. With the help of three different sequence markers covering different evolutionary timescales, the time of origin of this genus and the time of the major radiation was estimated via molecular clock analyses. Morphological, ecological and geographical data are mapped on the phylogenetic tree to analyse the direction of speciation (from forest to savannah or vice versa) as well as the influence of habitat fragmentation and climatic shifts on species divergences.

3. Summary of Results and Discussion

3.1. New markers – new approaches

(Giere & Hadrys 2006; Carballa *et al.* 2007; Hadrys *et al.* 2007a; Hadrys *et al.* 2007b; Damm & Hadrys 2009a; Damm & Hadrys 2009b; Damm *et al.* 2009b)

In the different studies of this thesis several new marker systems and methods were successfully applied. In the first four, more technical related studies a new method for the isolation of microsatellite loci was developed and applied to all four odonate species (*Trithemis arteriosa*, *Orthetrum coerulescens*, *Ischnura hastata* and *Anax imperator*). Analyses of allele frequencies, Hardy-Weinberg-Equilibrium (HWE) and linkage disequilibrium revealed the applicability of the isolated microsatellite loci of each species.

Ultimately, a panel of twelve microsatellite loci for *A. imperator* and *A. parthenope* could be used for paternity studies and comparative analyses of their sperm precedence patterns (6.1). Nine microsatellite loci for *I. hastata* (6.2) and *O. coerulescens* (6.3) are now available for further analyses of parthenogenesis and for monitoring studies in conservation genetics. The ten developed microsatellites for *T. arteriosa* were successfully used to analyse the population genetic structure of a desert inhabiting dragonfly (see 3.2, 6.4 & 6.5).

This population genetic study (3.2 & 6.5) also showed the applicability of the different tested markers. The mitochondrial marker ND1 revealed a high variability between and within the analysed populations and is therefore a suitable marker in population genetic studies in odonates. Also COI showed high genetic variation within analysed populations of different species (see 3.3 & 6.6). While the ITS I - II regions did not show enough genetic variability the microsatellite flanking region of a microsatellite locus (TartR04) isolated for *T. arteriosa* turned out to be a useful nuclear sequence marker. With newly developed statistical approaches haplotypes could be defined and therefore allow a direct comparison of the results with both microsatellites and mtDNA. Its application further enables the revision of microsatellite results and could unravel sex-specific behavioural traits when compared to mtDNA (3.2 & 6.5)

The presented study in 3.3 and 6.6 shows that both the taxonomic circle and the character-based barcoding approach are able to unambiguously discover new species also in extreme examples where both new entities are morphologically cryptic and regionally sympatric as in the described case study of the two cryptic *Trithemis* species.

3.2. Population genetic structure and diversity in a desert-inhabiting dragonfly

(Damm & Hadrys 2009b and references therein)

The first assessment of the population structure of a desert inhabiting dragonfly species revealed contrasting patterns between the analysed mtDNA (ND1) and the two nuclear markers (microsatellites and TartR04). While all three markers showed high genetic variability within the populations a high structuring between the populations was only observed with the mtDNA. According to the different modes of inheritance of nuclear and mitochondrial genes these contrasting patterns suggest sex-biased dispersal (see 6.5).

The mtDNA sequences revealed 90% private haplotypes which demonstrates a restriction in gene flow at the maternal lineage while the bi-parentally inherited markers showed high levels of gene flow by sharing most of the haplotypes. This pattern therefore

indicates male-biased dispersal. Such a mating system, where males disperse to actively search new territories and females are philopatric to save energy for foraging, mating and oviposition, might be evolved as a special adaptation to the challenging habitat conditions in arid regions.

Furthermore the genetic diversity patterns of the three markers clearly indicate high genetic variability at population sites with stable habitat conditions. Localities which are affected by drought or human impact show lower genetic diversities at least in the mtDNA. High genetic diversity was found in the northern Namibian populations where sufficient rainfall allowed the establishment of stable permanent water bodies and therefore large populations. MtDNA variability was low in the southern Namibian populations as well as the Kenyan populations which are influenced through periodically recurring times of drought and habitat disturbance through humans or larger animals. In general, all three marker sets show surprisingly higher genetic diversities in the arid Namibia than in the more tropical Kenya. This indicates that opportunistic odonate species in Namibia - despite of the problems of heat and rare water resources - are able to establish large and viable populations at habitats with a long-term stability. In Kenya species diversity in general is higher, which increases interspecific competition and in addition predation through fish might be more common than in Namibia.

By combining the distribution of genetic diversities with the population genetic structure another interesting pattern was observed. The highest differences of genetic diversities and substructures between mtDNA and nuclear DNA were found in populations which are affected by habitat instability. This leads to the conclusion of an increasing migration of the males in times of weak habitat conditions. If the habitat is stable like in North Namibia males are not forced to search for new territories.

This study demonstrates that *T. arteriosa*, a key species for permanent water bodies, is able to establish viable populations also in desert regions. The genetic diversities of the analysed populations highly correlate with the stability of water resources. Their dispersal potential allows long distance migration also covering large, not inhabitable areas. The combination of both mtDNA and nuclear markers revealed asymmetric philopatry with a male-based dispersal, the first case of male-based dispersal in a dragonfly species. This life-history trait might have been evolved due to the special requirements of desert inhabiting dragonfly species.

3.3. Cryptic speciation in *Trithemis* species complex

(Damm & Hadrys 2009a; Damm & Hadrys 2009c; Damm *et al.* 2009b and references therein)

While the above described population genetic study was able to reveal special behavioural traits by the application of genetic markers, the discovery of a cryptic speciation process in the genus *Trithemis* highlights the importance of including genetic data into taxonomic research. The two new species have only been discovered through the initially applied population genetic analyses (see 6.6).

T. stictica was found in Namibia, South Africa, Kenya, Tanzania and Ethiopia, but is absent in the region of the Zambezi and Okavango floodplains where population sites were inhabited by the two new species *T. morrisoni* and *T. palustris*. *T. stictica* could be distinguished from the latter two through differences in morphology (structure of the secondary genitalia and eye colouration), geography and genetic data, but the new species are difficult to delineate using only traditional characters.

The application of the taxonomic circle as an analytical process to discover new species proved to be a promising tool for modern taxonomic research. The five important components of the circle (morphology, ecology, geography, reproductive isolation and genetics) covering the different species concepts were tested and results showed that four components (reproductive isolation, high genetic differences, size differences and the occupation of different habitats) confirm the hypothesis of two new *Trithemis* species.

After applying the newly developed character-based DNA barcoding the different diagnostic characters concerning genetics, morphology, geography and ecology were incorporated into an elaborative data matrix. The incorporation of traditional characters allows the discrimination of the two species by not only genetic data but also morphology (size) and ecology (habitat) and therefore adds crucial information to conservation management. Such a comprehensive database can provide both rapid species identification and discovery (see 6.6).

After confirming the species discovery hypothesis a species description of *T. morrisoni* and *T. palustris* was done with a detailed delineation of males and females of all three species (see 6.7).

This species complex is the first example of morphologically cryptic species in odonates and further allows studying two different speciation processes in closely related species (see 6.8). Molecular clock analyses dates the split between *T. stictica* and the ancestor

of *T. morrisoni* and *T. palustris* to the Pliocene 3.5 Mya. This is the time of the major climate changes on the African continent with increasing aridity, rainforest fragmentation, river connection changes and desiccation of rivers and lakes. The Okavango and Zambezi floodplains were directly affected by aridification and are currently surrounded by dry savannahs and the Kalahari Desert. The increasing aridity might have forced a range shift in *T. stictica* and only populations which were able to adapt to these changing conditions survived, therefore suggesting allopatric speciation.

The divergence of *T. morrisoni* and *T. palustris* occurred in the Pleistocene 1-2.4 Mya. Assigning one of the main modes of speciation to this case study is a difficult task. Regarding the historical geography of the species distributional range and the high recent dispersal potential and migration rates of both species, a real allopatric speciation seems to be very unlikely. Several criteria for a potential sympatric speciation were analysed and results confirmed the criteria of (i) largely overlapping ranges, (ii) complete reproductive isolation, (iii) the sister species status and (iv) a recent panmictic distribution of *T. morrisoni* and *T. palustris*. Nevertheless, parapatric speciation might also be possible. While *T. morrisoni* and *T. palustris* inhabit different ecological niches, speciation was likely accompanied or even caused by a historical habitat shift. Excluding times of restricted or interrupted gene flow in the past is not easy to verify and therefore a proposed alternative model of “divergence-with-gene-flow” might be a promising explanation for the speciation of the two new species.

With the discovery of the first two cryptic dragonfly species this study highlights the importance of analysing the processes underlying diversifications and furthermore suggests that cryptic speciation in odonates might occur more often than previously thought.

3.4. Phylogeographic analyses of the genus *Trithemis*

(Damm *et al.* 2009a and references therein)

An integration of genetic, morphologic, geographic and ecological data like in the study of the speciation processes of the cryptic *Trithemis* species allows a deeper understanding of speciation processes also at the genus level. The first comprehensive phylogenetic analysis of an African dragonfly genus dates its origin to the late Miocene (6-9 Mya) with both molecular clock analyses and fossil records (see 6.9). The majority of species divergences took place in a very short timeframe in the Pliocene approximately 4-5 Mya, where most of the extant species evolved. The topology of the phylogenetic trees revealed by analyses of three

sequence markers (ND1, 16S and ITS I&II) showed very short branches at the time of the major divergence which leads to the assumption of a rapid radiation in the Pliocene, the time in which the African continent was influenced by severe climatic changes with increasing aridity.

The most basal *Trithemis* species are best adapted to arid environments and therefore the suggested primary habitat of this genus might be open savannah. While the red species seem to be evolved in a very short time period without close sister-species groups, the blue and dark species cluster together in three highly supported clades. By mapping their ecological requirements onto the tree three differing strategies of adaptation to deal with environmental changes and increasing competition were found. In one clade the species moved back into forested habitats with ecological progression towards forest by stepwise occupation of, adaptation to and speciation in increasingly closed habitats. The species of the second clade favour elevated open habitats in the highlands from the Cape of South Africa to Kenya. The species of the third clade occupied lowland habitats of 'mixed' flow, like channels in swamps and calm stretches and by-waters of streams across the Congo-Zambezi watershed. All red *Trithemis* species inhabit exclusively open savannah habitats and developed special adaptations to the arid climate. In the time of savannah expansions they were able to expand their ranges and are today the most widespread species of this genus.

Colouration therefore seems to be an indicator for ecological requirements rather than displaying phylogenetic relationships. The red colour is found in species inhabiting open habitats, while the dark species mostly occur at cooler or forested habitats. The open land dark species developed a reflective waxy body coating called pruinosity, which reflects light to avoid extreme exposure of the sun.

While many species got extinct in the changeable climatic past, the genus *Trithemis* might have had a selection advantage. It profited from the unoccupied habitats due to savannah expansions which finally resulted in the evolution of a great variety of different niche adaptations and mainly forced speciation in form of allopatry.

In sum, the changes of climate and environment benefitted dry-adapted open-land species. The great success of the genus seems to be related to their savannah origin (which favoured opportunistic species with great dispersal ability) and to their high adaptive potential. Until today *Trithemis* species often dominate the odonate communities at a great variety of different freshwater habitats in Africa.

4. Conclusions

This thesis presents a variety of new insights into adaptations, life history traits, speciation processes and biogeography of African dragonflies at each analysed level. The newly applied markers and approaches provide useful tools in conservation genetics and species discovery. The studies demonstrate the importance of applying molecular techniques to conservation biology, modern taxonomy, biodiversity research, speciation analyses and phylogeography. The continuing development of new informative molecular markers, computer-based algorithms and high throughput detection methods allows analyses on different evolutionary timescales and a fast assessment of biodiversity and conservation patterns. Hereby the recent focus is on the increasing integration of traditional and molecular disciplines. In particular, the combination of ecological, morphological, behavioural and genetic information allows comprehensive analyses for ecological and evolutionary questions.

Studies of species populations need the traditional ecological background. But without the use of genetic markers certain aspects such as gene flow and migration rates are very difficult to observe, particularly in species with high dispersal abilities like odonates. Analysis of species interactions with different environments is essential when preserving species and/or ecosystems of high conservation value. Different ecological conditions could lead to differences in dispersal behaviour and may result in changes of the population structure.

For the assessment of biodiversity, genetic markers provide rapid identification tools for species, but their success is sometimes limited, e.g. in species discovery. Therefore, DNA-based species discovery should always be supported by independent evidence gained from other disciplines. The increasing number of (sometimes questionable) cryptic species shows that a convincing framework is needed, which integrates the most important aspects of the different species concepts. The taxonomic circle applied in this thesis represents an analytic approach to prove a species discovery hypothesis. Here at least two of the five components of the circle (morphology, geography, ecology, reproductive isolation, genetics) have to corroborate the hypothesis of a new species. This framework provides the possibility of species discovery in a convincing way, although certain aspects such as sample size, the applied genetic marker and the geographical range of the sampling have to be considered in the decision making process.

The integration of multiple disciplines also greatly enhances the DNA barcoding potential. While DNA barcoding is a promising tool for assessing biodiversity, the discussions

surrounding the barcoding initiative suggest that the procedure should possibly be revised. The character-based barcoding applied here allows the establishment of a comprehensive database which includes genetics (the barcode fragment COI and any other marker), morphology, geography and ecology of the query species. Such a barcode is able to provide both rapid species identification and discovery, as shown here in *Trithemis*.

Nevertheless, when applying molecular methods, the choice of the genetic marker and algorithm for the analyses is of particular importance to every analysed level. The application of multiple markers (mitochondrial and nuclear) and different algorithms is a good and conservative way to avoid misleading assignments and conclusions. Applying nuclear and mitochondrial markers also allows supporting evolutionary hypotheses independent of the mode of inheritance. In population genetic studies, sequence markers as well as microsatellites provide the possibility to analyse population structures on two different scales. While mtDNA data give important information about the geographical distribution on a large scale, microsatellites are irreplaceable for genetic diversity assessments and long-term monitoring of specific population sites. Results of fine-scale analyses using microsatellites should be integrated into conservation management due to their usefulness as rapid detectors of habitat changes. Nevertheless, microsatellite analyses are highly dependent on sample sizes. But often sufficient sample sizes are difficult to obtain e.g. of endangered species or at localities with a low species abundance. The integration of a second non-coding nuclear marker system, like the here applied microsatellite flanking region, offers the possibility to independently verify the results of the microsatellites. Furthermore the application of different marker sets could provide additional insights into special life history traits if the results of nuclear and mtDNA are contradictory. In the case of *T. arteriosa*, we were able to reveal a potential male-biased dispersal as a consequence of the extreme climate in Namibia.

Understanding the processes of speciation is one major task in evolutionary biology. Because of its complexity many mechanism remain unclear and often a specific mode of speciation could not be assigned. This is also shown in the detection of the first cryptic species in odonates which also highlights that speciation without accompanied phenotypic changes can also occur in animal groups which were previously not considered to evolve cryptic species. The first comprehensive phylogenetic study of a dragonfly genus in Africa allowed us to reconstruct the biogeographical history of the genus and the speciation processes of the African dragonflies in general. The understanding of macro- and microevolutionary processes lying behind species adaptation and diversification is of great importance to analyse and estimate current speciation potential.

The integration of biogeography, morphology, genetics and ecology could assist us to evaluate how changes in major environmental parameters like climate and geology influenced the evolution of species in the past and which consequences we might expect for the future. In conclusion, the incorporation of different disciplines at any kind of level from population to phyla is of particular importance to understand the processes governing biodiversity and can help to rapidly detect the consequences of prospective environmental changes.

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6. Publications and manuscripts upon which this thesis is based

6.1 Hadrys H., Timm J., Streit B. & S. Giere (2007). A panel of microsatellite markers to study sperm precedence patterns in the emperor dragonfly *Anax imperator* (Odonata: Anisoptera). *Molecular Ecology Notes* **7**, 296-298.

6.2 Carballa O.L., Giere S., Cordero A. & H. Hadrys (2007). Isolation and characterization of microsatellite loci to study parthenogenesis in the citrine forktail, *Ischnura hastata* (Odonata : Coenagrionidae). *Molecular Ecology Notes* **7**, 839-841.

6.3 Hadrys H., Wargel A., Giere S., Kraus B. & B. Streit (2007). A panel of microsatellite markers to detect and monitor demographic bottlenecks in the riverine dragonfly *Orthetrum coerulescens* F. *Molecular Ecology Notes* **7**, 287-289.

6.4 Giere S. & H. Hadrys (2006). Polymorphic microsatellite loci to study population dynamics in a dragonfly, the libellulid *Trithemis arteriosa* (Burmeister, 1839). *Molecular Ecology Notes* **6**, 933-935.

6.5 Damm, S. & H. Hadrys (2009). Odonata in the desert - Population genetic structure of a desert inhabiting dragonfly (*Trithemis arteriosa*) suggests male-biased dispersal. In preparation for Molecular Ecology.

6.6 Damm, S., Schierwater, B. & H. Hadrys (2009) An integrative approach for species discovery - From character-based DNA-barcoding to ecology. *Molecular Ecology*, submitted.

6.7 Damm, S. & H. Hadrys (2009). *Trithemis morrisoni* sp. nov. & *T. palustris* sp. nov. from the Okavango and Upper Zambezi floodplains previously hidden under *T. stictica* (Odonata, Libellulidae). *International Journal of Odonatology*, **12** (1), 131-145.

6.8 Damm, S. & H. Hadrys (2009). Cryptic speciation via habitat shift - A case study on the Odonate genus *Trithemis* (Odonata: Libellulidae). In preparation for the Proceedings of the Royal Society Biological Science B.

6.9 Damm, S., Dijkstra, K.-D. B. & H. Hadrys (2009). Red drifters and dark residents: Africa's changing environment reflected in the phylogeny and ecology of a Plio-Pleistocene dragonfly radiation (Odonata, Libellulidae, *Trithemis*). *Molecular Phylogenetics and Evolution*, submitted.

A panel of microsatellite markers to study sperm precedence patterns in the emperor dragonfly *Anax imperator* (Odonata: Anisoptera)

Heike Hadrys,*† Janne Timm,* Bruno Streit‡ and Sandra Giere*

**ITZ, Ecology and Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany,*

† Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, 06520-8104, USA,

‡ Department of Ecology, Evolution and Diversity, J.W. Goethe-Universität, D-60054 Frankfurt am Main, Germany

This is the author's version of a work originally published by Wiley-Blackwell in:
Molecular Ecology Notes (2007) Volume 7, Pages 296-298; available under DOI: 10.1111/j.1471-8286.2006.01585.x.

Abstract

Odonates were the first group of organisms where sperm competition and last male sperm precedence have been identified. With the development of 10 microsatellites for the emperor dragonfly *Anax imperator,* the function and priority patterns of the multiple sperm storage organs of females can be studied and compared between species in natural populations. In addition, two microsatellite loci developed for the sister species *Anax parthenope,* are also highly polymorphic in *A. imperator*. For the presented 12 microsatellite loci, the number of alleles per locus ranged from two to 24. Observed heterozygosity ranged from 0.07 to 0.88.

Keywords: Aeshnidae, Odonata, microsatellites, sperm competition, cryptic female choice, sexual selection

Since the discovery of sperm competition, odonates (dragonflies and damselflies) have been paradigms for studies about the evolution of mating systems (Waage 1979). In recent years, there is a fast-growing body of evidence that not only males, but also females, could bias the outcome of sperm competition by cryptic female choice (e.g. Cordero Rivera *et al*. 2004). The methodological progress to obtain direct measures of paternity under natural conditions via microsatellites opens the potential to determine the mechanism of sperm handling by females (Fincke & Hadrys 2001). We seek to develop microsatellites for the Aeshnid species *Anax imperator* in order to study the sperm precedence mechanism and to compare it with the sister species *Anax parthenope* (Hadrys *et al*. 1993; Siva-Jothy & Hadrys 1998; Fincke & Hadrys 2001). Despite their close relationship, both species differ widely in their mating system traits related to sperm competition.

Tissue samples of 92 *A. imperator* individuals were collected in Namibia, France and Germany by noninvasive sampling (Hadrys *et al*. 2005). Genomic DNA was isolated using a modified phenol-chloroform extraction protocol (Hadrys *et al*. 1993). The microsatellite loci for *A. imperator* were detected and isolated using the slightly modified enrichment technique of Fischer & Bachmann (1998). Briefly, DNA was digested using the three restriction enzymes, *Rsa*I, *Hae*III and *Alu*I (Gene Craft). Two oligo adapters (Edwards *et al*. 1996) were ligated to the digested DNA fragments followed by the hybridization to two biotinylated probes $(GA)_{10}$ and $(AC)_{10}$. Ligated DNA fragments containing potential repeat motifs were bound to streptavidincoated magnetic beads and isolated using a magnet. Furthermore, a polymerase chain reaction (PCR) with the microsatellite-enriched eluate as template was employed in order to increase the template quantity. Hereby, 2.5 pmol of one adapter was used as a primer in a final reaction volume of 50µL 1× PCR buffer (Invitrogen), containing 1.5 mM MgCl, 0.8 mM of each dNTP, 0.5 U *Taq* DNA polymerase (Invitrogen). PCR cycling conditions were as follows: 94° C for 5 min, 35 cycles of 94° C for 1 min, 56° C for 1 min, 72° C for 2 min and a final elongation for 5 min. The enrichment process with the magnetic beads and PCR amplification were repeated once. The resulting PCR products were ligated into pGEM-T vectors (Promega) and transformed into competent *Escherichia coli* cells (TOP10; Invitrogen). Plasmids from positive clones were amplified using T7 and SP6 primers. Ninety-four of the resulting amplification products were subjected to Southern blot analyses with the two 3′ biotin-labelled probes $(GA)_{10}$ and $(AC)_{10}$. Thirty-six products were selected for sequence analyses on a MegaBace 500 using ET Terminator Mix (Amersham). Seventeen sequences contained a repeat motif of more than six repeat units for which fluorescence-labelled primers for microsatellite typing were designed. Initial PCRs were

performed in a 25 μL reaction volume containing 5–10 ng template DNA, 1× PCR buffer (Invitrogen), 2 mM MgCl, 5 pmol of each primer, 0.1 mM of each dNTP and 0.5 U *Taq* DNA polymerase (Invitrogen). PCR cycling conditions were as follows: 93° C for 3 min, followed by 35 cycles of 30 s at 93° C, 20 s at primer-specific annealing temperatures (Table 1), 40 s at 72° C and a final elongation of 2 min at 65° C. Automated genotyping was performed on an ABI 310 automated sequencer. The GENESCAN-500 ROX Size Standard from Applied Biosystems was used to determine the allele sizes. Data analysis was performed using GENESCAN (Applied Biosystems). GENEPOP 3.4 (Raymond & Rousset 1995) was used to estimate expected and observed heterozygosities, to test for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) using default values for the Markov chain parameters.

Initial assessment revealed that seven of the 17 loci amplified and genotyped were either monomorphic or showed no distinct amplification products. Two additional microsatellite loci, originally derived from the sister species *A. parthenope* (Ap7/8-E201 and Ap7/8-E202 in Table 1), showed successful cross-species amplification for *A. imperator*. These loci were detected and isolated following the RAMS protocol by Ender *et al.* (1996). Table 1 summarizes the genotyping results for all 12 loci. Most loci show a high genetic variability exhibiting 2 to 24 alleles in the genotyped individuals.

Genotype frequencies, tested multiple times for conformance to HW expectations, revealed significant deviations from HWE for five of the 12 loci. However, in separate population tests across Europe and Africa, only locus AiL04 showed significant heterozygote deficiencies in the majority of the 14 analysed populations, suggesting a rather population-specific pattern than the presence of null alleles. Significant LD was detected for a single pairwise comparison (AiB03 vs. AiL04; $P = 0.037$). The developed panel of microsatellites for *A. imperator* will be an essential tool to study the potential of females influencing sperm precedence patterns. Furthermore, initial tests between populations and species indicating their potential for population-level as well as cross-species studies.

Table 1 Characteristics of 12 polymorphic microsatellite loci in the dragonfly *Anax imperator* (Ai). Shown are GenBank Accession numbers, locus name, primer sequence, primer specific annealing temperature (°C), allele size range (bp), repeat motif, number of alleles per locus (N_A), expected (H_E) and observed (H_O) heterozygosity rates. Significant departure from Hardy-Weinberg equilibrium is indicated by asterix when $P = 0.05$. Note that the loci Ap E202 and Ap E201 originally derived from microsatellite screening in the sister species *Anax parthenope.*

Accession nos	Locus	Primer sequence (5' – 3')	Ta	Allelsize range	Repeat motif	N_A	$N_{ind.}$	H_e	H_o
DQ793120	Ap E202	f-19mer: HEX-TCTCGCACTGACCATTGTG r-18mer: CTTCTTCCCAACGAAAGC	60°C	156bp-178bp	$(TC)_2TT$ $(TC)_{11}(AC)_8$	10	90	0.76	0.70
DQ793121	Ap E201	f-17mer: FAM-GCTGCAGGATCGAACTG r-20mer: AGTAGGGAGAACATAATCCC	64°C	78bp-94bp	$(CA)_3CTTA$ $(CA)_7$	8	14	0.77	0.71
DQ793122	AiB03	f-20mer: HEX-GGAGAATTTCCGAATTTGAG r-20mer: GCTCGAGAGCGTTTATAAGG	52°C	217bp-293bp	$(AG)_{20}$	24	80	0.91	0.85
DQ793123	AiG03	f-20mer: FAM-CTTACGCGTGGACTCACTGC r-19mer: GAAGTCCCCTCTTCCACTG	56°C	220bp-256bp	$(TA)_3(TG)_9$	7	14	0.62	0.50
DQ793124	AiH04	f-20mer: FAM-TATGCGTCGACTCGATCACT r-23mer: TGCCTCTCAATAATTGTTTGTTT	57°C	117bp-125bp	$(TC)_9$	6	56	0.80	0.77
DQ793125	AiI04	f-21-mer:HEX-TTTTGCATGAGAATCCAGCTT r-20-mer:TTCCGAAGGAATATAGA	57°C	166bp-180bp	$(GT)_8GC$ $GT(GC)_5$	8	58	0.84	0.85
DQ793126	AiJ04	f-20-mer: FAM-TGGCTAATTGGGACTTCTGG r-20-mer:TCCGTTCCCACACGTTTAAT	57°C	240bp-244bp	$(GT)_2(GA)_2$ $C(AG)_7$	3	16	0.53	0.25***
DQ793127	AiK04	f-24-mer:HEX-GACTTCAAGAATTAACTCCACCAA r-26-mer:TTTTATGAATAGGTGACAATTCAGTG	57°C	184bp-190bp	$(AC)_7TA$ $(CA)_2(TA)_7$	2	27	0.50	0.85*
DQ793128	AiL04	f-20-mer:FAM-CGTGCACGGTAACTCTCTCC r-20-mer:TCAGGGTTAAAAGCACTCGT	57°C	214bp-260bp	$(CA)_6(TACA)_3(CA)_5(TA)_7$	15	37	0.90	0.60***
DQ793129	AiM04	f-20-mer:HEX-GATGGCGATAATAGCCCAAG r-20mer:GCCACTGAATAGCACTGCAC	57°C	213bp-223bp	$(AC)_{10}$	6	38	0.84	0.45***
DQ793130	AiN04	f-20mer:FAM-AGAGTGAGTCCGTTGGGTTG r-20mer: GATCACGCGACGATAGGTTT	57°C	169bp-179bp	$(GA)_{11}$	6	57	0.79	0.88
DQ793131	AiP04	f-21mer:FAM-CGAAACAGTTGGACCTGAACG r-20mer:AGGGGCAACTATTCCAAACA	57°C	223bp-231bp	$(GA)_9$	5	68	0.61	0.50*

Acknowledgements

This work was supported by grants from the German Science Foundation (DFG Ha 1947/2-1-2-3), the Federal Government (BMBF, BIOLOG Africa, BIOTA S08) and the HSP-program of Hessen. We thank Bernd Schierwater for comments and F. Suhling for providing samples. We also thank the staff of the ecological field station 'Bahnhof Schapen' for providing specimens.

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Isolation and characterization of microsatellite loci to study parthenogenesis in the Citrine Forktail *Ischnura hastata* (Odonata: Coenagrionidae)

Olalla Lorenzo Carballa[‡], Sandra Giere[*], Adolfo Cordero[‡] and Heike Hadrys[*†]

[‡] *Evolutionary Ecology Group, Dept. Ecology and Animal Biology, Universidad de Vigo. EUIT Forestal, Campus Universitario, 36005, Pontevedra, Spain.*

[*] *ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany*

[†] *Yale University, Dept. of Ecology and Evolutionary Biology, New Haven, CT, 06520-8104, USA*

This is the author's version of a work originally published by Wiley-Blackwell in:
Molecular Ecology Notes (2007) Volume 7, Pages 839-841; available under DOI: 10.1111/j.1471-8286.2007.01722.x.

Abstract

The Citrine Forktail *Ischnura hastata* is an american damselfly species, widely distributed, with only-female populations also found at the Azores islands. Here we report the development of nine microsatellite loci for this species. The number of alleles per locus ranged from six to 11, with an observed heterozygosity ranging from 0.245 to 0.737. Eight of the nine loci successfully amplified in a sample of parthenogenetic females from the Azores. The developed microsatellite system will be an useful tool to investigate population structure, as well as the number of clones, the type of parthenogenesis and the origin of the parthenogenetic populations of this species.

Keywords: Odonata, damselflies, *Ischnura hastata*, microsatellites, parthenogenesis

The Citrine Forktail *Ischnura hastata* is a damselfly species, widely distributed in North and South America, the Caribbean and Galapagos islands (Dunkle 1990). As generally known for Odonata (damselflies and dragonflies), only bisexual populations have been described for the above regions. At the Azores islands, however, all populations found exclusively consist of female individuals. This is the first case of parthenogenesis described in this insect order (Cordero et al. 2005). Here we report on the development of a microsatellite system to study the genetic diversity within and among bisexual and parthenogenetic populations, and to further explore the origin and type of parthenogenesis in *I.hastata*.

Genomic DNA was extracted from thoracic muscle following a CTAB-based protocol, modified from Doyle and Doyle (1987). Microsatellite loci were isolated using the modified enrichment technique of Fischer and Bachmann (1998). DNA was digested with two restriction enzymes (*Alu*I and *Rsa*I) and ligated to two oligo adapters (Oligo A: 5'-CTCTTGCTTACGCGTGGACTA- 3' and Oligo B: 5'- TAGTCCACGCGTAAGCAA-GAGCACA- 3') (Edwards *et al.* 1996). Two 3'-biotinylated oligo probes [$(GA)_{10}$ and $(AC)_{10}$] were hybridized to the digested DNA. DNA fragments containing the potential repeat motifs were selectively retained using streptavidine-coated magnetic beads (Promega). The microsatellite enriched eluate was used as a template in a polymerase chain reaction (PCR) with 10 pmol of the Oligo A adapter as a primer and containing in a final volume of 50 µL 1x buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.8 mM of each dNTP and 2.5 U of *Taq*DNA polymerase (Invitrogen). PCR cycling conditions were 94 ºC for 5 min, 40 cycles at 94 ºC for 1 min, 56 ºC for 1 min, 72 ºC for 2 min and a final elongation step of 72 ºC for 5 min. The enrichment process with the magnetic beads and the subsequent PCR were repeated once, according to Giere and Hadrys (2006). The enriched library was ligated into pGEM-T vectors (Promega) and transformed into competent *Escherichia coli* cells (TOP10) according to Sambrook *et al.* (1989). A total of 211 positive clones were selected for PCR amplification using T7 and Sp6 primers. Seventy-six products were sequenced on a MegaBACE 500 using ET Terminator Mix from Amersham. Eight sequences revealed no repeat motifs, and eighteen were excluded from further analysis due to either too small or too complex repeat units. Ultimately, 16 sequences that contained a repeat motif of more than six repeats were used for primer design using Primer3 (Rozen & Skaletsky 2000). Each of the forward-primers was labelled with a fluorescent dye (HEX™ or 6-FAM™) for microsatellite typing. PCR was performed in a 25 µL reaction volume containing 1µL DNA (5-10 ng of genomic DNA), 1x buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 5 pmol of each primer, 0.1 mM of each dNTP and 0.75 U of *Taq*DNA polymerase (Invitrogen). PCR cycling conditions were 93 °C for 3

min followed by 35 cycles 93 ºC for 30 s, 25 s at primer-specific annealing temperatures (Table 1), 30 s at 72 ºC and a final elongation step of 72 ºC for 5 min. Automated genotyping was performed on a MegaBACE 500 automated DNA sequencer.

Table 1: Characteristics of nine microsatellite loci for the coenagrionid damselfly *Ischnura hastata* (Ihas). Listed are GenBank Accession numbers, locus name, primer sequence, primer specific annealing temperature (ºC), allele size range (bp) and repeat motif.

Accession nos	Locus	Primer (5'-3')	T_a (ºC)	Allele size range (bp)	Repeat motif
EF088818	Ihas01	f-20-mer: FAM-TGTGCACGCTACCCTATCTA r-20-mer: CTGTCGCTCTTCTGTGATTG	53	155-167	$(TC)_9$
EF088819	Ihas05	f-20-mer: HEX-TCACAACACTTCCTCCTCCT r-20-mer: GAAATCTCAAGGGGGAAAAT	53	213-229	$(CT)_8$
EF088820	Ihas08	f-20-mer: FAM-CCACCTTTATTGCCTTTCAC r-20-mer: CGATCGGACACTTCAAATCT	58	186-202	$(AG)_9$
EF088821	Ihas09	f-20-mer: FAM-CTTCGAAATGATTCGACCTC r-21-mer: GGAAGTCGAGGTGTAAAAGGT	60	175-199	$(CT)_{11}$
EF088822	Ihas10	f-20-mer: FAM-GCTGCACTACAAAGCCATCT r-20-mer: AATAGGAAGGGGACCTCAAC	60	157-173	$(CT)_9$
EF088823	Ihas11	f-19-mer: FAM-TCCAGGAAAAGCCATTAGG r-20-mer: CTTCCACTCCTTCCACACTC	58	165-187	$(TG)_7$
EF088824	Ihas13	f-20-mer: HEX-CAGTCACCGTCAACTGTTTG r-20-mer: TTAGTTGCCGGAGAAGAGTC	58	245-265	$(AC)_7A$ (AC)
EF088825	Ihas15	f-20-mer: HEX-ACAACTCTCGATGACACACG r-20-mer: GATGTATGAAGGGCTCCAAG	58	221-233	$(CT)_9$
EF088826	Ihas16	f-24-mer: HEX-TCTACCCACCCTCTATATTCCTGA r-19-mer: CCCCCGTACAGTCCCTACC	50.8	167-187	$(TC)_{14}$

The ET-550 Size Standard (Amersham Biosciences) was used to determine allele sizes. Data analysis was performed using the GENETIC PROFILER, version 1.2 (Amersham Biosciences). GENEPOP version 3.4 (Raymond & Rousset 1995) was used to estimate observed (H_O) and expected (H_E) heterozygosity, deviations from Hardy-Weinberg equilibrium (HWE) and to test for linkage disequilibrium (LD).

A total of 63 individuals of *Ischnura hastata* (representing two bisexual populations from Florida (n=27) and Mexico (n=37)) were genotyped for each locus. Of the 16 loci tested, two appeared to be monomorphic, one was not suitable as it produced dubious amplification patterns, and four primer pairs did not amplify any product. Nine loci were polymorphic, showing a high genetic variability. The results of the genotyping are summarized in Table 2.

Table 2. Genotyping results for the samples of *I. hastata* from North America. Listed are: locus name, allele size range (bp), number of alleles per locus (NA), number of individuals genotyped (Nind), expected (HE) and observed (HO) heterozygosity rates and P value of the departure from Hardy-Weinberg equilibrium.

Locus	N_A	N_{ind}	H_E	H_O	*P* value
Ihas01	6	53	0.409	0.245	0.0077*
Ihas05	8	61	0.757	0.623	0.0535
Ihas08	9	39	0.848	0.692	0.0020*
Ihas09	10	52	0.788	0.673	0.0399*
Ihas10	8	38	0.795	0.684	0.1417
Ihas11	11	19	0.906	0.737	0.0025*
Ihas13	10	52	0.813	0.712	0.3997
Ihas15	7	38	0.702	0.658	0.3552
Ihas16	11	60	0.658	0.667	0.3172

*Indicates significant heterozygote deficiency (P<0.05)

The number of alleles per locus ranged from six to 11, with an overall number of 80 alleles. The observed heterozygosity ranged from 0.245 to 0.737. Deviation from HWE was detected for loci Ihas01, Ihas08, Ihas09 and Ihas13. However, when populations were treated separately, only loci Ihas01 and Ihas09 in Mexico population and locus Ihas10 in Florida population revealed significant heterozygote deficiencies. No LD was detected between any pair of loci.

The developed microsatellite system was further tested in a sample of parthenogenetic females from the Azores. Eight of the nine loci were checked up to now, and all show successful amplification products in these samples. However, the results of the genotyping revealed a significant lower genetic variability compared with the sexual populations. Of the 8 loci tested, five were polymorphic and three were monomorphic. The overall number of alleles was 13, and the number of alleles per locus ranged from one in the monomorphic loci to 2 for the polymorphic loci. Observed heterozygosities ranged from 0 to 1 (Table 3). Only one clone (eight-locus genotype) was detected among the parthenogenetic individuals, which can be due either to a low clonal variability of the parthenogenetic populations, or to a small sample size. Increasing the number of parthenogenetic individuals genotyped could lead to the detection of more clonal copies at these populations.

In summary, the developed microsatellite system for *I. hastata* will be an essential tool to study the genetic structure of bisexual and parthenogenetic populations, to determine the number of clones and to detect the type and origin of parthenogenesis in the Azorean populations of this species.

Table 3 Genotyping results for the parthenogenetic female samples of *Ischnura hastata*. Listed are locus name, allele size range (bp), number of alleles per locus (N_A), number of individuals genotyped (N_{ind}), expected (H_E) and observed (H_O) heterozygosity rates and P value of the departure from Hardy-Weinberg equilibrium.

Locus	Allele size range (bp)	N_A	N_{ind}	H_E	H_O	*P* value
Ihas01	159	1	20		0	
Ihas05	221-225	2	78	0.503	1	0.0000*
Ihas08	192-194	2	18	0.514	1	0.00002*
Ihas09	187-191	2	18	0.514	1	0.00001*
Ihas10	159	1	11		0	
Ihas11	165-171	2	17	0.515	1	0.0001*
Ihas13	245-251	2	28	0.509	1	0.0000*
Ihas15	233	1	21		0	

*Indicates significant heterozygote deficiency (P<0.05)

Acknowledgments

This work was supported by a research grant of the Spanish Ministry of Education and Science to ACR (CGL2005-00122) and a predoctoral grant to OLC. We thank Bernd Schierwater for providing the laboratory facilities and the staff of the ITZ in Hannover for their help during the lab work. We also thank, Laura Sirot (Florida) and Sandy Upson, Doug Danforth, Robert Behrstock, and Alejandro Córdoba-Aguilar (México) for their help during field work, and Dennis Paulson for providing samples.

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A panel of microsatellite markers to detect and monitor demographic bottlenecks in the riverine dragonfly *Orthetrum coerulescens* F.

Heike Hadrys,*† Antonia Wargel,* Sandra Giere,* Bernd Kraus‡ and Bruno Streit‡

**ITZ, Ecology and Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany*

†Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06520–8104, USA,

‡Department of Ecology, Evolution and Diversity, JW Goethe-Universität, D-60054 Frankfurt am Main, Germany

This is the author's version of a work originally published by Wiley-Blackwell in:
Molecular Ecology Notes (2007) Volume 7, Pages 287–289; available under DOI: 10.1111/j.1471-8286.2006.01582.x

Abstract

Odonates (dragonflies and damselflies) are important indicators for monitoring anthropogenic impacts on freshwater ecosystems. We developed a panel of microsatellite loci for the keeled skimmer *Orthetrum coerulescens*, a libellulid dragonfly inhabiting small streams. By using two different isolation techniques, nine microsatellite loci have been isolated. Screening of 209 individuals resulted in an overall number of 88 alleles, ranging from three to 19 alleles per locus. The observed heterozygosity ranged from 0.37 to 0.83. One locus showed significant deviation from Hardy–Weinberg equilibrium.

Keywords: dragonflies, long-term monitoring, microsatellites, Odonata, *Orthetrum coerulescens*

Odonates restricted to riverine habitats are especially prone to environmental changes (Corbet 1999). Dredging, canalization, siltation of streambeds and pesticide pollution are common threads to larvae and imagos. A species especially affected by dredging of their breeding habitat, is the keeled skimmer, *Orthetrum coerulescens*. Populations of this species are often established along irrigation ditches, as those are the only viable breeding habitats left in developed landscapes. Our objective was to develop a panel of microsatellite loci for *O. coerulescens*, which will prove useful to detect and monitor the impact of environmental changes on this species.

Tissue samples of 209 *O. coerulescens* individuals were collected at several breeding sites in southern France, Germany and Italy by nondestructive sampling (Fincke & Hadrys 2001). Genomic DNA was isolated from single legs following a protocol by Hadrys *et al*. (1992). Seven microsatellite loci (Ocoe A03; Ocoe E04; Ocoe F04; Ocoe G04; Ocoe H04; Ocoe J04; Ocoe K04) were isolated with the slightly modified enrichment technique from Fischer & Bachmann (1998). DNA was digested with three restriction enzymes (*Rsa*I; *Hae*III; *Alu*I) and ligated to two oligo adapters (Oligo A 5′-CTCTTGCTTACGCGTGGACTA-3′ and Oligo B 5′-TAGTCCACGCGTAAGCAAGAGCAAGAGCACA-3′) using a T4-Ligase (Edwards *et al*. 1996). The digested DNA was hybridized with two 3-biotinylated oligo probes $(GA)_{10}$ and $(AC)_{10}$. DNA fragments containing the potential repeat motifs were selectively retained using a biotin-streptavidin reaction with magnetic beads (Streptavidin MagneSphere Paramagnetic Particles; Promega). Polymerase chain reactions (PCR) were carried out on a GeneAmp 2700 (Applied Biosystems) to increase the quantity of the resulting microsatellite- enriched eluate by using 2.5 pmol Oligo A adapter as a primer. PCRs were performed in a total volume of 50- μL buffer (Invitrogen), containing 1.5 mm MgCl2, 0.8 mm of each dNTP and 0.5 U *Taq* DNA polymerase (Invitrogen) with the following cycling conditions: 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and a final elongation for 5 min. The biotin–streptavidin reaction and PCR were repeated once. After purification with a gene cleaning kit (GeneClean, Qbiogen) the enriched library was ligated into pCRII-TOPO vectors (Invitrogen) and transformed into competent *Escherichia coli* cells (TOP10; Invitrogen). A total of 64 positive clones were subjected to PCR amplification using T7 and SP6 primers. Twelve amplification products with a size range from 500 to 1000 bp were subsequently sequenced on an Amersham Bioscience MegaBACE 500 sequencer. Primers for seven loci were designed using the software primer 3 (Rozen & Skaletsky 2000) by labelling each of the forward-primers with a fluorescent dye (HEX or FAM).

Two loci (OrAB; OrM2) have been isolated using the randomly amplified microsatellites (RAMs) technique as described in detail by Ender *et al*. (1996) and Hadrys *et al*. 2005. Briefly, genomic DNA was subjected to random amplified polymorphic DNA (RAPD)-PCR using 71 random 10-mer primers (Kits A, B, C, F; Operon Technologies). PCRs were performed in a total volume of 25 μL containing 0.5 ng template DNA, 2 mm MgCl2, 5 pmol random primer, 0.35 U *Taq* polymerase (Silverstar), 0.25 mm each dNTP, 1× buffer (Eurogentec). The amplification conditions were 2 min at 90 °C followed by 40 cycles 20 s at 92 °C, 15 s at 38 °C, a ramp of 0.5 °C/s, 15 s at 72 °C followed by 2 min at 72 °C (e.g. Hadrys *et al*. 1992). RAPD profiles were blotted onto positively charged nylon membranes and hybridized overnight to four digoxigenin-labelled oligonucleotides $(GA)_{10}$, $(GT)_{10}$, $(CA)_{10}$ and $(ATT)_{10}$. Twenty-six RAPD fragments of sizes 200–1000 bp with a strong hybridization signal were re-amplified in a second PCR step using the same PCR conditions and primers as before. Re-amplification of 11 fragments either failed or resulted in multiple banding patterns. Consequently they were excluded from further analyses. Cloned directly into the pGEM-T Vector (Promega) were 10–20 ng of each of the 15 remaining amplification products. Plasmids were grown in transformed JM109 *E. coli cells* and sequenced following Ender *et al*. (1996). From the 15 clones sequenced, eight included microsatellite motifs of more than six repeat units length. Primers were designed and fluorescent dye-labelled as described above.

Polymorphism at the overall 15 potential microsatellite loci was assayed in 209 individuals. Amplification for microsatellite typing was carried out in a total reaction volume of 25 μL containing 1× PCR buffer (Invitrogen) and using 0.5 ng DNA as template, 0.5 U *Taq* DNA polymerase (Invitrogen), 2 mm MgCl2, 5 pmol primer, 0.1 mm dNTPs and 1 μg bovine serum albumin (BSA). PCR cycling conditions were as follows: an initial denaturation of 3 min at 93 °C followed by 35 amplification cycles (30 s/92 °C; 35 s/primer-specific annealing temperatures; 30 s/72 °C) and a 5-min final elongation at 72 °C. Microsatellite genotyping was performed using an ABI PRISM 310 automated DNA sequencer. Observed and expected heterozygosities, deviations from Hardy–Weinberg equilibrium (HWE) and test for linkage disequilibrium (LD) was calculated using genepop version 3.4 (Raymond & Rousset 1995).

Table 1 Characteristics of nine microsatellite loci isolated from *Orthetrum coerulescens*. Given are the locus name and GenBank Accession numbers; primer sequence; annealing temperature (T_a); allele sizes in bp; the core motive; number of alleles per locus (N_A); observed heterozygosity (H_o); expected heterozygosity (H_e); the number of individuals analysed (N_{ind}). Significant departure from Hardy-Weinberg equilibrium is indicated by asterisk when $P = 0.05$.

Locus name Access. nos.	**Primer sequences (5'-3')**	***Ta* (C°)**	**$MgCl_2$ (mM)**	**Allele size range (bp)**	**Repeat motiv**	N_A	H_E	H_O	N_{ind}
Ocoe E04	F: FAM-CTGTGAGCCTAGAGGATGGT	57	2,5	220-228	$(TG)_7$	7	0,41	0,37	205
DQ786767	R: CACTAACTTTTTCCCCTGGT								
Ocoe F04	F: HEX-AAAAATTCGAAATGCCGTTA	57	2,5	202-242	$(AG)_3T$	19	0,86	0,77	209
DQ786768	R: CTTGGCGTGACCTCACTAAT				$(GA)_{11}$				
Ocoe G04	F FAM-ACACAATCTGCGTTAGTTCG	54	3,0	245-275	$(CT)_{10}$	16	0,75	0,83	206
DQ786769	R: TTGTCACCGTTTTATTGCAG								
Ocoe H04	F: HEX-TGGTCCTTGAGTTGACCATA	57	3,0	228-238	$(AC)_6$	7	0,65	0,66	206
DQ786770	R: TCCTTCTGGTTGGGGTATTA								
Ocoe J04	F: HEX-TAAAGTGGAGGTGAAGCACA	54	2,5	275-295	$(CT)_8$	11	0,65	0,5	202
DQ786771	R: AAAAGAGTCGACAAAGG								
OrAB	F: HEX-AGCGAGAAGTCGTTCG	52	2,5	151-159	$(CT)_{10}$	7	0,56	0,6	202
DQ786772	R: CGTCATCGTTATATCACCG								
OrM2	F: FAM-TTTTGCCCTTCTCTGC	52	2,0	227-243	$(CA)_7$	14	0,85	0,77	176
DQ786773	R: GGTGAGAGTCCGATAACG								
OcoeA03	F: FAM-AAGAGCGCCAAAGAGAAGTA	57	2.5	206-212	$(TG)_8$	3	0,68	0,69	23
DQ846696	R: GGGTCTCAAATAATTACCATTT								
OcoeK04	F: FAM-CAAAGATAATGATGGTGTGTG	55	2.5	139-147	$(TG)_9$	4	0,73	0,62*	23
DQ846697	R: GGGAATCGATCTCTTGCTTA								

Six out of the 15 loci appeared to be either monomorphic or showed no clear amplification product. Nine loci were polymorphic with the number of alleles per locus ranging from three to 19. Characteristics of the nine microsatellites are shown in Table 1. Expected and observed heterozygosities ranged from 0.41 to 0.86 and from 0.37 to 0.83, respectively. Deviation from HWE was only detected for locus Ocoe K04. No LD was detected after Bonferroni correction for multiple comparisons. Cross-species amplification and genotyping of all loci in 10 more species of the genus *Orthetrum* revealed promising results for six of the nine microsatellites tested (Table 2). In sum, with both isolation protocols used in this study polymorphic microsatellites have been detected. The main difference between the two techniques used is the amount of genomic DNA in the first steps of the protocol. While the enrichment protocol needs a high amount of genomic DNA for construction of the library, the RAMs protocol needs only 5–10 ng per reaction. The microsatellites described here show high levels of variation making them suitable to estimate allelic and genetic diversities among and within populations over years and individuals.

Table 2 Results of cross-species amplification with *Orthetrum coerulescens* microsatellite marker for 10 *Orthetrum* species. Shown are the loci (E04-OrM2) with successful amplification and genotyping results (below listed as allele sizes) in at least one of the tested species.

Species	E04	F04	G04	J04	OrAB	OrM2
O. brachiale	210/222	212	245	-	150/152	229
O. crysostigma	-	214	245	283/285	152	223/231
O. julia	210/222	206/212	249/252	-	-	209
O. caffrum	224	214	243/247	277	153	217/223
O. hintzi	-	208/210	243/245	283/291	152	233
O. ictomeralis cinctifrons	224/227	210	253/255	-	154	235
O. machadoi	222	-	243/247	-	152	-
O. robustrum	222	214	245/247	-	152	-
O. stemale kalai	-	-	243	-	-	-
O. trinacria	-	-	253/255	-	-	-

Acknowledgements

The project was funded by the German Science Foundation (DFGHA 1947 2-3) and HSP-Program of Hessen. We thank Werner Schroth for technical help, Adolfo Cordero for providing samples and Bernd Schierwater for comments. We also thank the staff of the ecological field station “Bahnhof Schapen” for providing specimens.

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Polymorphic microsatellite loci to study population dynamics in a dragonfly, the libellulid *Trithemis arteriosa* (Burmeister, 1839)

Sandra Giere[*] & Heike Hadrys[*,†]

[*]*ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany*

[†]*Yale University, Department of Ecology and Evolutionary Biology, New Haven, CT, 06520-8104, USA*

This is the author's version of a work originally published by Wiley-Blackwell in:
Molecular Ecology Notes (2006) Volume 6, Pages 933–935; available under DOI: 10.1111/j.1471-8286.2006.01405.x

Abstract

One of the most widely distributed dragonfly species in Africa is the red-veined-dropwing *Trithemis arteriosa.* It is an indicator for permanent water bodies, which are freshwater ecosystems of high environmental value especially in arid regions. For studies to determine population structures, assess species viability and monitor environmental changes, a panel of ten polymorphic microsatellite loci was developed. The number of alleles per locus ranged from four to 12, with an observed heterozygosity ranging from 0.149 to 0.843.

Keywords: conservation genetics, dragonflies, microsatellites, Odonata, *Trithemis arteriosa*

Despite the increasing importance of odonates (damselflies and dragonflies) as key taxa for identifying driving factors controlling biodiversity and defining conservation units of freshwater ecosystems, only three microsatellite systems - exclusively for damselflies - have yet been developed. (Fincke & Hadrys 2001, Hadrys *et al*. 2005, Watts *et al*. 2004, Keat *et al*. 2005). We here report on the development of the first microsatellite system for a dragonfly species.

The red-veined-dropwing *Trithemis arteriosa* is one of the most widely distributed dragonfly species in Africa. Its distribution ranges from the semi-arid to tropical and humid regions (Pinhey 1970). It is an indicator species for perennial water bodies like reedy pools, streams or swamps, freshwater resources of high environmental and socioeconomic value. The application of sensitive genetic methods offers the potential of fast detection of environmental changes in these important wetlands areas.

Our objective was to develop a panel of polymorphic microsatellite markers for *T. arteriosa* to study and monitor the genetic diversity within and among populations. Therefore, research sites were chosen along different environmental and geographical gradients across Namibia and Kenya.

Microsatellite loci were isolated using a modified enrichment technique described in Fischer & Bachmann (1998). Genomic DNA was extracted with a phenol-chloroform-extraction protocol (Hadrys *et al*. 1992). DNA was digested with the two restriction enzymes ALU I and RSA I. DNA fragment size ranged from 500 to 1200 bp. The fragments were ligated to two oligo adapters (Oligo A: 5` CTC TTG CTT ACG CGT GGA CTA 3` and Oligo B: 5` TAG TCC ACG CGT AAG CAA GAG CAC A 3` (Edwards *et al*. 1996). Two 3`biotinylated oligo probes [$(GA)_{10}$ and $(GT)_{10}$] were hybridized to the digested DNA. Fragments with the potential repeat motifs were isolated using streptavidin-coated magnetic beads (Promega). The microsatellite enriched eluate was used as a template in a polymerase chain reaction (PCR) with 2.5 pmol of the Oligo A adapter as a primer and containing in a final volume of 50 µl 1x Buffer (Invitrogen), 1.5 mM MgCl, 0.8 mM of each dNTP, 0.5 U Taq DNA Polymerase (Invitrogen). PCR cycling conditions were 94 °C for 5 min, 35 cycles of 94 °C 1 min, 56 °C 1 min, 72 °C 2 min and a final elongation for 5 min. The enrichment process with the magnetic beads and the PCR was repeated. PCR-products were ligated into pCR®II-TOPO® vectors (Invitrogen) and transformed into competent *Escherichia coli* cells (TOP10). Colonies with inserts were amplified using T7 and SP6 primers. A total of 180 positive clones were chosen for PCR amplification. Eighty of the amplification products (size

range from 500 to 1000 bp) were selected for a Southern Blot analyses with the biotin-labelled probes $(GA)_{10}$ and $(GT)_{10}$. Twenty products with a strong hybridization signal were sequenced on a MegaBACE500 using ET Terminator Mix from Amersham. All products contained microsatellite sequences and were used for primer design. Each of the forward-primers was labelled with a fluorescent dye (HEX™ or FAM™) for microsatellite typing. PCR was performed in a 25 µl reaction volume containing 1 µl DNA (5-10 ng genomic DNA), 1 x Buffer (Invitrogen), 2 mM MgCl, 5 pmol of each primer, 0.1 mM of each dNTP and 0.5 U Taq DNA polymerase (Invitrogen). PCR cycling conditions were: 93 °C for 3 min, followed by 35 cycles of 30 s at 93 °C, 20 s primer specific annealing temperatures (Table 1), 40 s 72 °C and a final elongation of 2 min. Automated genotyping was performed on a MegaBACE500 automated sequencer. The ET-550 Size Standard (Amersham) was used to determine the allele sizes. Data analysis was performed using the Genetic Profiler, version 1.2 (Amersham Bioscience). GENEPOP 3.4 (Raymond & Rousset 1995) was used to estimate expected (H_E) and observed (H_O) heterozygosity deviations from Hardy-Weinberg equilibrium and to test for linkage disequilibrium.

A total of 122 individuals of *T. arteriosa* (representing 12 populations in Namibia and Kenya) were genotyped for each locus. Seven out of the 20 loci appeared to be monomorphic, three primer pairs did not amplify any product, but ten loci were polymorphic. Table 1 summarizes the results of the genotyping. All loci show a high genetic variability. The number of alleles per locus ranged from 4 to 12 with an overall number of 90 alleles. The observed heterozygosity ranged from 0.149 to 0.843. Genotype frequencies were tested multiple times for conformance to Hardy-Weinberg expectations and revealed always significant deviations from Hardy-Weinberg equilibrium (HWE) for seven of the loci. However, when population sites were tested separately, only locus TartM04 reveals significant heterozygote deficiencies in the majority of the 12 populations, which is possibly due to null alleles. The other loci displayed heterozygote deficiencies only in one up to three populations. Significant linkage disequilibrium was only detected for the pair TartL04 and TartS04 ($p \leq 0.03$) across all populations and within populations for the pair TartM04 and TartQ04 ($p \leq 0.01$) in one Kenyan population. Although our preliminary analyses revealed a similar high level of allelic and genetic diversity in Kenyan and Namibian populations, 10 out of 12 populations show private alleles suggesting possible processes of genetic drift and/or isolation. In sum, the developed microsatellite system will be useful for a variety of population genetic studies in *Trithemis arteriosa* for monitoring freshwater ecosystems.

Table 1 Characterisation of ten polymorphic microsatellite loci for the libellulid dragonfly *Trithemis arteriosa.* Shown are GenBank Accession nos., locus name, primer sequence, annealing temperature (°C), allele size range (bp), repeat motif, number of alleles per locus (N_A), expected (H_E) and observed (H_O) heterozygosity rates and P-value of the departure from Hardy-Weinberg equilibrium.

GenBank Accession nos.	Locus	Primer (5' – 3')	T_a (°C)	Allele size range (bp)	Repeat	N_A	H_e	H_o	P-value
DQ406677	Tart B04	f-20-mer: HEX-CCGAAAGTCTCTGAGGCAAC r-22-mer: GGAAAAATATCCCTTGCAGTCA	57°C	250-258	$(CA)_3T(CA)_2$ $(CA)_6$	4	0.482	0.730	0.000
DQ406678	Tart C04	f-20-mer: FAM-TTTGCCTCAGAGAATGTTCC r-20-mer: AGGTTTCGCGGATCATTAAA	57°C	218-226	$(CA)_8$	5	0.629	0.879	0.023
DQ406679	Tart I04	f-21-mer: FAM-TTTTCAGGAGGAGGGTTTAAT r-21-mer: CCTAGGATGTAGCGAAACAAA	57°C	155-177	$(CT)_9$	12	0.801	0.545	0.000
DQ406680	Tart L04	f-20-mer: FAM-AGATAGGTGCAGAAGGAACG r-20-mer: TCCAAAGAGGCCATTTACTC	55°C	184-192	$(CT)_8$	6	0.293	0.259	0.243
DQ406681	Tart M04	f-20-mer: HEX-GCCAAATGACCACCTACTTT r-20-mer: CACTTCTTTGGAAAACACGA	55°C	250-272	$(GT)_7(TAA)_5$	8	0.790	0.376	0.000
DQ406682	Tart N04	f-20-mer: FAM-TGATGAACAATGGAAAGGTG r-20-mer: CAAAAGGCGAAAAAGTCTGT	55°C	199-211	$(GT)_7AT(GT)_5$	12	0.731	0.719	0.042
DQ406683	Tart P04	f-19-mer: FAM-AGAAAATCCGGCTGAAAAG r-22-mer: TTTCTTTCATTTCAGGTGAGTG	55°C	284-312	$(AC)_8$	12	0.621	0.533	0.097
DQ406684	Tart Q04	f-20-mer: HEX-CGCTTTCTCTTTCTCTCCTG r-20-mer: AAATCGACCAGAAAGAGTCG	55°C	233-273	$(GT)_8$	12	0.774	0.610	0.000
DQ406685	Tart R04	f-20-mer: FAM-TCCAGAGTTTCGTCATTTCA r-20-mer: ATCGAAACCATGGTCGTTTA	55°C	294-300	$(AT)_3C(AT)_3G$ $(CA)_7$	7	0.245	0.149	0.000
DQ406686	Tart S04	f-20-mer: HEX-TTCATTTCATTGGTGCCATA r-20-mer: GACTCTTCGATGCGAGTGTA	55°C	253-271	$(GT)_8$	12	0.838	0.843	0.052

Acknowledgements

This work was supported by a German Federal Government grant (BMBF, BIOLOG Africa, BIOTA S08). We thank Bernd Schierwater for comments, the staff from the "Ökologische Forschungsstätte Schapen" and V. Clausnitzer and F. Suhling for providing samples.

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Odonates in the desert:

Population genetic structure of a desert inhabiting dragonfly (*Trithemis arteriosa*) suggests male-biased dispersal

Sandra Damm* & Heike Hadrys†

*ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany

†Yale University, Department of Ecology and Evolutionary Biology, New Haven, CT, 06520-8104, USA

This work is prepared for submission to *Molecular Ecology*.

Abstract

Water-dependent species inhabiting desert regions seems to be a contradiction in terms. Nevertheless many species have evolved survival strategies for arid conditions. In Odonates (dragonflies and damselflies), both larvae and adults need very different and complex water associated habitats. The present study investigates the genetic diversity, population structure and dispersal behaviour of a desert inhabiting dragonfly species, the Red-veined Dropwing (*Trithemis arteriosa*). Eight populations from the arid Namibia and four population sites in the more tropical Kenya were analysed using nine microsatellite loci, two non-coding nuclear fragments and the mtDNA fragment ND1. Microsatellite analyses as well as the nuclear fragment reveal a high allelic diversity in all populations and with nearly no genetic sub-structuring. In contrast, ND1 sequence analyses show sub-structuring and exhibits, except of two main haplotypes, only private haplotypes. The conflicting patterns of nuclear markers versus a mitochondrial sequence marker can be explained by a male-biased dispersal in this species. Results indicate that migration of male is dependent on the environmental stability of the habitat, but females are philopatric. This life history trait would allow females to save energy for mating and oviposition, a possible adaptation to the demanding environment of desert regions. Both results give first direct insights into the dispersal behaviour and pathways of a desert inhabiting, strongly water dependent flying insect.

Keywords: dragonflies, desert regions, microsatellites, mtDNA, non-coding nuclear marker, dispersal pathways, sex-biased dispersal

Introduction

Dispersal is one of the key processes allowing for the survival of species in fragmented landscapes and extreme environmental conditions, but also the "decision" to disperse can have far reaching consequences for the fitness of individuals like e.g. founding new populations (Clobert *et al.* 2001). Considering potential benefits as well as the substantial risks associated with dispersal it is highly plausible that dispersal might be depending on actual environmental conditions (Bowler & Benton 2005; Gros *et al.* 2008). Analyzing the population structure of key taxa in extreme environments could therefore help to understand the dispersal strategies by taking into account the stability of habitat situations. We here analyzed the genetic diversities and population structure of a desert-inhabiting dragonfly to investigate the dispersal strategies of a water-associated insect in desert environments.

Desert regions are one of the most challenging environments for living organisms. With no more than 100 - 500 mm precipitation per annum water is the most limited resource in desert and semi-desert regions (Shmida 1985). Despite of these extreme conditions several species have evolved strategies for survival like adaptations for water conservation or heat tolerance (e.g. Ward 2009). Namibia is one of the most arid countries in the world. Most of the landscape is characterised by desert, semi-desert or dried savannah with only three permanent rivers at the borders of the country (Mendelsohn *et al.* 2002). Although water is the most limited resource, episodic rainfall may establish temporary rivers or ponds and in mountainous regions, small springs and streams provide permanent natural water bodies (Curtis *et al.* 1998). Besides the three permanent rivers all other rivers are ephemeral and are dry throughout most of the year (Mendelsohn *et al.* 2002). The only exception are several rare but permanent water ponds along the river course resulting from resurgence of underground water dependent on geology or topology (Martens & Dumont 1983; Jacobsen *et al.* 1995).

Nevertheless, water resources are rare and sometimes separated by large uninhabitable areas. Studies of genetic diversity, population structures and dynamics for desert inhabiting species are limited and mainly focus on mammals or other terrestrial organisms (e.g. Hurtado *et al.* 2004; Lorenzen *et al.* 2008; Sole *et al.* 2008). So far only less is known about the genetic consequences of the limited availability of water bodies for freshwater associated organism living in desert regions.

Odonates (dragonflies and damselflies) are highly dependent on water bodies with a complex life cycle composed of an aquatic larval and a terrestrial imago stage. They are highly mobile insects with the Anisoptera (dragonflies) in particular have the power to fly

over long distances. But the dispersal potential of both dragonflies and damselflies species differs significantly in correlation with specific habitat preferences (Corbet 1999). While some are migratory species and dispersed across whole continents (e.g. *Anax junius* or *Libellula quadrimaculata*), others are dependent on highly specialised habitats (e.g. *Megaloprepus caerulatus* or *Trithemis hartwigi*) (Fincke & Hadrys 2001; Freeland *et al.* 2003; Artiss 2004; Dijkstra 2007; Groeneveld *et al.* 2007).

In arid regions some species groups evolved real desert endemics like in reptiles or mammals (Griffin 1998; Simmons *et al.* 1998), but the majority of the desert-inhabiting odonates are widely distributed across the African continent. They have evolved ecological strategies enabling them to survive under arid conditions (Suhling *et al.* 2003; Johansson & Suhling 2004). Most of them are more or less opportunistic in habitat preferences and a short larval development enables some species to breed also in ephemeral water bodies during the rainy season (Suhling *et al.* 2005; Suhling & Martens 2007). For Namibia 126 of an estimated 850 afrotropical odonate species have been identified with the highest species diversity in the more humid and tropical parts of Namibia in the North (Dijkstra 2003; Suhling *et al.* 2006). Here perennial and running waters allow more tropical species to inhabit the region. In the arid parts of Namibia species diversity is poor and, in contrast to other animal groups, no endemic dragonfly species has been identified up to date.

To investigate the dispersal strategies and genetic effects of dealing with rare water resources as water dependent species in desert regions, the population structure of the Red-veined Dropwing *Trithemis arteriosa* (Burmeister 1839; Libellulidae) was analysed. Its distribution ranges from the semi-arid to tropical and humid regions across the African continent (Pinhey 1970). *T. arteriosa* occurs only at perennial waters with emergent vegetation (Suhling *et al.* 2006) for which it can be regarded as valuable bioindicator species (Clausnitzer 2003). In Namibia population sizes differ widely depending on the stability of the habitat and water resource. As a consequence of the dry climate, Namibia's freshwater systems are particularly threatened by both aridification and the impact of human activities (overuse of water, water pollution, extraction of groundwater for irrigation) (Barnard 1998). Therefore the application of sensitive genetic methods to monitor indicator species may be a powerful tool for rapidly assessing environmental changes in these important wetland areas. Identification of dispersal pathways may further help to identify population sites of high conservation value.

In order to explore the population structures and genetic diversities of *T. arteriosa* in Namibia three different genetic marker systems were used; microsatellites, mtDNA and

nuclear sequence markers. For additional comparative analyses we also include populations from the more tropical Kenya. This way we will gain first insights into the genetic consequences of a strongly water-associated insect inhabiting desert landscapes.

Materials and methods

Study sites and sample collection

Samples of adult *T. arteriosa* individuals (n=129), representing twelve distinct geographical populations in Namibia and Kenya (see Table 1, Figure 1), were collected and stored in 75% ethanol. All sampled individuals are males, because females mostly stay apart from the waterside and are often difficult to identify (Corbet 1999; Suhling & Martens 2007). Due to the species habitat preferences all study sites are permanent water bodies, but abundances of *T. arteriosa* differ as a consequence of type and quality of the habitats. The most northern population site is located in the Baynes Mountains. Here the species established a medium sized population at a natural spring. The sites Palmwag and Ongongo are located in North-West Namibia. These populations were found at small ponds inside a dry riverbed, where *T. arteriosa* was able to establish quite large populations. Waterberg is situated in the Northeast where *T. arteriosa* was found at an artificial stream in a low abundance. The population site Rehoboth is located at the artificial lake Oanob which provides water for the urban area around Rehoboth in South-central of Namibia. Despite of this rather atypical habitat, *T. arteriosa* established a medium-sized population. The population sites Tsauchab and Neuras are both located south of the great central Namibian escarpment. While Tsauchab is again a permanent spring in a dry ephemeral river course with a high abundance of *T. arteriosa*, the Neuras population is influence by human disturbance and only a small number of individuals were found. The most southern population site is located at a natural spring in the dry Fish River bed with again a higher population size (see Figure 1).

For a comparative analysis, four population sites in the more tropical region of Kenya were added to the study. Although Kenya possesses arid regions, it contains many more natural and permanent water resources than Namibia, for example the small, natural Lake Chala in the South of Kenya. The other population sites (Pemba River, Mzima Springs and Nairobi National Park) are permanent rivers and streams with riverine vegetation (Figure 1). Although water stability is higher than in Namibia, these localities are often used as watering

places by mammals such as elephants or hippos and *T. arteriosa* established populations of medium size.

All samples were collected using a non-destructive method (Hadrys *et al.* 1993). The samples were stored at 4°C in ≥70% ethanol for consecutive DNA extraction. Extraction of total genomic DNA was carried out using a modified phenol-chloroform protocol (Hadrys *et al.* 1992) and stored at -20°C.

Table 1 Sampling locations with abbreviations and geographical coordinates as well as number of analysed individuals (n) of the red-veined dropwing, *Trithemis arteriosa* from Namibia and Kenya.

Country	Abbrev.	Locality	Latitude	Longitude	n
Namibia	BayMt	Baynes Mountains	17.231 S	12.805 E	8
	Palm	Palmwag	19.887 S	13.937 E	19
	Ong	Ongongo	19.140 S	13.820 E	10
	Wb	Waterberg	20.483 S	17.235 E	9
	Reho	Rehoboth	23.301 S	17.031 E	11
	Neur	Neuras	24.463 S	16.228 E	11
	Tsau	Tsauchab	24.503 S	16.115 E	16
	FishR	Fishriver	24.498 S	17.863 E	9
Kenya	Pem	Pemba River	04.183 S	39.400 E	12
	Mzi	Mzima Springs	02.967 S	38.017 E	8
	NNP	Nairobi National Park	01.400 S	36.900 E	8
	LCh	Lake Chala	03.317 S	37.700 E	8

Genetic analyses

For genetic analyses four different markers were chosen, the mitochondrial gene ND1 (NADH dehydrogenase subunit 1), ITS I-II (internal transcribed spacer region I and II including the intermediate 5.8S), a non-coding nuclear fragment TartR04 (microsatellite flanking region) and a set of nine microsatellite loci (Giere & Hadrys 2006).

A 610 bp fragment of ND1 was amplified and sequenced according to Rach *et al.* (2008). The ITS I-II region was amplified with primers based on known insect sequences from GenBank. The forward primer (ITS-Odo fw: 5`CGT AGG TGA ACC TGC AGA AG 3`) is located within the 18S rDNA and the reverse primer (ITS-Odo rev: 5`CTC ACC TGC TCT GAG GTC G 3`) within the 28S rDNA region. Amplification was successful under the following conditions: Initial denaturation for 3 min by 95°C, 35 cycles of 95°C for 30 sec, 60°C for 40 sec and 30 sec at 72°C and a final extension at 72°C for 3 min. The final volume of 25 µl contained 1× amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 5 pmol each primer, and 0.75 U *Taq* DNA polymerase (Invitrogen). For amplification of a

301bp fragment of TartR04, primers and PCR regime as described in Giere & Hadrys (2006) were used.

Purified PCR products were sequenced in both directions on an automated sequencer (MegaBACE500; Amersham Bioscience) using the ET Terminator Mix from Amersham Bioscience following the manufacturer's protocol. DNA sequences of both directions were assembled and edited using SeqmanII (version 5.03; DNAStar, Inc). Consensus sequences were aligned using Clustal X version 1.8 (Thompson *et al.* 1997). To reconstruct the gametic phases in heterozygote individuals for the nuclear markers, the Bayesian statistical method implemented in the program PHASE version 2.1 (Stephens *et al.* 2001) was used. Ten independent runs were conducted to infer the best reconstructed haplotypes with a posterior probability greater than 95% as suggested by the authors. Haplotype definition for ND1 and calculations of variable nucleotide positions were performed with Quickalign (Müller & Müller 2003). Sequences of each haplotype are available in GenBank under Accession nos FJ471463-FJ471481 (ND1), XXX (ITS) and XXX (TartR04).

In addition nine microsatellite loci described in Giere & Hadrys (2006) were used for genotyping. Amplified fragments were analysed on a MegaBACE500 (Amersham Bioscience) automated sequencer. Allele sizes were determined using the internal size standard ET-550 (Amersham Bioscience). Data analyses were performed using the Genetic Profiler software (version 1.2; Amersham Bioscience). MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004) was used to test for null alleles and allelic dropout using 1000 Monte Carlo simulations and a Bonferroni corrected 95% confidence interval.

Statistical analyses

Genetic diversity. The genetic variation among mtDNA and nuclear sequences was quantified as haplotype diversity (*h*) and nucleotide diversity (π) and estimated using DNASP version 4.0 (Rozas *et al.* 2003). For the microsatellites single locus statistics including number of alleles, allele frequencies and allelic richness were calculated using FSTAT version 2.9.3.2 (Goudet 2001). Observed (H_O) and expected (H_E) heterozygosities were calculated using GENEPOP version 4.0 (Rousset 2008). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using the Markov chain method implemented in GENEPOP. Associated probability values were corrected for multiple comparisons using a Bonferroni adjustment for a significance level of 0.05 (Rice 1989). The entire dataset and the individual locality were tested for selective neutrality using Tajima`s *D* (Tajima 1989) and Fu`s *Fs* (Fu 1997) using ND1 and nuclear sequences. If Tajima`s *D* and Fu`s *Fs* are found to be

significantly negative, it would suggest the presence of selection or the occurrence of population growth.

Population structure. ARLEQUIN version 3.0 (Excoffier *et al.* 2005) was used for all markers to estimate genetic differentiation between populations (F_{st}) and to conduct exact tests of population differentiation (Raymond & Rousset 1995). Hierarchical structuring of genetic variation was determined using analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) as implemented in ARLEQUIN. AMOVA estimates the amount of genetic variation attributable to genetic differentiation among predefined groups (Φ_{CT} and θ_{CT} for mtDNA and nuclear markers, respectively), among localities within groups (Φ_{SC} and θ_{SC}), and among localities relative to the total sample (Φ_{ST} and θ_{ST}). Analysing the distribution of variation five different groups of localities were compared as described in Table 3.

Statistical parsimony haplotypes networks were constructed for ND1 and nuclear sequences using the 95% parsimony criterion as implemented in the TCS version 1.13 program (Templeton *et al.* 1992; Clement *et al.* 2000). Such genealogical network provides a better representation of gene genealogies at the population level and allows to resolve also relationships at the lower intraspecific level.

For the microsatellites the population structure was estimated with the model-based Bayesian approach implemented in STRUCTURE version 2.1 (Pritchard *et al.* 2000). Ignoring prior population notation, individuals were placed into *K* populations, which were genetic clusters with distinctive allele frequencies. Individuals were assigned probabilistically to populations, with membership coefficients summing to 1 across clusters. To provide the correct estimation of *K*, the ΔK statistic was used (Evanno *et al.* 2005). Runs with values of *K* from one to twelve, corresponding to the numbers of sampled populations, were repeated 20 times. Using the admixture model with correlated frequencies, runs had a burn-in period of 10^5 steps followed by 10^6 Markov chain Monte Carlo replicates.

Mantel test was performed to test for a correlation between geographic and genetic distance and as well as F_{st}-values using the program IBDWS version 2.6 (Rousset 1997; Jensen *et al.* 2005). Default settings were used, including 1000 randomizations.

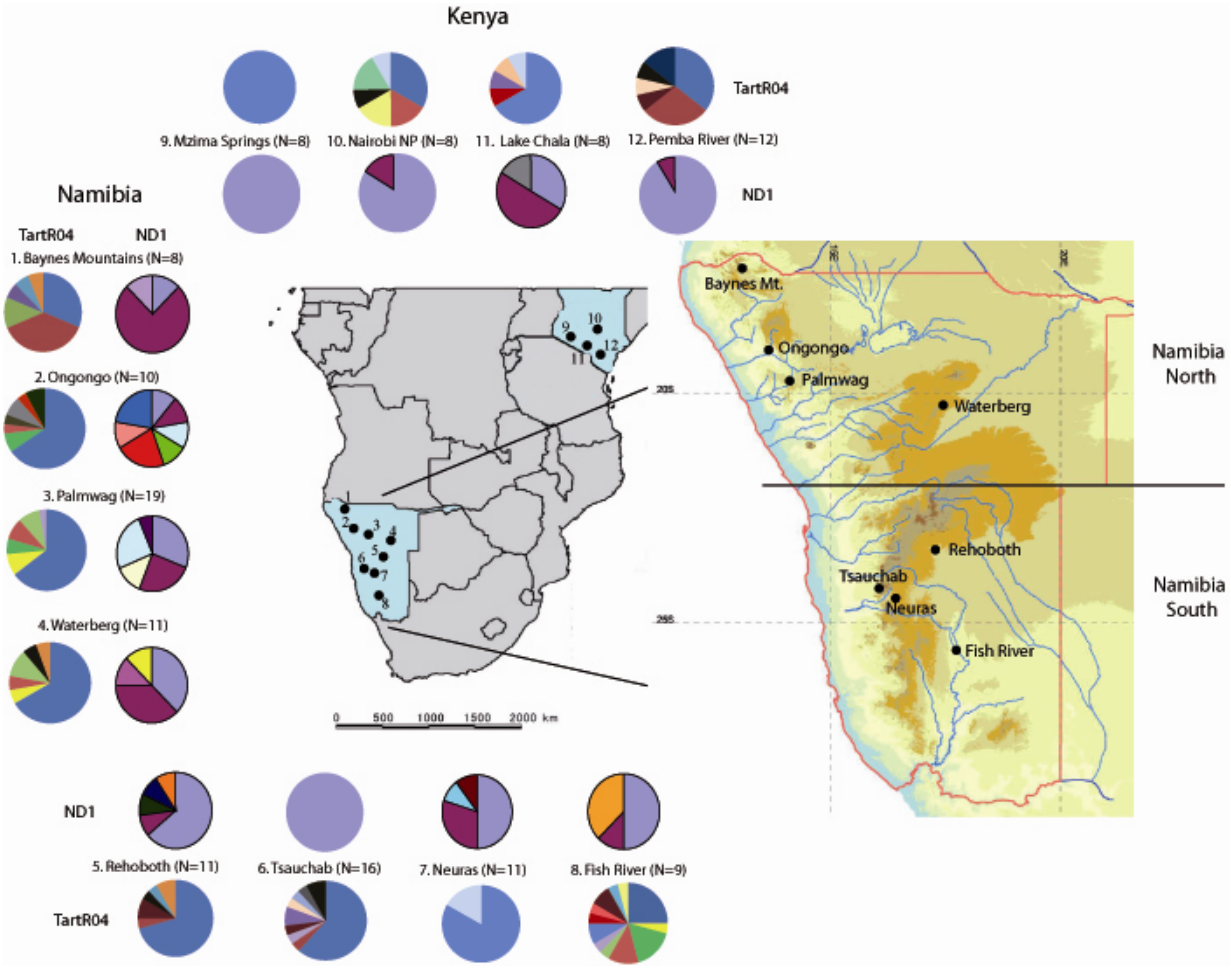

Figure 1 Sampling localities in Namibia and Kenya (left) with a detailed map of Namibia (right) illustrating the ephemeral river catchments and the geological relief. N= numbers of individuals for each population. Pie charts display the haplotypes frequencies of ND1 and TartR04 found for each analysed population of *T. arteriosa*.

Results

Genetic variation

ND1. Sequences of a 481-bp fragment of ND1 were obtained from all 129 individuals. Across the whole data set, 20 variable sites were identified resulting in 19 different haplotypes. No deletions or insertions were observed. Two common haplotypes (ART1 and ART2) were found in 69 % of all individuals. Haplotype ART1 occurred in all and haplotype ART2 in 10 (except of Tsauchab and Mzima Springs) of the analysed populations. The other 16 haplotypes were private for one specific population (see Figure 2a). Nucleotide sequence

diversity (π) ranged from 0 to 0.99% (Table 2). The populations Tsauchab and Mzima Springs exhibit only ART1 and therefore π and haplotype diversity (*h*) are zero (Table 2). The highest π was observed for Ongongo (0.99%) followed by the populations of Waterberg (0.97%) and Nairobi National Park (0.97%). The highest *h* was found in Ongongo (0.94) followed by Palmwag (0.81) and Waterberg (0.79). Both tests for selective neutrality (Fu`s *Fs* and Tajima`s *D*) were not significantly different from 0 in any analysed population suggesting selective neutrality of the observed nucleotide polymorphism. Only population Pemba had a significant negative *D* (-1.94, p=0.009), which might be caused by a recent population expansion.

ITS I-II. This region revealed a 600 bp fragment with only low genetic variability. In total, two positions with gaps and two positions with substitutions were found which occur in more than one sequenced individual. In addition at five positions single substitutions were found. This low variability showed also no indication of geographical correlation. Therefore this marker is not suitable for population genetic analyses in the studied species and was leaved out for further analyses.

TartR04. In contrast to ITS I-II, the 301 bp fragment of the nuclear microsatellite flanking region TartR04 showed 16 polymorphic site and nine gaps. Two gaps are single deletions and the other seven gaps resulted of a seven bp long insertion in five individuals occurring in different population. Using the program PHASE 2.1 (Stephens *et al.* 2001) 29 haplotypes (including gaps and polymorphic sites) could be inferred with a posterior probability of 95%. One haplotype (R04-1) occurred in all populations, followed by a second (R04-4) which was present in nine out of the twelve populations (except of Mzima Springs, Neuras and Lake Chala). 13 haplotypes were shared by at least two populations, while 16 haplotypes were private (Figure 2b). Nucleotide (π) and haplotype diversity (*h*) ranged from 0.1 to 0.89% and 0.29 to 0.91, respectively with the highest value of both π and *h* found for Nairobi National Park (0.89%, 0.86) and Fish River (0.77%; 0.91) (Table 2). Test for selective neutrality revealed significant negative *Fs* values for three populations, Tsauchab (-4.60 $p<0.001$), Waterberg (-2.51 p=0.02) and Lake Chala (-2.47, p=0.006).

Microsatellites. In total 85 alleles were scored for the twelve analysed populations and the number of alleles per locus ranged from four to twelve. Allelic diversity ranged from 3.22 to 5.56 averaged for the nine loci. Allelic richness, which is based on the smallest sample size,

ranged from 3.06 to 4.0 per population and locus (Table 2). The number of alleles found in populations ranged from 29 in Mzima to 50 in Palmwag. The highest number of private alleles was three and occurred in the Namibian populations Tsauchab, Neuras and Rehoboth as well as in the Kenyan population Pemba River. Observed heterozygosities across all loci ranged from 0.48 to 0.65 (Table 2).

Eight of the nine loci showed no evidence for null alleles. For the locus TartM04 the null alleles test observed a significant value in six populations and deviations from the Hardy-Weinberg equilibrium in eight populations ($P<0.01$). Consequently this locus was excluded from further analyses. Furthermore, three populations (Lake Chala, Waterberg and Tsauchab) showed a significant deviation from Hardy-Weinberg equilibrium ($p<0.01$) indicating a heterozygote excess. For all combinations of pairs no significant linkage disequilibrium was found. When linkage disequilibrium was tested for single populations only at Lake Chala, two locus combinations (P04/N04, $P=0.034$ and S04/N04, $P=0.02$) showed a significant value.

Population structure

Two parsimony networks illustrate the genealogical relationships between the haplotypes of ND1 and TartR04 (Figure 3a and b). For ND1 two haplogroups can be defined. Haplogroup I includes the most common haplotype ART1 in its central position and nine other haplotypes are separated from ART1 by only one to three mutation steps. Haplogroup II includes nine haplotypes separated by one to three mutation steps with haplotype ART2 in its central position. Both haplogroups contain population sites from North and South Namibia as well as Kenya.

The haplotype network of TartR04 is dominated by one common haplotype (R04-1) occurring in all populations. 19 further haplotypes are separated from R04-1 by one to two mutation steps. A second haplotype (R04-4) separated by three mutations steps from the most common haplotype was found in nine of the twelve populations and is connected with four further haplotypes separated by one to two mutation steps to R04-4. One group of five haplotypes is separated by at least eight mutations steps from the R04-1. This group contains individuals which have the seven bp insert as described above. Different from ND1 13 haplotypes of TartR04 are shared by at least two populations.

Table 2 Mitochondrial DNA (ND1), nuclear sequence marker (TartR04) and nuclear microsatellite diversity in twelve *T. arteriosa* populations: Number of haplotypes; nucleotide diversity in % (π); haplotype diversity (*h)*; standard deviation (SD); Tajima`s D (*D*); Fu`s Fs (*Fs*); number of alleles (*n)*; number of alleles per locus (A/locus); allelic richness corrected for sample size (AR); observed heterozygosities *(H_o)*; expected heterozygosities *(H_e)*. Significant values are marked with an asterisk.

	ND1					TartR04					Microsatellites				
Locality	Haplotypes Total/ Private	% $\pi \pm$ SD	$h \pm$ SD	*D*	*Fs*	Haplotypes Total/ Private	% $\pi \pm$ SD	$h \pm$ SD	*D*	*Fs*	*n* Total/ Private	A/ locus	AR_c	H_o	H_e
BayMt	3 / 1	0.73 ± 0.3	0.46 ± 0.2	0.04	2.95	6 / 2	0.66 ± 0.1	0.78 ± 0.07			39 / 1	3.89	3.61	0.64	0.66
Palm	5 / 2	0.79 ± 0.13	0.81 ± 0.05	1.45	2.16	6 / 0	0.45 ± 0.08	0.57 ± 0.09	0.86	-0.67	50 / 1	5.56	3.50	0.5	0.59
Ong	7 / 4	0.99 ± 0.21	0.94 ± 0.07	0.38	-1.33	7 / 4	0.53 ± 0.12	0.57 ± 0.1	-0.46	0.11	38 / 2	4.22	3.44	0.49	0.61
Wb	4 / 2	0.97 ± 0.1	0.79 ± 0.11	1.70	2.03	6 / 1	0.33 ± 0.1	0.56 ± 0.13	-0.47	-2.51*	39 / 2	4.33	3.56	0.48*	0.60
Reho	5 / 3	0.47 ± 0.18	0.62 ± 0.16	-0.66	-0.07	6 / 2	0.36 ± 0.1	0.50 ± 0.12	-0.99	0.31	46 / 3	5.11	4.00	0.65	0.74
Neur	4 / 2	0.78 ± 0.17	0.71 ± 0.12	0.75	2.12	2 / 0	0.1 ± 0.04	0.29 ± 0.12	0.02	0.46	40 / 3	4.44	3.54	0.62	0.62
Tsau	1 / 0	0	0	0	0	9 / 2	0.43 ± 0.09	0.62± 0.11	-0.96	-4.60*	46 / 3	5.11	3.77	0.55*	0.65
FishR	3 / 1	0.94 ± 0.15	0.68 ± 0.12	2.27	3.71	12 / 3	0.77 ± 0.07	0.91 ± 0.04	0.16	-2.44	35 / 0	3.89	3.23	0.52	0.61
Pem	2 / 0	0.24 ± 0.19	0.17 ± 0.13	-1.94*	2.76	6 / 1	0.60 ± 0.1	0.81 ± 0.10	0.38	-0.62	48 / 3	5.33	3.84	0.55	0.65
Mzi	1 / 0	0	0	0	0	1 / 0	0	0	0	0	29 / 1	3.22	3.22	0.56	0.55
NNP	2 / 0	0.97 ± 0.45	0.67 ± 0.31	0	2.88	6 / 1	0.89 ± 0.1	0.86 ± 0.07	0.51	0.87	35 / 1	3.89	3.06	0.48	0.67
LCh	3 / 1	0.84 ± 0.24	0.73 ± 0.15	0.94	2.47	5 / 0	0.30 ± 0.1	0.58 ± 0.16	-1.38	-2.47*	39 / 1	4.33	3.69	0.58*	0.66

Table 3 Distribution of genetic variance via hierarchical AMOVA. For nuclear and mitochondrial markers (ND1, TartR04 and microsatellites) five different groupings were tested. Kenya (represented by the populations Pem, Mzi, NNP, LCh), Namibia North (BayMt, Palm, Ong, Wb) and Namibia South (Reho, Neur, Tsau, FishR). For abbreviations see Table 1. Significant *P*- values are displayed with * $P< 0.05$, ** $P< 0.001$ and *** $P< 0.0001$.

	ND1		TartR04		Microsatellites	
Source of variation	Variation	Fixation	Variation	Fixation	Variation	Fixation
Model 1 (without grouping)						
among populations	20.03%	F_{ST}= 0.200***	7.07%	F_{ST}= 0.07***	3.02%	
within populations	79.97%		92.93%		96.98%	F_{ST}= 0.030***
Model 2 (Namibia) (Kenya)						
Among groups	-1.9%	F_{CT} = -0.018	-1.71%	F_{CT} = -0.017	-0.02%	F_{CT} = -0.000
Among populations within groups	21.05%	F_{SC}= 0.205**	7.76%	F_{SC}= 0.076***	3.03%	F_{SC}= 0.030***
Within populations	80.85%	F_{ST}= 0.191**	93.95%	F_{ST}= 0.061***	96.99%	F_{ST}= 0.030***
Model 3 (Namibia North) (Namibia South) (Kenya)						
Among groups	5.87%	F_{CT} = 0.058	-1.88%	F_{CT} = -0.019	-0.09%	F_{CT} = -0.001
Among populations within groups	15.44%	F_{SC}= 0.154	8.44%	F_{SC}= 0.083***	3.08%	F_{SC}= 0.031***
Within populations	78.58%	F_{ST}= 0.213	93.44%	F_{ST}= 0.066***	97.01%	F_{ST}= 0.030***
Model 4 (Namibia South, Kenya) (Namibia North)						
Among groups	12.38%	F_{CT} = 0.123*	-0.77%	F_{CT} = -0.008	-0.1%	F_{CT} = -0.001
Among populations within groups	12.5%	F_{SC}= 0.142***	7.51%	F_{SC}= 0.074***	3.07%	F_{SC}= 0.031***
Within populations	75.12%	F_{ST}= 0.248***	93.27%	F_{ST}= 0.067***	97.03%	F_{ST}= 0.030***
Model 5 (Namibia North, Kenya) (Namibia South)						
Among groups	0.62%	F_{CT} = 0.006	-1.46%	F_{CT} = -0.014	-0.05	F_{CT} = -0.001
Among populations within groups	19.66%	F_{SC}= 0.198***	7.89%	F_{SC}= 0.078***	3.04	F_{SC}= 0.030***
Within populations	79.71%	F_{ST}= 0.203**	93.57%	F_{ST}= 0.064***	97.01	F_{ST}= 0.030***

By means of AMOVA a significant overall Φ_{ST}- and θ_{ST}-value was detected when comparing genetic variation among all populations for all three markers (ND1: 0.200**; TartR04: 0.07*** and microsatellites 0.03***) (Table 3). Hierarchical analysis of AMOVA revealed for all markers the highest variation within rather than among populations for all models tested (ND1: 75.12 to 80.85%; TartR04: 92.93 to 93.57%; microsatellites: 96.98 to 97.03%). The variation among and within populations in the different defined groups as well as within the populations showed nearly no differences for TartR04 and the microsatellites. This resulted in the same level of significant θ_{SC}– and θ_{ST}-values (TartR04: 0.061 to 0.083***; microsatellites: 0.030 to 0.031***) while the θ_{CT}-values are not significant. In contrast to that the variation in ND1 among groups varied between -1.9 to 12.38 % with Model 4 (Kenya & Namibia South / Namibia North) showing also a significant Φ_{CT}-value (0.123, P=0.001) indicating a sub-structuring between these two groups. Here also the Φ_{ST}-value was the highest (0.248, P<0.0001). For the other models the Φ_{ST}- value ranged from 0.191 to 0.213 indicating a substructure within the populations of each group.

Pairwise Φ comparisons (ND1) varied widely. Of the 66 population comparisons, 20 showed significant Φ_{ST}-values after Bonferroni corrections (values ranging from 0.012 to 0.758). While some of the high values might have been caused by low nucleotide diversities (Tsauchab and Mzima Springs) the main significant Φ_{ST}-values were found between northern Namibian populations and Kenya. Pairwise θ_{ST} comparisons for TartR04 showed 25 significant pairwise comparisons out of 66 (values ranging from 0.075 to 0.364). Here two populations (Fish River and Baynes Mountains) showed the majority of the significant θ_{ST}-values to nearly all other populations. For the microsatellites pairwise θ_{ST} comparisons showed 27 significant θ_{ST}-values, which were slightly higher than in the AMOVA analyses (ranging from 0.019 to 0.103). The highest θ_{ST}-value was found between Fish River and Lake Chala with 0.103 (P<0.0001). The most significant values were found between northern and southern populations of Namibia and again between Fish River and the other populations.

According to the found structuring between northern Namibian populations to southern Namibian and Kenyan populations in Φ_{ST} and θ_{ST}, the exact test of population differentiation was analysed (i) for population comparison and (ii) for groups of populations (North Namibia vs. South Nambia vs. Kenya). Microsatellites revealed no significant differentiation for both population and group comparison. ND1 and TartR04 showed the same pattern of population pairwise differentiation as revealed by Φ_{ST}- and θ_{ST}-values. Testing the differentiation of the three predefined groups, both markers showed a significant differentiation between North Namibia and South Namibia as well as Kenya while the latter were not significant different.

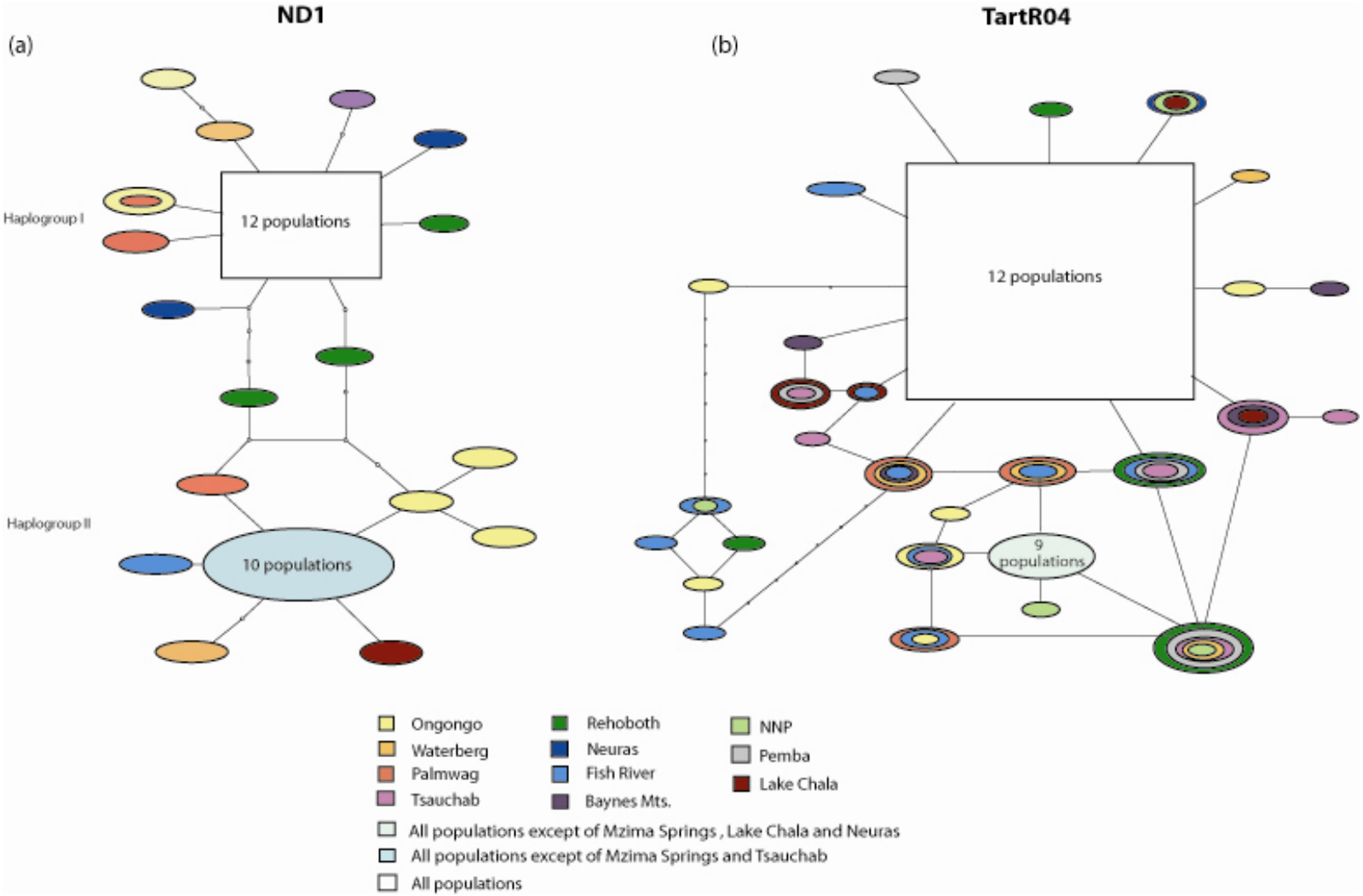

Figure 2 Mutational haplotype networks of (a) ND1 and (b) TartR04 sequences based on statistical parsimony. Shown are the genealogical relationships between the haplotypes in twelve populations of *T. arteriosa.* Haplotypes considered to be ancestral are depicted as rectangles, all other haplotypes as circles. Missing mutational steps connecting haplotypes are represented by small non-coloured dots. Haplotypes connected by a single line differ in one mutational step. The size of the rectangle and circles correlates with haplotype frequency within each network. The different colours represent different populations.

The model-based clustering method implemented in STRUCTURE (Pritchard *et al.* 2000), which assigns all individuals to *K* clusters without predefined populations was run for (i) all populations separately and (ii) for five predefined groups according to exact population differentiation results of ND1 and TartR04 (North Namibia, South Namibia, Kenya and the two highly differentiated populations Fish River and Baynes Mountains). This was done to allow higher sample sizes for each geographical region. For both approaches *K*=3 produced the highest value of Δ*K*. Nevertheless, a high degree of overlap among individuals from different populations and regions were found indicating high gene flow between the populations.

Mantel tests for the three marker showed no significant correlation between geographic and genetic distances (ND1: r= -0.0897, one-sided p= 0.7410; TartR04: r= 0.0470, one-sided p= 0.6510; microsatellites: Nei`s distances: r= 0.1274, P= 0.1880; θ_{ST}: r=0.0142, P=0.4440).

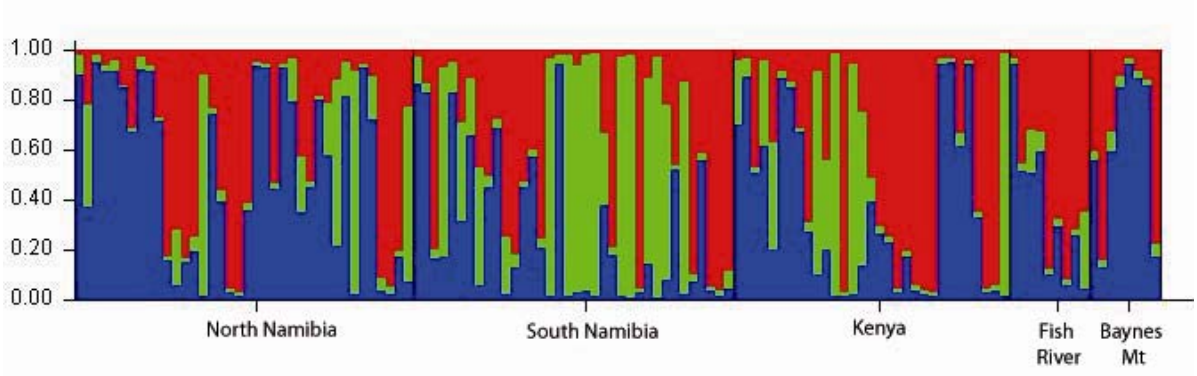

Figure 3 Bayesian analysis of the nuclear genetic structure of *T. arteriosa* populations based on eight microsatellite loci. Each vertical bar represents an individual and is partitioned into one to three coloured segments indicating the individual membership in the three genetic groups found by STRUCTURE. Regional origin of the individuals is indicated by regarding the two populations Fish River and Baynes Mountains separately according to their high θ_{ST}-values to the other populations.

Discussion

A basic requirement to understand the evolution of life history traits and patterns of biodiversity, both within and among ecosystems, is to follow population structures and dispersal strategies of selected key taxa. Estimates of gene flow and genetic diversity are therefore a *sine qua non*. The application of the two nuclear markers revealed highly similar results of the population structure of the desert inhabiting dragonfly *T. arteriosa* and indicates high levels of gene flow between populations. In contrast to that the mtDNA marker ND1 showed nearly exclusive private haplotypes in each population indicating reduced gene flow between populations.

Genetic diversity

The ribosomal ITS I-II was successfully used in population genetic studies before (e.g. Gomez-Zurita & Vogler 2003; Bower *et al.* 2009), but for the dragonfly species analysed here only low genetic diversity was found. Nevertheless, the other three marker systems showed a high genetic diversity within *T. arteriosa*. With up to seven (ND1) and twelve (TartR04) haplotypes and fifty alleles, high nucleotide and haplotype diversities were found in nearly all populations. Interestingly, in ND1 the highest sequence diversities were found in the northern Namibian populations (Palmwag, Ongongo and Waterberg), while in the microsatellites and TartR04 no pronounced difference could be observed. The number of ND1 haplotypes was lowest in the Kenyan populations, in which for TartR04 only two private haplotypes were found. The high mtDNA and nuclear diversities in the northern part of Namibia lead to the assumption that these populations have been in Hardy-Weinberg equilibrium (HWE) for a long time period. In contrast, lower mtDNA diversities in the southern populations of Namibia, but the comparably same amount of TartR04 haplotypes and the high number of private alleles suggest a past population decrease as a cause of more instable habitat conditions.

The Namibian Tsauchab population exhibits only one mtDNA haplotype although the number of analysed individuals was high. In contrast, a high number of microsatellite alleles (n = 46) and TartR04 haplotypes (nine including two private) was found. The loss of mtDNA diversity might be a response to climatic fluctuations resulting in a desiccation of water resources and a repeated decline in population size at this population site. This is also supported by a significant Fu`s *Fs* value (-4.60, $p<0.001$) in TartR04 indicating a recent bottleneck or population expansion (Tajima 1989; Fu 1997). The population Mzima in Kenya

exhibited in all three markers a low genetic diversity which might have been caused by a recent population decline. The two populations Fish River (most southern) and Baynes Mountains (most northern) have a high TartR04 haplotype diversity and show different haplotype frequencies (ND1 and TartR04) in comparison to the other populations. This might be caused through additional genetic input from populations of the adjacent countries South Africa and Angola.

When comparing genetic diversities of *T. arteriosa* in the dry country Namibia and the more tropical Kenya, lower diversities in the more demanding habitats of Namibia with isolation and reduced water resources are expected. Interestingly, our study revealed rather the opposite. The populations in Namibia have in comparison to Kenya a higher number of private haplotypes or alleles in all three markers. The four Kenyan populations exhibit only the two most common haplotypes in ND1 and most of the haplotypes of TartR04 are shared with the southern Namibian populations. This could be caused by several reasons. Both Lake Chala (in TartR04) and Pemba (in ND1) showed a significant negative Tajima *D* or Fu`s *Fs*. A population decline might therefore reduce the genetic diversities and most common haplotypes are favoured. Due to the more stable habitats the Kenyan populations might be more influenced by a higher amount of predators for the larvae (e.g. fish, frogs), interspecific competition, mammals or human habitat disturbance which resulted in smaller population sizes. In Namibia the two populations Neuras and Waterberg with the most human influence have also only a small population size which indeed resulted in lower genetic diversities.

Population differentiation

Results of population structure analyses revealed different patterns when comparing mtDNA and nuclear markers. Nuclear markers showed nearly no population substructure which suggest a high level of gene flow between the analysed populations. With geographical distances of up to 2600 km (south Namibia – Kenya) the dispersal ability of *T. arteriosa* seems to be very high. Only the two populations Baynes Mountains and Fish River showed a higher differentiation to the other populations indicated by pairwise θ_{ST}–values. AMOVA analyses of TartR04 and the microsatellites revealed that the great majority of variability (around 95%) was found within populations. The TCS-network of TartR04 and the STRUCTURE analyses in the microsatellites showed no geographical correlation of haplotypes or allele frequencies and nearly half of the TartR04 haplotypes are shared by at least two populations (see Figure 3b). In contrast, ND1 exhibited only private haplotypes (except of the two main haplotypes) and a population substructure between North and South Namibia was

found in AMOVA, pairwise Φ_{ST} and the exact test of population differentiation (see Table 3). But the majority of ND1 variability was found within populations due to the high amount of private haplotypes.

The restricted gene flow between the North and the South of Namibia can be explained by some remarkable geographic structures. While north-west Namibia is more or less plain, central Namibia consists of a plateau with a height ranging from 900 to 1300 m above sea level. Here also some of Namibia's mountains are situated with altitudes up to 2000 m. These highlands are potential barriers for flying insects, even if they are excellent flyers like dragonflies. Here the populations Tsauchab and Neuras are situated (within Naukluft Mountains). Rehoboth and Fish River are also situated south of the main central escarpment. The partly great canyon of the ephemeral Fish River has, in contrast to the other river catchments, a north-south direction and originates at the southern Namibian border at Orange River. Migration might therefore be southwards along the Fish River Canyon in the direction of South Africa. While the populations Ongongo and Palmwag are stable, the southern Namibian population are more effected by drought through periodical absence of rain in the rainy season (Mendelsohn *et al.* 2002).

Interestingly the Kenyan populations are rather more similar to the southern Namibian populations than to the northern supported by both ND1 and TartR04. The migration of *T. arteriosa* from Kenya to Namibia might follow the coastline of southern Africa with the coastal wind and enter Namibia from South Africa. The northern Namibian populations have genetic exchange rather with the populations from Angola and Zambia. Individuals of populations inbetween Namibia and Kenya as well as of South Africa had to be included to answer this question more clearly, but preliminary analyses of other species (*Orthetrum crysostigma* and *Orthetrum julia*, unpublished data) revealed the same picture.

Contrasting patterns via sex-biased dispersal?

Although comparisons of mtDNA, nuclear sequence markers and microsatellites are complicated because of their different characteristics (allelic variation at specific loci versus mtDNA sequence variation) similar patterns of genetic differentiation are expected if gender-based dispersal can be excluded (Bos *et al.* 2008; Lukoschek *et al.* 2008). In *T. arteriosa*, mtDNA revealed, except of the two main haplotypes, only private haplotypes in each locality. In contrast, microsatellites alleles and nuclear haplotypes were shared between populations of all analysed regions indicating no genetic differentiation. Because microsatellite analyses require high sample sizes to assure that the genetic diversity of a population is covered (e.g.

Waples 1998), the non-coding nuclear sequence marker TartR04 was included which confirmed microsatellite results.

One reason for the different population structure could be the fourfold-reduced effective population size of the only maternal inherited mtDNA in comparison to the diploid/ bi-parentally inherited nuclear markers (Birky *et al.* 1989). Thus, theoretically mtDNA may show higher levels of differentiation at a mutation-drift equilibrium compared to microsatellites, although mutation rates for microsatellites are higher. But due to the random mating assumption this generalisation is also discussed to be incomplete in natural populations (Chesser & Baker 1996). The existence of a high number of mtDNA haplotypes in *T. arteriosa* in general would suggest that at least some of these haplotypes are shared with other populations in the context of the high gene flow revealed by nuclear markers.

Therefore a second, highly promising explanation for the incongruence of mtDNA and nuclear data is sex-biased dispersal behaviour. Male-biased dispersal could homogenize allele frequencies among populations at biparentally (nuclear), but not maternally (mitochondrial) inherited genetic markers (e.g. Prugnolle & de Meeus 2002). Therefore, sex-specific dispersal can lead to incongruent results of analyses on population structures when comparing nuclear with mitochondrial markers. Male-biased based dispersal is well studied in different vertebrate species like e.g. mammals (e.g. Mesa *et al.* 2000), birds (e.g. Gibbs *et al.* 2000; Dallimer *et al.* 2002), and fishes (e.g. Cano *et al.* 2008). Three main categories of differential migration between sexes could be classified: (i) the resource competition hypothesis (Greenwood 1980), (ii) the local mate competition hypothesis (Perrin & Mazalov 2000) and (iii) the inbreeding avoidance hypothesis (Pusey 1987; Perrin & Mazalov 2000).

In dragonflies it is well known that in the majority of species females stay away from the waterside and arrive only for mating and oviposition, while male dragonflies compete for mating opportunities at the water (e.g. Corbet 1999; Suhling & Martens 2007). Competition in large populations with spatial limitations leads to evasion to new water resources and therefore dispersal (Perrin & Mazalov 2000). Also the costs for dispersal might differ in genders resulting in the dispersal of only one sex (Gros *et al.* 2008). For females, staying at the breeding sides and saving energy for mating and oviposition is of special importance when one regards their exhausting habitat conditions in an arid region such as Namibia. Such a mating system, where males disperse to search for new territories and mating partners and females are philopatric, has many advantages under challenging habitat conditions. This is also described in some well studies desert-inhabiting fruit flies (Markow & Castrezana 2000). Sex-biased dispersal is therefore a promising explanation for the different dispersal patterns in

T. arteriosa. Because of their high mobility, dispersal patterns in dragonflies are difficult to assess and without genetic information often impossible (Holland *et al.* 2006). To date, only one study has addressed sex-biased dispersal in odonates by comparing different damselfly species based on the capture-mark-recapture (CMR) method (Beirinckx *et al.* 2006). However CMR in general has many limitations and for migration estimates over long distances it is unfeasible.

Nevertheless, migration rates of males in *T. arteriosa* seem to be correlated with the environmental situation of the habitat at the specific localities. While smaller population sites (Neuras, Waterberg, Mzima Springs) exhibit a lower genetic diversity and share most of their nuclear haplotypes with other populations, the populations with a long-term stable history have a higher genetic diversity and a higher amount of private nuclear haplotypes and alleles (Ongongo, Fish River, Baynes Mountains, Rehoboth). Therefore a decrease of food and/or mating resources might have led to dispersal, which in fact is male-biased facultative migration. This picture is best seen in the Tsauchab population where only the most common ND1 haplotype was found. Here recurrent drying of the water resource leads to a nearly complete migration of the males. While females do not migrate and stay at their breeding sites, the maternal inherited haplotypes in ND1 stay private for the specific locality and in founder event this resulted in the occurrence of a low amount of maternal lineages as shown for the Tsauchab population.

Conservation implications

One major problem in population genetic studies is the availability of enough samples to correctly evaluate population structures. This is especially true for endangered species or species in extreme environmental situations where sometimes only small and/or isolated populations could be established. But for conservation management analysing patterns of dispersal and genetic diversities are of high importance particularly in these species groups. The application of mtDNA and microsatellites in population genetic studies have proved to be powerful (e.g. Avise *et al.* 1987; Goldstein & Schlötterer 1999). But especially microsatellite analyses are highly dependent on the number of analysed individuals of a given population. In our study the use of a third marker system, a non-coding nuclear sequence marker, resulted in congruent patterns to the microsatellites and was able to verify the preliminary results (Zhang & Hewitt 2003). While the ribosomal ITS I-II reveals only little intraspecific variation, a microsatellites flanking region might be very promising marker in population genetic studies. Although sample sizes are small in some populations due to low species abundance, the

observed patterns in genetic diversity were rather correlated with the stability of water resources than with sample size (Tsauchab, Pemba River, Ongongo). Also merging populations of geographical regions did not change the overall picture. So using non-coding nuclear region as a complement to mtDNA or/and microsatellites might allow to reconstruct population genetic structure also in smaller sample sizes.

By applying the three different marker systems we could show that a desert-inhabiting species dependent on perennial waters is able to establish viable populations with high genetic diversities despite of their isolated situation. The Namibian environment requires populations to deal with heat and rare, mostly ephemeral water resources. In the desert, dispersal ability is of high importance as populations are always at risk of a spatial or total desiccation of water resources either by human impact or natural causes. While some species are obligatory migrants, others may disperse for foraging, reproduction or seasonally induced reasons. For conservation management, knowledge about the dispersal behaviour and pathways of a species is of great importance. In *T. arteriosa*, a key species for permanent water bodies, genetic analyses indicate a male-biased dispersal which seems to be dependent on the stability of the habitat. While for females philopatry seems to be a fitness-advantage, males are forced to migrate in times of drought or habitat disturbance to search for other suitable habitats. Regarding the differences of genetic variability in species with sex-biased dispersal including both mtDNA and nuclear markers is important for conservation genetic studies. While nuclear markers might show a high genetic diversity the maternal lineage could be impoverished (like shown for Tsauchab and the Kenyan populations).

Overall the results provide crucial information about dragonflies in the desert. The combined analyses of two different nuclear markers with mtDNA revealed a larger-scale picture of population dynamics in *T. arteriosa* by not only identifying high gene flow between populations but also environmental dependent sex biased dispersal.

Acknowledgements

The work was supported by the program BIOTA South (S08) of the German Federal Ministry of Education and Research (BMBF). We are grateful to Frank Suhling and Viola Clausnitzer for providing us specimens. We thank Eugene Marais (National Museum of Namibia) for his support and all collaborators for helping collect samples during our stay in Namibia.

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Supplementary data

Table S1 Haplotype frequencies from the 19 ND1 haplotypes found in twelve analysed populations of *T. arteriosa* in Namibia and Kenya.

	BayMt	**Palm**	**Ong**	**Wb**	**Reho**	**Tsau**	**Neur**	**FishR**	**LCh**	**NNP**	**Mzi**	**Pem**
ART 1	0.125	0.312	0.111	0.375	0.636	1	0.5	0.5	0.333	0.833	1	0.917
ART 2	0.75	0.25	0.111	0.375	0.091	-	0.3	0.125	0.5	0.167	-	0.083
ART 3	-	0.125	-	-	-	-	-	-	-	-	-	-
ART 4	-	0.25	0.111	-	-	-	-	-	-	-	-	-
ART 5	-	0.062	-	-	-	-	-	-	-	-	-	-
ART 6	-	-	-	0.125	-	-	-	-	-	-	-	-
ART 7	-	-	-	0.125	-	-	-	-	-	-	-	-
ART 8	0.125	-	-	-	-	-	-	-	-	-	-	-
ART 9	-	-	0.111	-	-	-	-	-	-	-	-	-
ART 10	-	-	0.222	-	-	-	-	-	-	-	-	-
ART 11	-	-	0.111	-	-	-	-	-	-	-	-	-
ART 12	-	-	0.222	-	-	-	-	-	-	-	-	-
ART 13	-	-	-	-	-	-	-	0.375	-	-	-	-
ART 14	-	-	-	-	0.091	-	-	-	-	-	-	-
ART 15	-	-	-	-	0.091	-	-	-	-	-	-	-
ART 16	-	-	-	-	0.091	-	-	-	-	-	-	-
ART 17	-	-	-	-	-	-	0.1	-	-	-	-	-
ART 18	-	-	-	-	-	-	0.1	-	-	-	-	-
ART 19	-	-	-	-	-	-	-	-	0.167	-	-	-

Table S2 Haplotype frequencies from the 29 TartR04 haplotypes found in twelve analysed populations of *T. arteriosa* in Namibia and Kenya.

	BayMt	**Palm**	**Ong**	**Wb**	**Reho**	**Neur**	**Tsau**	**FishR**	**Pem**	**Mzi**	**NNP**	**LCh**
R04_1	0.312	0.647	0.654	0.667	0.708	0.833	0.615	0.250	0.357	1.000	0.333	0.667
R04_2	-	0.088	-	0.056	-	-	-	0.042	-	-	-	-
R04_3	-	0.059	0.077	-	-	-	-	0.167	-	-	-	-
R04_4	0.375	0.088	0.039	0.056	0.042	-	0.039	0.125	0.286	-	0.167	-
R04_5	0.125	0.088	-	0.111	-	-	-	0.042	-	-	-	-
R04_6	-	0.029	-	-	-	-	0.039	0.042	-	-	-	-
R04_7	-	-	0.039	-	-	-	-	-	-	-	-	-
R04_8	-	-	0.077	-	-	-	-	-	-	-	-	-
R04_9	-	-	0.039	-	-	-	-	-	-	-	-	-
R04_10	-	-	0.077	-	-	-	-	-	-	-	-	-
R04_11	-	-	-	-	-	-	-	0.083	-	-	-	-
R04_12	-	-	-	-	-	-	-	0.042	-	-	-	0.083
R04_13	-	-	-	-	-	-	-	0.042	-	-	-	-
R04_14	-	-	-	-	0.083	-	0.039	0.083	0.071	-	-	-
R04_15	-	-	-	-	-	-	-	0.042	-	-	-	-
R04_16	-	-	-	-	-	-	-	0.042	-	-	0.167	-
R04_17	0.063	-	-	-	-	-	0.077	-	-	-	-	0.083
R04_18	-	-	-	-	-	-	0.039	-	0.071	-	-	0.083
R04_19	-	-	-	-	-	-	0.039	-	-	-	-	-
R04_20	-	-	-	-	-	-	0.039	-	-	-	-	-
R04_21	-	-	-	0.056	0.042	-	0.077	-	0.071	-	0.083	-
R04_22	-	-	-	0.056	-	-	-	-	-	-	-	-
R04_23	-	-	-	-	-	-	-	-	0.143	-	-	-
R04_24	-	-	-	-	-	-	-	-	-	-	0.167	-
R04_25	-	-	-	-	-	0.167	-	-	-	-	0.083	0.083
R04_26	0.063	-	-	-	-	-	-	-	-	-	-	-
R04_27	0.063	-	-	-	-	-	-	-	-	-	-	-
R04_28	-	-	-	-	0.042	-	-	-	-	-	-	-
R04_29	-	-	-	-	0.083	-	-	-	-	-	-	-

Table S3 Pairwise population F_{st}-values for (a) ND1 sequences, (b) TartR04 and (c) eight microsatellite loci. Significant F_{st}-values based on 10000 permutations are displayed in bold.

		BayMt	Palm	Ong	Wb	Reho	Neur	Tsau	FishR	Pem	Mzi	NNP	LCh
(a)	BayMt	*											
	Palm	0,239	*										
	Ong	0,041	0,125	*									
	Wb	0,008	**0,012**	-0,012	*								
	Reho	**0,432**	-0,005	**0,290**	0,139	*							
	Neur	0,212	-0,056	0,115	-0,029	-0,027	*						
	Tsau	**0,758**	**0,225**	**0,598**	**0,504**	0,131	**0,277**	*					
	FishR	0,024	0,028	0,021	-0,085	0,156	-0,012	**0,522**	*				
	Pem	**0,584**	0,077	**0,423**	0,289	-0,040	0,075	0,013	0,307	*			
	Mzi	**0,617**	0,100	**0,427**	**0,311**	-0,004	0,114	0,000	**0,330**	-0,093	*		
	NNP	0,395	-0,056	**0,232**	0,079	-0,130	-0,084	0,153	0,098	-0,103	-0,034	*	
	LCh	-0,133	0,134	-0,022	-0,085	**0,331**	0,097	**0,726**	-0,067	**0,509**	**0,546**	0,280	*

		BayMt	Palm	Ong	Wb	Reho	Neur	Tsau	FishR	Pem	Mzi	NNP	LCh
(b)	BayMt	*											
	Palm	**0,112**	*										
	Ong	**0,140**	-0,004	*									
	Wb	**0,113**	-0,032	-0,009	*								
	Reho	**0,169**	0,004	-0,002	-0,014	*							
	Neur	**0,279**	0,050	0,041	0,037	0,000	*						
	Tsau	**0,112**	0,000	-0,004	-0,018	-0,012	0,048	*					
	FishR	0,027	**0,093**	**0,099**	**0,099**	**0,133**	**0,225**	**0,086**	*				
	Pem	-0,022	**0,082**	**0,093**	0,078	**0,108**	**0,228**	0,058	0,009	*			
	Mzi	**0,364**	0,095	0,091	0,097	0,069	0,063	**0,103**	**0,293**	**0,318**	*		
	NNP	0,013	**0,086**	**0,089**	**0,075**	0,114	0,210	0,059	0,004	-0,013	**0,324**	*	
	LCh	0,131	-0,008	-0,017	-0,024	-0,016	-0,001	-0,036	**0,093**	0,079	0,113	0,066	*

		BayMt	Palm	Ong	Wb	Reho	Neur	Tsau	FishR	Pem	Mzi	NNP	LCh
(c)	BayMt	*											
	Palm	**0,029**	*										
	Ong	**0,038**	0,029	*									
	Wb	**0,039**	0,030	0,051	*								
	Reho	-0,003	**0,050**	0,032	0,019	*							
	Neur	0,020	**0,029**	**0,032**	**0,029**	0,020	*						
	Tsau	0,027	**0,019**	**0,035**	-0,008	0,019	0,022	*					
	FishR	**0,049**	**0,061**	0,019	**0,064**	**0,054**	**0,052**	0,023	*				
	Pem	0,016	0,020	**0,038**	-0,001	-0,006	-0,006	-0,001	**0,044**	*			
	Mzi	0,028	0,047	**0,049**	0,030	0,001	0,001	0,021	**0,077**	-0,012	*		
	NNP	0,037	0,020	0,052	0,021	**0,049**	**0,049**	-0,017	0,030	0,012	0,036	*	
	LCh	**0,046**	**0,038**	**0,054**	0,031	0,028	0,028	**0,047**	**0,103**	0,029	**0,070**	**0,055**	*

An integrative approach to species discovery: from character-based DNA barcoding to ecology

Sandra Damm[1], Bernd Schierwater[1,2] & Heike Hadrys[1,3]

[1]*ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, 30559 Hannover, Germany*

[2]*Division of Invertebrate Zoology, American Museum of Natural History, New York, NY 10024, USA*

[3]*Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, 06520-8104, USA*

This work is submitted to *Molecular Ecology*.

Abstract

Modern taxonomy requires an analytical approach incorporating all lines of evidence into decision-making. Such an approach can enhance both, species identification and species discovery. The character-based DNA barcode method provides a molecular dataset that can be incorporated into classical taxonomic datasets. This way the discovery and delineation of a new species can include not only a descriptive organismal but also an analytical molecular taxonomical framework. We here illustrate such a corroborative framework in a dragonfly model system to unravel the existence of two new, but visually cryptic species.

In the African dragonfly genus *Trithemis* three highly distinct genetic clusters can be detected which cannot be identified by using classical taxonomic characters. In order to test the hypothesis of two new species, DNA-barcodes from different sequence markers (ND1 and COI) were combined with morphological, ecological and biogeographic datasets. Phylogenetic analyses and incorporation of all datasets into a scheme called taxonomic circle highly supports the species discovery hypothesis of two new species.

According to this case study we suggest that an analytical approach to modern taxonomy which integrates datasets from different disciplines will increase the ease and reliability of both species discovery and species assignment.

Keywords: character-based barcoding; Odonata; new (cryptic) species; taxonomic circle; integrative approach; conservation genetics

Introduction

"It is clear to us that genomic information should be an active component of modern taxonomy, but DNA should not be the sole source of information retrieval" (DeSalle *et al.* 2005). The use of DNA sequence data in taxonomy dates back almost three decades ago (e.g. Fox *et al.* 1980; Paquin & Hedin 2004; Cardoso & Vogler 2005). It is widely accepted that a species identification system based on DNA sequences can be a rapid, reliable and consistent method, which is especially important for crisis disciplines like conservation biology and biodiversity research (Vogler & DeSalle 1994; DeSalle & Birstein 1996; Goldstein & DeSalle 2000; DeSalle *et al.* 2005; DeSalle 2006; Vogler & Monaghan 2007). The recent introduction of DNA barcoding, as a fast identification method for assessing biodiversity of known species, has created excitement about a new, powerful tool for taxonomy (e.g. Hebert & Gregory 2005; Vences *et al.* 2005; Clare *et al.* 2007; Pfenninger *et al.* 2007). However, problems arise when new, unidentified species are discovered, in other words, when specimens come from the major part of biodiversity that has not been described yet (DeSalle 2006; Rubinoff 2006). DNA barcoding studies have mainly been focusing on distance-based methods to identify and delimitate species (e.g. Hebert *et al.* 2003). This however can proof difficult for various reasons. For example, substitution rates of mtDNA vary between different groups of species resulting in a broad overlap of intra- and interspecific distances (Will & Rubinoff 2004; Hickerson *et al.* 2006). Consequently Hebert *et al.* (2004b) proposed a threshold of 3% mtDNA distances and the 10x rule to delimitate species. Such thresholds may work for some animal groups but not for all, resulting in the discovery of a number of equivocal cryptic species and more criticism about DNA barcoding in species discovery (Hebert *et al.* 2004a; Lefebure *et al.* 2006).

As a fruitful site effect of this discussion a hot debate arouse about the importance of defining and outlining new ways to modernize taxonomy (Savolainen *et al.* 2005; Rubinoff *et al.* 2006; Vogler & Monaghan 2007; Cardoso *et al.* 2009). Researchers agree that ideally in modern taxonomy all disciplines should interact in species discovery and it should be possible to use the different data sets to test, corroborate, refine and revise species delimitation via a feedback loop (Vogler & Monaghan 2007) or a taxonomic circle (DeSalle *et al.* 2005). This however proves difficult when genetic distances are combined with taxonomy.

A recently applied new technique, the character-based DNA barcode method, characterizes species through a unique combination of diagnostic characters rather than genetic distances (DeSalle *et al.* 2005; Rach *et al.* 2008). This way species boundaries can be

defined by a diagnostic set of characters which can be increased to any level of resolution by applying multiple genes (Rach *et al.* 2008). Another advantage of character-based barcoding is the fact that DNA characters can be combined with characters from other disciplines, e.g. ecology, morphology, geography and behaviour which allows to establish a comprehensive database to test new species hypotheses based on an analytical rather than descriptive approach.

An analytical discovery process is especially important when traditional taxonomy fails to identify a species but genetic evidence is obvious, i.e. in the discovery of "cryptic species". The taxonomic circle introduced by DeSalle *et al.* (2005) describes a way in which different datasets can interact to discover new species. In this scheme a genetically, morphologically or geographically discovered entity can only be raised to species status when at least two disciplines support the species discovery hypothesis. The advantage of this corroborative approach is the reliability on at least two different datasets of qualitatively different characters. Although this scheme displays the evolutionary process in a highly oversimplified way, it demonstrates that species discovery could be based on the biological and evolutionary species concepts.

In a case study on odonates (dragonflies and damselflies) we apply the scheme of a taxonomic circle to prove the discovery of the first two "cryptic" species. Odonates are highly mobile organisms and their complex life cycle - aquatic larval stages and terrestrial adults - and species-specific habitat requirements make them excellent indicators for assessing biodiversity and wetland health (Corbet 1999; Stoks *et al.* 2005; Hadrys *et al.* 2006; Groeneveld *et al.* 2007). Their complex reproductive system and behaviour is unique in the animal kingdom and has made them model organisms for a variety of evolutionary studies (Waage 1979; Hadrys *et al.* 1993; Hadrys *et al.* 2005; Turgeon *et al.* 2005; Cordoba-Aguilar 2008). Despite the lead of odonate research in the insect orders, the expected head start for integrating genetic tools into modern conservation and taxonomical research did not occur. The specificities that make odonates particularly valuable for biodiversity assessment on the one hand also make them technically difficult to study on the other hand.

Despite a variety of phylogenetic and population genetic studies and an estimated high number of still undescribed species, so far species discovery is based solely on classical taxonomic descriptions and no cryptic odonate species is discovered yet (Misof *et al.* 2000; Weekers *et al.* 2001; Stoks *et al.* 2005; Hadrys *et al.* 2006; Hasegawa & Kasuya 2006). We here report the first species discovery hypothesis in odonates based on genetic evidence using ND1 (NADH dehydrogenase 1) and COI (cytochrome c oxidase subunit I) DNA sequence

marker and incorporation of morphology, ecology and biogeography. In the libellulid dragonfly *Trithemis stictica*, only the integration of all datasets into one character-based matrix ultimately allows both, species discovery and species assignment in a straightforward manner. Such a "total evidence" barcode can be of direct importance to conservation management.

Material and methods

Field studies and geography

The genus *Trithemis* (Libellulidae) is worldwide distributed and includes 40 described species (Pinhey 1970). These species show a great variety of habitat specificities ranging from habitat generalists dispersed throughout Africa, to regionally restricted specialists. *Trithemis stictica* (Burmeister 1839) is a generalist and a common species in Sub-Saharan Africa. It inhabits swamps, pools or streams in open and forested areas and depends on permanent waterbodies with a high degree of vegetation (Pinhey 1970). In Namibia, one of the most arid countries in the world such waterbodies are rare. From 133 monitored localities, *T. stictica* was only found in two regionally restricted areas, the Naukluft Mountain region in western-central Namibia and the Caprivi Stripe, with the Okavango and Kwando River in the north-eastern corner. Between 2000 and 2006, 108 samples of *T. stictica* were collected from 14 localities in Namibia, Botswana (Okavango Delta), Zambia (Zambezi River), South Africa (Western Cape, Royal Natal Park), Tanzania (East Usambara Mountains), Kenya (Kiboko River, Nairobi National Park) and Ethiopia (Ambo) to broadly cover its geographical distribution (see Table 1 and Figure 1). Habitat parameters were mapped for each location. For comparative phylogenetic analyses five closely to distantly related *Trithemis* species were also sampled and included into the study.

Table 1 Population sites (country, locality, abbreviation) and number (n) of analysed individuals of *T. stictica* (Clade 1, 2 and 3) as well as five other *Trithemis* species.

Species	Country	Locality	Abbr.	n
T. stictica	Namibia	Naukluft	Nauk	8
	Namibia	Zebra River	Zebra	9
	Namibia	Popa Falls	Popa	32
	Namibia	Andara	And	3
	Namibia	Rundu	Rund	4
	Namibia	Kwando River	Kwan	7
	Botswana	Okavango Delta	Bot	11
	Zambia	Zambezi River	Zam	17
	Kenya	Kiboko River	KR Ken	5
	Kenya	Nairobi NP	NNP Ken	1
	Tanzania	East Usambara Mt.	Tanz	5
	South Africa	Western Cape	WC SA	2
	South Africa	Royal Natal Park	RN SA	3
	Ethiopia	Ambo	Eth	1
T. annulata	Namibia	Rehoboth		2
	Namibia	Popa Falls		3
T. furva	Ethiopia	Nekemte		3
	South Africa	Wakkerstrom		2
T. grouti	Liberia	Gola Forest		2
	Liberia	Lorma Nat. Forest		3
T. nuptialis	Congo	Lingomo		1
	Congo	Lukomete		1
T. kirbyi	Namibia	Tsaobis		3
	Namibia	Waterberg		2

DNA extraction and Sequencing

Total genomic DNA was isolated from leg tissue using a modified phenol-chloroform extraction (Hadrys *et al.* 1992). For initial population genetic analyses the mitochondrial marker ND1 was used. A 610 bp fragment was amplified using the primer pair P 850 fw and P 851 rev (Abraham *et al.* 2001). The amplification product includes the $tRNA^{Leu}$ and a 3` partial fragment of the 16S rDNA fragment and the ND1 gene region. The PCR thermal regime was performed as described in Rach *et al.* (2008). A second marker, the suggested universal barcode region COI, was used on a subset of individuals covering the previously identified genetic clades (five individuals of each clade). Here a 630 bp fragment was amplified using universal primers (Hebert *et al.* 2003). PCR conditions were as follows: 3 min initial denaturation at 95° C, followed by 35 cycles of 95° C for 30 s, 50° C for 40 s and 72° C for 40 s, and 2 min extension at 72° C. PCR was carried out in a total volume of 25 µl, containing 1X amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 7.5 pmol each primer, and 0.75 U Taq DNA polymerase (Invitrogen).

Cycle sequencing of purified PCR-products was done using the ET Terminator Mix from Amersham Bioscience and sequenced on an automated sequencer (MegaBACE 1000; Amersham Bioscience). Sequences were assembled and edited using Seqman II (vers. 5.03; DNAStar, Inc). Consensus sequences were aligned by means of MUSCLE 3.6 (Edgar 2004). Sequences of each haplotype of all species were deposited into GenBank under accession numbers FJ358436-FJ358482.

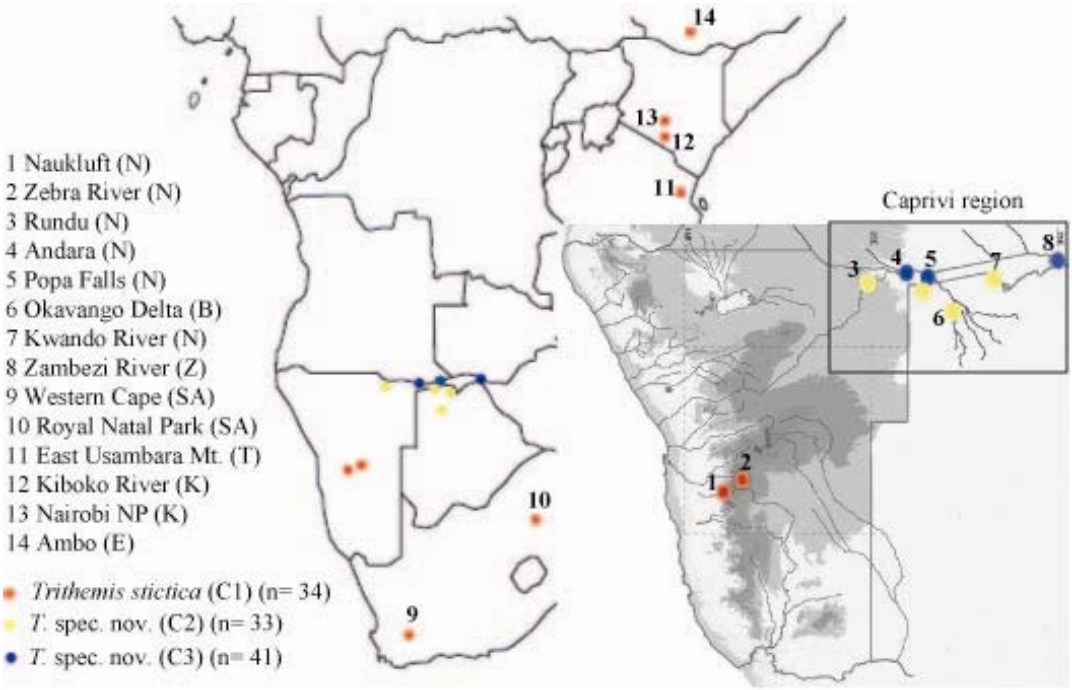

Figure 1 Map of the analysed sample sites of *T. stictica* (C1= clade 1), *T.* spec. nov. (C2= clade 2) and *T.* spec. nov. (C3= Clade 3). The true *T. stictica* is distributed across Southern Africa with five different countries included in this study (N: Namibia; SA: South Africa; T: Tanzania; K: Kenya; E: Ethiopia), while *T.* spec. nov. (C2) and (C3) are restricted to the Caprivi region, at the borders between Namibia (N), Botswana (B) and Zambia (Z).

Genetic distance and phylogenetic analysis

Number of haplotypes and variable nucleotide positions were calculated using Quickalign (Müller & Müller 2003). Pairwise genetic distances for ND1 and COI were calculated using the Kimura-2-Parameter distance model implemented in PAUP vers. 4.0b10 (Swofford 2002). For estimation of gene flow between populations F_{st}-values were computed in ARLEQUIN vers. 3.1 (Excoffier *et al.* 2005) and tested for significance by permuting haplotypes between samples (10,000 replicates).

For phylogenetic analyses two different tree building methods, Bayesian (BA) and Maximum Parsimony (MP) were compared. Using the Akaike Information Criterion in

Modeltest 3.7 (Posada & Crandall 1998) the TrN+I model for ND1 was selected and the GTR model for COI for BA performed with MrBAYES vers. 3.1.2 (Huelsenbeck & Ronquist 2001). The most appropriate parameters for among site variation, base frequencies and discrete gamma distribution were employed and Marcov-Chain Monte-Carlo posterior probabilities determined. The Marcov-Chain Monte-Carlo search was performed with four chains for 1,500,000 generations and trees were sampled every 750th generation. MP analyses were performed as implemented in PAUP vers. 4.0b10 (Swofford 2002). Here, a heuristic search for each marker was employed using TBR branch swapping and random addition of taxa for 100 replicates. Bootstrap values were calculated based on 1,000 replicates (Felsenstein 1985).

Character-based barcode analysis

The identification of diagnostic characters within ND1 and COI sequences was performed in two steps. First, for pairwise comparisons of *T. grouti, T. nuptialis* and the three genetic *T. stictica* clades, the numbers of nucleotide substitutions distinguishing all individuals of one species or clade from the others were listed for each species pair. Nucleotide substitutions occurring only in single individuals of a species were ignored and only pure diagnostic characters mentioned (see Rach *et al.* 2008)

Second, employing the CAOS algorithm (Sarkar *et al.* 2002; Rach *et al.* 2008) a search for species specific combinations of character states for both markers was performed for the whole dataset (including the five *Trithemis* species and the three clades). Here, the most variable sites distinguishing between the species were chosen and the character states at these nucleotide positions were listed. This way, unique combinations of character states, "character-based DNA barcodes", were achieved. For a detailed description of character-based DNA barcoding using CAOS see Rach *et al.* (2008).

Morphological analyses

A total of 43 male specimens from Namibia (Zebra River, Kwando, Andara and Popa Falls), Botswana, Zambia, Kenya, Tanzania and South Africa were examined using a stereoscopic microscope, a scanning electron microscope (SEM) and a stage micrometer. Statistical tests were performed using SAS to test for Normality (Shapiro-Wilk test) and to analyse significance of morphological differences between the genetic entities (Wilcoxon test).

With the SEM (ETEC-AUTOSCAN) the secondary copulatory apparatus (SCA) of selected individuals of each locality were dissected, including the penis located in the inner

part of the SCA. The specimens, previously preserved in 80% ethanol, were dried under vacuum, sputter coated with gold and examined in the vacuum chamber of the SEM.

Results

Genetic distance patterns

The alignment of the ND1 marker contains sequences of all 108 individuals of *T. stictica*. The fragment of 496 bp harbours 62 variable and 60 parsimony informative sites. One deletion occurs at position 126 in the region of the $tRNA^{Leu}$ in 73 sequences (all from the Caprivi region which includes Popa Falls, Andara, Kwando, Rundu, Zambia and Botswana). In total, 26 haplotypes were identified with no haplotype shared by all localities. Genetic distances range from 0% to 9.0% (Table 2) with very high values between three groups of individuals resulting in three separate haplotype clades without intermediate haplotypes (Figure 2). The alignment of the COI marker contains 630 bp, including 67 variable and 59 parsimony informative sites. Nine different haplotypes were found and genetic distances range from 0 to 8.3% (Table 2). The individuals group together in the same three distinct clades as in ND1.

Table 2 Sequence divergence (in %) based on the Kimura-2-parameter of ND1 (above) and COI (below) of the three clades (C1=clade 1, C2=clade 2, C3=clade 3) of *T. stictica* and four *Trithemis* species.

ND1	C1 (*T. stictica*)	C2 (*T.* spec. nov)	C3 (*T.* spec. nov)	*T. grouti*	*T. nuptialis*	*T. annulata*	*T. furva*
C1 (*T. stictica*)							
C2 (*T.* spec. nov)	9.0						
C3 (*T.* spec. nov)	8.5	5.0					
T. grouti	6.8	8.1	8.1				
T. nuptialis	2.2	7.6	8.7	7.0			
T. annulata	10.6	6.5	7.3	10.0	9.4		
T. furva	9.1	8.0	8.3	10.2	8.3	8.3	
COI	C1 (*T. stictica*)	C2 (*T.* spec. nov)	C3 (*T.* spec. nov)	*T. grouti*	*T. nuptialis*	*T. annulata*	*T. furva*
C1 (*T. stictica*)							
C2 (*T.* spec. nov)	7.9						
C3 (*T.* spec. nov)	8.3	5.7					
T. grouti	3.3	8.9	8.9				
T. nuptialis	3.3	9.5	9.3	1.0			
T. annulata	9.1	10.6	11.4	8.1	8.5		
T. furva	9.7	10.1	10.4	10.1	10.6	9.3	

All localities, except for one, could be assigned to one of the three clades. The first genetic clade consists of localities separated by long distances, South Africa, Ethiopia, Tanzania, Kenya and two sites in central Namibia, the Naukluft and Zebra River region (red dots in Figure 1). The second clade contains regionally restricted individuals from the Caprivi region, the localities Okavango Delta in Botswana, Kwando River, Rundu and a part of the Popa Falls individuals in Namibia (yellow in Figure 1). The remaining Popa Falls individuals belong to the third clade together with individuals of the sites Zambezi River (Zambia) and Andara, again all from the Caprivi region (blue in Figure 1). Genetic distances between the clades are very high. Between the first and the second clade it is 9.0% in ND1 and 7.9% in COI and between the first and third clade it is 8.5% in ND1 and 8.3% in COI. The regionally restricted clades 2 and 3 with individuals of the Caprivi region are separated by 5.0% in ND1 and 5.7% in COI (see Table 2). In contrast genetic distance within clades is low and ranges from 0 to 1%. At one site in the Caprivi region, Popa Falls, individuals of clade 2 and 3 occur sympatrically. Interspecific genetic distances between the five known *Trithemis* species included in this study range from 1.9 to 10.6% in ND1 and 1.0 to 11.4% in COI (Table 2). Here e.g. the genetic distances between clade 1 and the known species *T. nuptialis* (2.2% in ND1, 3.3% in COI) and *T. grouti* (6.8% in ND1, 3.3% in COI) is lower than to clade 2 and 3.

Comparisons of F_{st}-values reveal high genetic substructuring between the populations, but without geographical correlation. Grouping individuals according to their genetic clade, the F_{st}-values between these groups range from 0.906 to 0.960 in ND1 and from 0.921 to 0.984 in COI. These high levels of F_{st}-values suggested that there is no gene flow neither between the population sites of the Caprivi region (clades 2 and 3) nor between the Caprivi region and clade 1 (Namibia Naukluft, Kenya, Tanzania, South Africa and Ethiopia).

Phylogenetic analyses

For both markers (ND1 and COI) Maximum Parsimony (MP) and Bayesian analyses (BA) reveal the same topology, which mirrors the picture from the distance analyses, where individuals are grouped into three clusters (Figure 2).

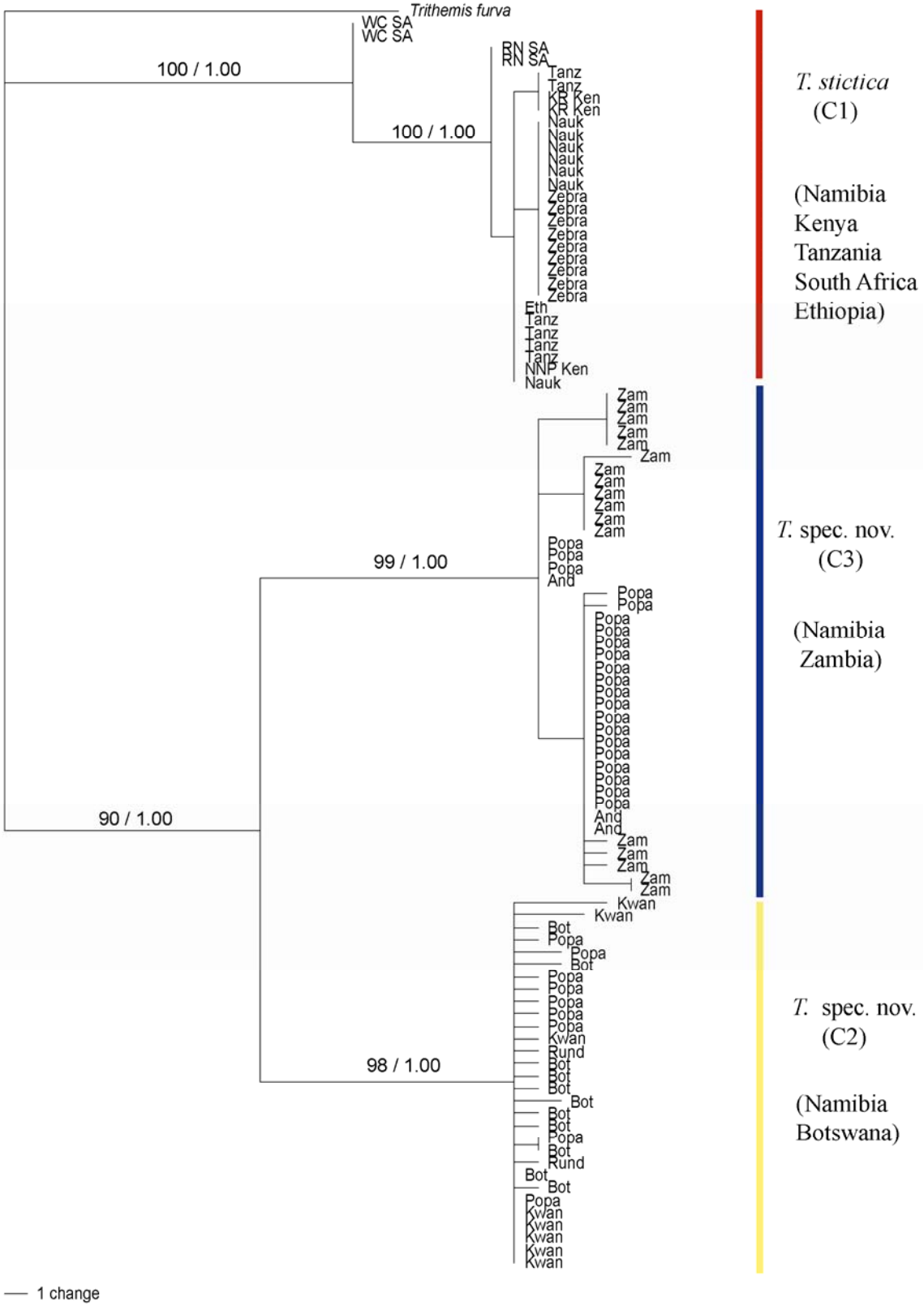

Figure 2 Maximum Parsimony tree (ND1) of all individuals sampled under the species name of *T. stictica.* Included are posterior probabilities and bootstrap values. A clustering of the individuals into three separate clades is highly supported. Clade 1 consists of individuals of the real *T. stictica*, and Clade 2 and Clade 3 are the putative new species. Locality abbreviations are congruent with table 1.

In order to position the genetic clades of *T. stictica* in a phylogenetic tree, MP and BA analyses of the three clades together with two closely and three more distantly related *Trithemis* species were performed. The resulting trees show a clear separation of clade 1 (red) from clade 2 and 3 (yellow and blue, Figure 3). Clade 1 groups together with *T. grouti* and *T. nuptialis,* which is congruent with the classical taxonomic position of *T. stictica* (Pinhey 1970). Based on this tree topology clade 1 is identified as the originally described *T. stictica*. A sister group (sister species) relationship between the putative new species (clade 2 and 3) is highly supported (PP=1.00; 100% bootstrap).

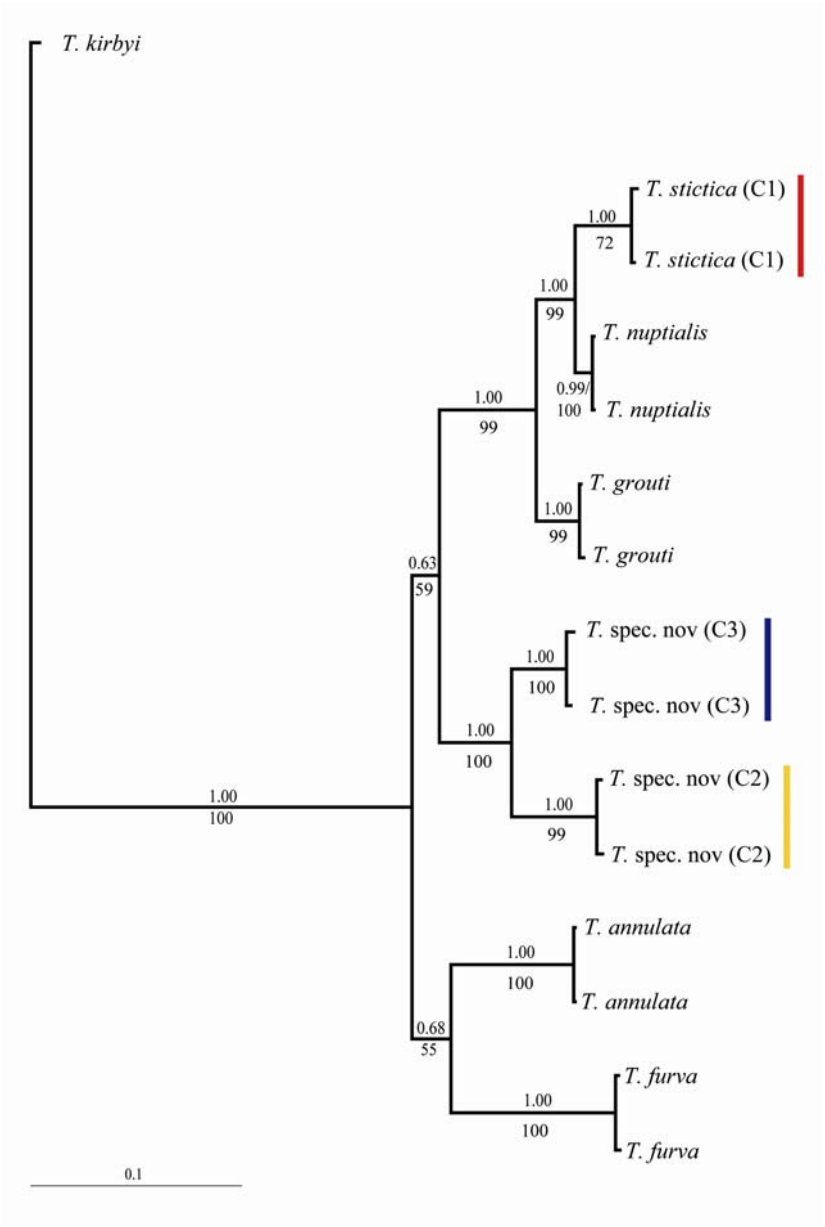

Figure 3 Bayesian tree of selected *Trithemis* species based on a concatenated matrix of COI and ND1. Posterior probabilities and bootstrap values are included. For the different species at least two individuals were incorporated as well as the two most common haplotypes of each newly found clade. *T. stictica* groups together with *T. nuptialis* and *T. grouti*, while *T.* spec. nov. (C2) and *T.* spec. nov. (C3) form two separate sister taxa.

Character-based DNA barcodes

Table 3 (a) lists the barcodes, i.e. species-specific nucleotide positions (pure diagnostic barcode characters), for the three *Trithemis* clades and two closely related species. The three clades are distinguishable by unambiguous barcodes. Clade 1 and clade 2 can be distinguished by 26 variable nucleotide positions (vnp's) in ND1 and 43 in COI, clade 1 and clade 3 by 27 positions in ND1 and 43 in COI and clade 2 and 3 by 13 positions in ND1 and 28 in COI. In contrast to this high number of vnp's, only four positions vary in ND1 and 19 in COI to distinguish *T. nuptialis* from clade 1. The comparison of clade 1 and *T. grouti* revealed 21 (ND1) and 20 (COI) different positions. Interestingly the vnp´s between clade 2 and 3 are nearly the same as between *T. grouti* and *T. nuptialis*, with around 30 variable positions in ND1 and 50 in COI.

For establishing character-based barcodes for all *Trithemis* species studied, 13 nucleotide positions of ND1 and 15 of COI were chosen. The particular nucleotide positions revealed the highest numbers of diagnostic characters (Table 3b). Regarding only these chosen positions, all species could be distinguished by at least four diagnostic characters in both markers.

Table 3 (a) Total number of pure diagnostic characters discriminating all individuals from a specific clade or species from each other in a pairwise comparison listed for *T. stictica* (C1=clade 1), the two putative new species *T.* spec. nov (C2=clade 2), *T.* spec. nov. (C3=clade 3) and two closely related sister species based on ND1 (422bp) and COI (630bp) sequences.

(a)

Pairwise comparison	**ND1**	**COI**
T. stictica (C1) / *T.* spec. nov. (C2)	26	43
T. stictica (C1) / *T.* spec. nov. (C3)	27	43
T. stictica (C1) / *T. nuptialis*	4	19
T. stictica (C1) / *T. grouti*	21	20
T. spec. nov. (C2)/ *T.* spec. nov. (C3)	13	28
T. spec. nov. (C2) / *T. nuptialis*	32	51
T. spec. nov. (C2) / *T. grouti*	30	49
T. spec. nov. (C3) / *T. nuptialis*	28	52
T. spec. nov. (C3) / *T. grouti*	30	50

Table 3 (b) Character-based DNA barcodes for seven *Trithemis* species, including *T. stictica* and T. spec. nov. (C2 & 3) for ND1 and COI. Shown are diagnostic character states at 13 selected nucleotide positions for ND1 and 16 for COI which are different in at least four positions per species combination.

(b)

ND1	**Nucleotide positions**												
Species	**101**	**132**	**135**	**152**	**185**	**191**	**245**	**287**	**290**	**326**	**342**	**355**	**419**
T. stictica (C1)	G	A	G	A	T	A	C	A	T	A	T	C	T
T. spec. nov. (C2)	C	G	A	A	G	T	T	A	C	G	T	C	T
T. spec. nov. (C3)	T	G	A	A	G	T	T	G	T	A	C	C	T
T. grouti	A	A	T	A	T	A	T	G	T	A	T	C	C
T. nuptialis	G	A	G	G	G	A	T	A	T	A	T	T	T
T. annulata	T	G	A	A	C	T	T	A	T	A	T	T	T
T. furva	T	T	A	A	C	C	G	T	T	T	C	T	G

COI	**Nucleotide positions**														
Species	**45**	**144**	**162**	**180**	**279**	**288**	**294**	**297**	**330**	**333**	**360**	**393**	**396**	**454**	**459**
T. stictica (C1)	C	C	A	C	T	A	A	T	T	G	T	A	A	A	T
T. spec. nov. (C2)	C	G	A	A	A	A	T	T	T	T	C	A	A	C	T
T. spec. nov. (C3)	A	G	A	A	G	G	C	T	T	G	T	G	A	C	T
T. grouti	A	G	G	C	T	A	A	T	T	G	T	A	A	T	C
T. nuptialis	C	G	G	C	T	A	A	C	C	G	T	A	A	A	T
T. annulata	A	T	T	C	A	A	A	T	T	A	T	A	C	A	T
T. furva	A	A	A	T	T	A	A	A	T	T	A	T	T	T	T

Morphological analyses

Originally all individuals collected in the field for population genetic studies were identified as *T. stictica*. After re-examination of selected 43 specimens slightly different colouration patterns of the abdomen and the thorax were found. These differences are not correlated to the genetic clades. Two phenotypic traits could be identified, however, which unambiguously separate individuals from different genetic groups: (i) eye colour and (ii) colouration of the base of the wings. All individuals of the two clades from the Caprivi region have two-coloured eyes and a yellow wingbase, where the specimens from clade 1 have single-coloured eyes and a clear wingbase (Damm & Hadrys 2009).

Less unambiguous, but still significant differences were obtained from more detailed measurements of different morphological traits (details see Table 4). Most important, SEM analyses of the secondary genitalia revealed differences in penis morphology. The shape of the two cornuti, located at the distal penis segment, is significantly different in two groups of individuals. The cornuti of all individuals from clade 1 (Kenya, Tanzania, South Africa, Ethiopia, Zebra River and Naukluft) are curved and at the end pointed as it is described for the holotype of the true *T. stictica* (Pinhey 1970). In contrast, the cornuti of clade 2 and 3 consistently have a different shape. The only difference so far between clade 2 and 3 is body

size. Abdomen and segment four are significantly shorter in clade 3 compared to clade 1 and 2. In sum, while the true *T. stictica* could be identified and delimitated morphologically from the other two clades by eye through wing colouration and penis structure, the differences between the putative new species (clade 2 and 3) are, except of slight size differences, cryptic Table 4).

Ecological pattern

Mapping the habitats of the sampled sites onto the phylogenetic trees reveals that the three genetic clades differ in their habitat preferences. Habitat sites of *T. stictica* (clade 1) were well-vegetated ponds, streams and rivers sometimes with a high degree of shade (Naukluft populations and all localities outside of Namibia). Individuals of clade 2 were exclusively found along the quite floating areas of the Okavango River, at the smaller Kwando River and in the Okavango Delta (see Figure 1). The waterbodies are open and the surrounding bank vegetation is dominated by grassland and reed. Most of the gallery forest along the Okavango is deforested. Clade 3 was discovered at two sites within the Nature Reserve Popa Falls (Okavango River, including Andara) and at the Zambezi River near Victoria Falls (Zambia). These sites have a mostly intact gallery forest along the river with higher trees and shady areas. Here the water flows very fast with rapids in-between. Interestingly, at one site in the Caprivi region, Popa Falls, a highly heterogeneous landscape, clade 2 and 3 occurred sympatrically. The flight season of all three species is between August/September and April/May and the two clades at Popa Falls were caught in the same season at the same time.

Table 4 Summary of diagnostic characters used in the taxonomic circle to proof the discovery of two new species. Shown are the diagnostic characters discriminating the true *T. stictica* from the two newly discovered *T.* spec. nov (Clade 2) and (Clade 3). Sequence divergence (Seq. div., %), number of variable nucleotide positions distinguishing all individuals of one species from all individuals of the others (diagnostics), significant morphological traits (length of hindwing (HW), length of the base of hindwing (Bs Hw), length of abdomen (AbdL), length of abdomen segment 4 (S4), distal penis segment (Cornuti)), F_{st}- values, and a simplified description of differences in ecological and biogeographical patterns (details see text).

	DNA				**Morphology**				**Reproductive Isolation**				**Ecology**	**Geography**
	Seq. div.		diagnostics		Size parameters				Cornuti shape	F_{st}				
	ND1	CO1	ND1	CO1	Hw	Bs Hw	AbdL	S4	differences	ND1	COI			
T. stictica / Clade 2	9.0	7.9	26	43	*	**	-	-	significant	0.960	0.984	*T. stictica*	open habitat	widespread
T. stictica / Clade 3	8.5	8.3	27	43	***	***	***	***	significant	0.944	0.966	Clade 2	swamp-like habitats	Caprivi region
Clade 2 / Clade 3	5.0	5.7	13	28	-	-	**	**	weak	0.906	0.921	Clade 3	fast running water	Caprivi region

Discussion

Application of DNA sequence data in taxonomy has come to a point where procedures need to be developed, which integrate genetic information into the classical taxonomic system. Particularly DNA-based taxonomy needs a corroborative framework. The fact that in morphology-based species delineation quantitative parameters have rarely been applied highlights the difficulty of obtaining quantitative appropriate characters in traditional taxonomy and also reflects the problem of subjectivity in current species descriptions (Cracraft 1992; Vogler 2006; Vogler & Monaghan 2007; Cardoso *et al.* 2009). While taxonomy by definition assesses the distribution of character variation, Vogler & Monaghan (2007) point out that neither the kind of variation nor the underlying biological process are of primary importance and therefore any kind of character is valuable for taxonomic classification. Here it would clearly be helpful to formalize processes that incorporate different sets of characters.

Our application of the taxonomic circle (DeSalle *et al.* 2005) to a case study in dragonflies suggests that this simple scheme is able to provide a framework for the discovery of new species. Our analyses of 108 *T. stictica* individuals combine genetic data with morphology, ecology and geography and lead to the discovery of two new species that have phenotypically been *cum grano salis* "cryptic".

The taxonomic circle

The genetic data provided the immediate and most obvious dataset suggesting the existence of two new *Trithemis* species. None of the other disciplines alone would have discovered the new species. This highlights the importance of DNA analyses for the discovery of new species, particularly at the level of so-called "cryptic species". On the other hand DNA approaches alone can hardly fullfil a species concept in a satisfying way. The taxonomic circle suggested by DeSalle *et al.* (2005) captures in a simplified way the components of such a modern taxonomic system: hypothesis testing, corroboration, reciprocal illumination and revision. In this scheme at least two of the five components of the circle (DNA, morphology, reproduction, ecology and geography) have to support the hypothesis of a new species. Any two of the five disciplines are sufficient to determine a species boundary and revise the species discovery hypothesis. In the case study presented here initially a DNA-based hypothesis is postulated and tested against the classical taxonomic components (see Figure 4). After testing the multiple DNA-based profiles of the new species against morphology,

ecology and geography we could leave the taxonomic circle, confirm our hypothesis, and also bridge gaps to both the biological and evolutionary species concept.

The initial molecular study started with one species (*T. stictica*) which revealed three genetic clusters. Therefore we analysed two different hypotheses with the help of the taxonomic circle (as displayed in Figure 4). In the first hypothesis we analysed if the two clades (2 and 3) from the Caprivi region can be delimitated from *T. stictica*. Fixed differences in morphology (eye and wing colouration, cornuti), geography and ecology corroborate the hypothesis of two separate entities. In the second hypothesis we tested if clade 2 and 3 can be raised to species status. A separate species status is supported by DNA (e.g. genetic isolation), morphology (fixed size differences) and ecology (niche separation). Thus, with three components supporting the hypothesis we must accept the hypothesis of two separated species. In sum, the significant genetic isolation of the two lineages, the ecological niche shift, the fixed size differences and the most likely reproductive isolation provide substantial corroborative evidence to support the hypothesis of two new sympatric *Trithemis* species in the Caprivi region (Figure 4b).

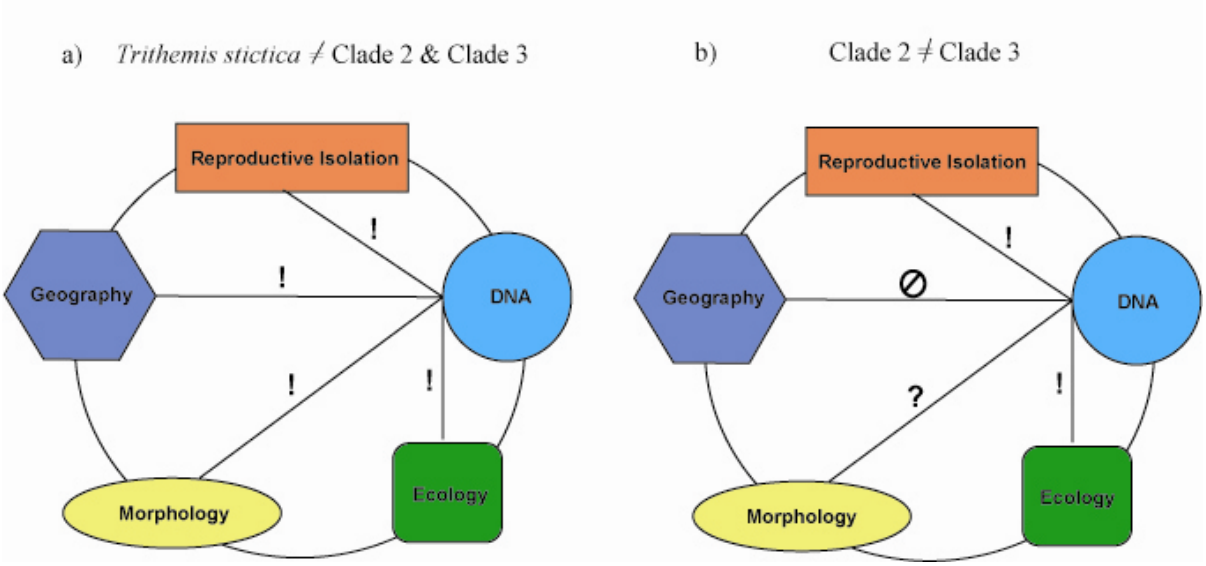

Figure 4 Taxonomic circles demonstrating an integrative species discovery approach. In this scheme a new taxon could be delineate if at least two disciplines corroborate and verify the hypothesis of a new taxon, which is indicated by an exclamation mark at the interior traversal line. In both circles species discovery hypothesis is based on DNA- evidence **a)** First hypothesis tests the distinctiveness of *T. stictica* and the two new clades. Here all components of the circle corroborate the hypothesis of new species. In **b)**, based on multiple DNA evidence, the hypothesis is tested if the two clades, *T.* spec. nov (C2) and (C3) are separate species. Here ecology and reproductive isolation corroborate the hypothesis of two new species in the genus *Trithemis*, while morphological characters differ only weakly.

In the above case study the taxonomic circle proved to be a valuable tool for the discovery of new species in one of the hardest of all possible cases, in sympatric and "cryptic species". In general, some aspects still need to be discussed. The here chosen components of the circle may work for most animal groups, but problems arise e.g. in microbial species due to the lack of geographical and morphological information for corroboration (DeSalle *et al.* 2005). In such problematic cases other components like additional gene regions or more ecological information could be incorporated to support or refute a species hypothesis. In addition, the quality of hypothesis testing relies on additional aspects like sample size, the chosen genetic marker and the geographical range for the sampling regime. Morphological as well as genetic variation also occur intraspecifically and are often correlated to geography. The optimal way would be to cover the whole distributional range of a hypothetical species. In most cases this will not always be possible, but highlights the importance of the integration of different disciplines in decision making. Often DNA data will suggest a separation, which then leads to more intensive and specific investigation at different organismal levels. Subsequently the taxonomic circle presents a practical framework which requires more than one line of evidence to support a species hypothesis. It provides sufficient strictness for species discovery by serving the bridge between traditional morphological and modern molecular approaches. We suppose that the *Trithemis* case study is just one example out of many yet undiscovered examples for the presence of valid species that at the organismal level are easily overlooked.

Advantages of character-based DNA barcoding

In this case study traditional DNA barcoding methods would have also discovered the two new *Trithemis* species. Sequence divergences between the relevant groups are in concordance with those of taxonomically well described *Trithemis* species and the 3% cut-off value and the 10x rule are fulfilled (Hebert *et al.* 2003; Hebert *et al.* 2004b). In many cases, however, distance methods relying on DNA data alone are ill suited for species delineation. The main reason is that substitution rates of mtDNA vary largely between different groups of species resulting in a broad overlap of intra- and interspecific distances (Will & Rubinoff 2004). In dragonflies a universal genetic distance cut-off value would not be applicable, since there are several examples in which intrapopulation variation exceeds divergences between species (Cordero Rivera *et al.* 2004; Svensson *et al.* 2006). Thus it seems understandable that "DNA barcoding" in general got criticized to fail in new animal species discovery (Hickerson *et al.* 2006).

The introduction of character-based DNA barcoding (Sarkar *et al.* 2002; Rach *et al.* 2008) seems to be a promising complement that avoids the problem of subjective distance thresholds. In the *Trithemis* study character-based DNA barcoding distinguished all three clades easily through the presence of diagnostic characters or specific combinations of character states. The established character-based DNA barcodes for all *Trithemis* species (using 13 character states of the ND1 and 15 of the COI sequences) represents unique and unambiguous combinations of character states for each species. In some cases the use of a single barcode marker may not be enough. For example in a former study the species pair *Aeshna grandis* and *Aeshna cyanea* differ only at one single position in ND1 (Rach *et al.* 2008). Here the application and combination of a second barcode marker, e.g. COI, is helpful (Rach *et al.* 2009, submitted). Another example is the genus *Calopteryx*, where several species show very low genetic distances and exhibit very few diagnostic character states (Rach *et al.* 2008), although the three sister species (*Calopteryx virgo, C. splendens* and *C. haemorrhoidalis*) can clearly be discriminated by morphology (Misof *et al.* 2000; Dumont *et al.* 2005). Such examples highlight the overall advantage of character-based barcoding, particularly the possibility to expand the DNA based barcodes with characters from other disciplines.

A character-based database can also contribute more directly to conservation biology, since in conservation management information about genetics, ecology and geography is equally important. In the here described *Trithemis* species complex the two new clades were hidden for a long time because the previously described habitat preferences of *T. stictica* (Pinhey 1970) seem to perfectly fit the habitats of the Caprivi region with its rivers Okavango, Kwando and Zambezi. Here the genetic data fueled the discovery of the new species and resolved differences in habitat choice. We can now map ecological characters to each of the three species.

While the character-based DNA barcode consists of fixed characters for each species the most critical parameters when establishing a barcode are sample size and the number of CAs (characteristic attributes). With the increasing number of analysed individuals the level of confidence of a CA to be fixed in a species also increases. Although there will be no absolute certainty for a given CA to be fixed, the reliability of a barcode increases with each independent CA added (Rach *et al.* 2008). In endangered or rare species with small population sizes, like in e.g. the rainforest damselfly *Megaloprepus caerulatus* (Fincke & Hadrys 2001), high sample sizes are not easy to obtain. Nevertheless, a DNA barcode of a single individual is still useful and provides important information for this species within a

group of interest. Incorporating characters from other disciplines will then increase the reliability in species identification. Criticism for the integrated approach may arise because the establishment of such a database might not be fast enough for conservation concerns. But DNA based identification will allow the first and quick decision and the background knowledge of non-DNA data can later on complement the database. Thus, DNA based information can be associated with biological information to incorporate also the evolutionary and taxonomically background (Vogler & Monaghan 2007).

Independent of the form, a reliable and fast method for species identification is needed for any kind of conservation management and biodiversity program. We suggest the integration of distinct DNA characters and traditional information like morphology, ecology and geography in a comprehensive barcode database which is all character based and allows fast species identification and discovery.

Cryptic speciation in dragonflies

The results of our study unravelled two new dragonfly species which at the organismal level appeared to be “cryptic” species. To our knowledge this is the first detection of speciation in dragonflies distributed in the same region without obvious reproductive barriers.

Odonates in general are not supposed to evolve “cryptic” species. Their ways to communicate are not based on invisible mechanism (e.g. smells or sounds) which are believed to be a major driving force for cryptic speciation. Nevertheless, their unique reproductive system and fast reaction to environmental change can promote speciation processes without accompanying morphological changes (Kirkpatrick & Ravigne 2002; McPeek & Gavrilets 2006; Svensson *et al.* 2006). Their complex reproductive system and a variety of sperm competition mechanism may allow the fast evolution of reproductive barriers via strong sexual selection (Waage 1979; Arnqvist *et al.* 2000; Cordoba-Aguilar *et al.* 2003; Cordero Rivera *et al.* 2004). Furthermore their fast reaction to environmental changes allows fast ecological shifts. In the presented study no immediately obvious differences in morphology were found between the two new *Trithemis* species and without the tests against all other datasets the species would have remained undetected. This example shows how important it is to combine different disciplines to determine species boundaries in modern taxonomy. A modern taxonomic system can be derived from both, quantitative data and expert opinion. Integration of datasets from different disciplines into one character based matrix ultimately allows species discovery and species assignment in a more straightforward way.

Acknowledgements

The work was supported by the Federal Government Research Program (BMBF) BIOTA South (S08). We are grateful to Jens Kipping, K.-D. B. Dijkstra, Frank Suhling, and Viola Clausnitzer for providing specimens. Many thanks also to Jessica Rach, who helped with the CAOS analyses. Sequences were generated in our laboratory.

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Trithemis morrisoni sp. nov. and *T. palustris* sp. nov. from the Okavango and Upper Zambezi Floodplains previously hidden under *T. stictica* (Odonata: Libellulidae)

Sandra Damm[1] & Heike Hadrys[1, 2]

[1]ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, 30559 Hannover, Germany.

[2]Yale University, Dept of Ecology and Evolutionary Biology, New Haven, CT, 06520-8104, USA.

This is the author's version of a work originally published in the *International Journal of Odonatology* (2009, Volume 12, Issue 1, Pages 131–145).

Abstract

During the course of a population genetic study of *Trithemis stictica* that included sites in Namibia, Kenya, Tanzania, Ethiopia, Botswana and Zambia, two undescribed libellulid species were discovered in the Okavango and Upper Zambezi Floodplains. These were both previously identified as *T. stictica*. We describe the two species, *T. morrisoni* sp. nov. (holotype ♂: Namibia, Nature Reserve Popa Falls, Okavango River at the rapids, 18°07´S, 21°40´E; iv 2007, leg. K.-D.B. Dijkstra; dep. in the National Museum of Namibia, Windhoek) and *T. palustris* sp. nov. (holotype ♂: Botswana, Okavango Delta, Moremi Game Reserve, 19°15´S, 23°20´E; ii 2007, leg J. Kipping; dep. in the National Museum of Namibia, Windhoek) and compare them with *T. stictica*.

Keywords: Odonata, dragonfly, Anisoptera, *Trithemis*, taxonomy, Africa, new species, genetics.

Introduction

The genus *Trithemis* Brauer is predominately distributed throughout Africa, including its islands, with a small number of species in Asia (Pinhey 1970). Altogether about 40 species are recognised. The species of the genus show a wide variety of habitat preferences, ranging from generalists to range-restricted specialists. Pinhey (1970) revised the genus, concentrating on the African species. Most of his material is kept in the Natural History Museum of Zimbabwe in Bulawayo (NMBZ). Additional taxonomic work was published by Clausnitzer (2001) and by Dijkstra (2007) who recently revisited Pinhey's collection.

Between 2001 and 2005 a field project mapping the odonates of Namibia was conducted (Suhling et al. 2006). Distribution patterns and dispersal strategies of several key species were studied with population genetic analyses (Hadrys et al. 2006; Dijkstra et al. 2007; SD, HH unpubl.). At the same time other associated projects provided insights in distribution patterns of the genus in neighbouring countries, e.g. from Botswana with the vast Okavango Delta swamps and its surroundings (Kipping 2003, in press). For population genetic studies, samples of *T. stictica* (Burmeister, 1839) were collected from 15 localities in Namibia, Botswana, Zambia, South Africa, Kenya, Tanzania and Ethiopia (Figure 1). While other *Trithemis* species occur throughout Namibia, *T. stictica* was exclusively found at isolated springs in the Naukluft Mountains and in the region of the Caprivi Strip with its surrounding river systems in Botswana and Zambia (Kipping in press; Suhling & Martens 2007). In other sub-Saharan African countries the species is common and inhabits swamps, pools or streams in open areas (Pinhey 1970).

The population genetic study discovered three distinct and completely reproductively and genetically isolated clades within what had been called *T. stictica* (SD, HH unpubl.). The genetic distances of four genetic markers between the clades are unequivocal at the species level. In a phylogenetic tree comparing several species of the genus *Trithemis*, the two newly discovered species are sister species, but are more distantly related to *T. stictica.* Molecular clock analyses suggest that the split between the two new species occurred about one million years ago (SD, HH unpubl.). Because the discovery of new species based solely on genetic data is controversial and in some cases clearly arguable (e.g. DeSalle et al. 2005; Hickerson et al. 2006), we took an integrative approach to species delimitation which includes morphological, ecological, geographical, and genetic characters (SD, HH unpubl.). In this analysis all evidence leads to the recognition of two new species. Since the phenotypes of the three species are very similar, they were first identified in the field as *T. stictica*. However,

detailed morphological analyses revealed significant differences. Here we describe the two new species *T. morrisoni* sp. nov. and *T. palustris* sp. nov. and their morphological differences with *T. stictica*.

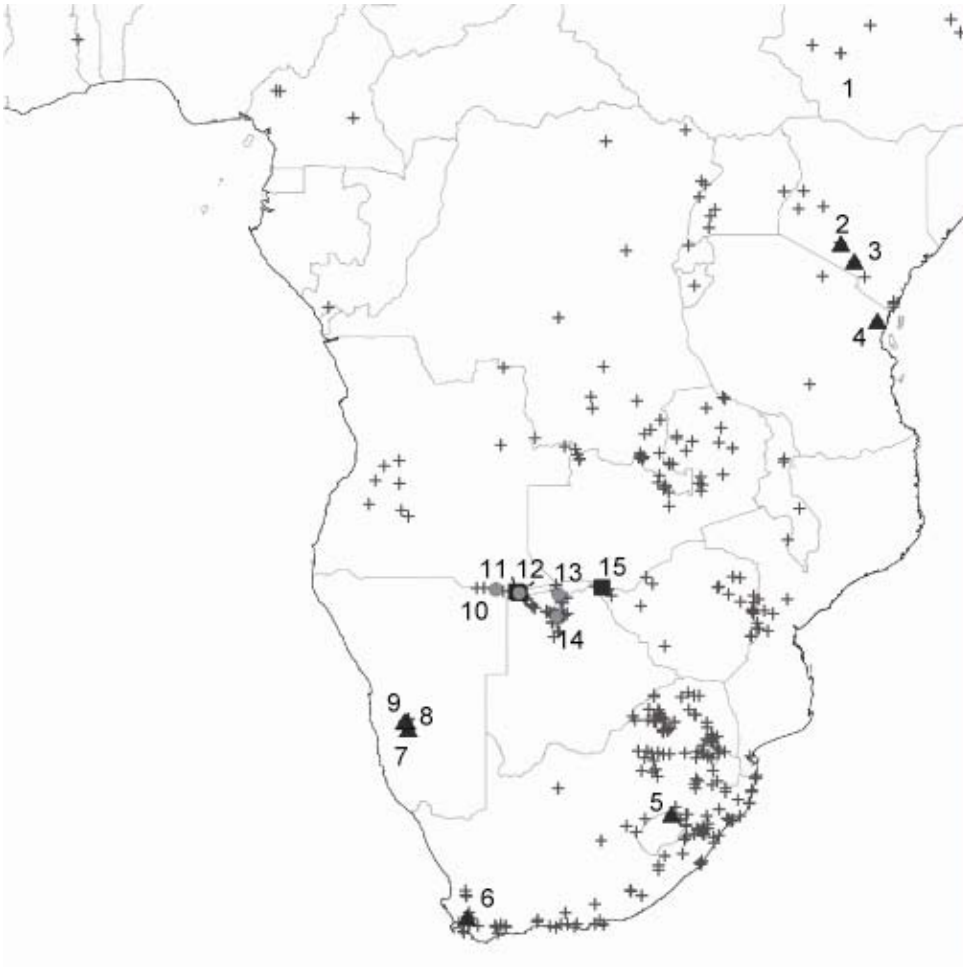

Figure 1 Distribution map of three *Trithemis* species — *T. morrisoni* sp. nov. (●), *T. palustris* sp. nov. (■) and *T. stictica* (▲). (+) displays all records of the *T. stictica* group (one of the three above species) which were not identified so far. Sites of analysed populations – 1: Ethiopia; 2: Nairobi NP, Kenya; 3: Kiboko River, Kenya; 4: Usambara Mt., Tanzania; 5: Royal Natal Park, RSA; 6: Western Cape, RSA; 7: Naukluft Mt. Tsams Ost, Namibia; 8: Naukluft Mt. Naukluft River, Namibia; 9: Naukluft Mt. Zebra River, Namibia; 10: Omatako River, Namibia; 11: Andara, Namibia; 12: Popa Falls, Namibia; 13: Kwando River, Namibia; 14: Okavango Delta, Botswana; 15: Zambezi River, Zambia.

Material and methods

Of 106 genetically analysed specimens, 43 males from Kenya, Tanzania, South Africa, Namibia, Botswana, and Zambia covering the three genetic groups were selected for morphological analyses. We examined the external appearance of the specimens: patterns of thorax and abdomen, wing venation, shape of secondary genitalia and appendices, pubescence, coloration of Pt, frons, vertex, eyes and patch of Hw, and we measured 11 phenotypic characters, e.g. the length of the Hw, abdomen and Pt of the Fw with a stereomicroscope, and analysed the male secondary genitalia with a scanning electron microscope (SEM). Statistical tests were performed using SAS, first to test for Normality (Shapiro-Wilk test) and then to analyse the significance of morphological differences between the genetic groups (Wilcoxon test). Additionally we examined seven females representative of each new species.

Colour plate I Male of *Trithemis morrisoni* sp. nov. — Bovu Island in Zambesi River, Zambia, 18 February 2006. Photo by Jens Kipping.

Trithemis morrisoni sp. nov.

(Figures 1, 2a-d, Plate I)

Trithemis stictica (Burmeister). — Pinhey (1970: 127-128, figures. 47, in part, notes on Victoria Falls dwarf series); — Kipping (2003); — Martens et al. (2003: in part).

Trithemis sp. nov. — Kipping (in press); — Kipping & Suhling (in press); — Suhling et al. (in press); — Suhling & Martens (2007: 233-234, in part).

Etymology

Named after the poet James Douglas Morrison and his passion for deserts and the hidden mysteries of nature.

Specimens studied

Total number of adult specimens examined: 12 ♂, 7 ♀. — **Holotype** ♂: Namibia, Nature Reserve Popa Falls, Okavango River at the rapids (18°07´S, 21°40´E), iv 2007, leg. K.-D.B. Dijkstra, K. Schütte, V.J. Kalkman; — **Paratypes**: 3 ♂: same data as holotype, iv 2003, leg. S. Damm; 2 ♂: ii 2004, leg. F. Suhling; 3 ♂: Namibia, near Catholic Mission Station Andara, Okavango River (18°01´S, 21°30´E), ii 2004, leg. F. Suhling; 3 ♂, 7 ♀: Zambia, Bovu Island, Zambezi River (17°29´S, 25°20´E), ii 2007, leg J. Kipping. The holotype will be deposited in the National Museum of Namibia, Windhoek. Paratypes will stay at University of Veterinary Medicine Hannover, ITZ, Ecology & Evolution, Germany.

Description of holotype male

Head: Labium yellow with a broad black band in the middle extending onto the posterior lobe and the anterior margins of the lateral lobes. Face yellow. Postclypeus with two central, separated black comma-shaped streaks. Frons and vertex metallic steel-blue. Antennae black. Labrum black with two lateral yellow spots. Occipital triangle black with two yellow posterior spots. Back of the head black with four yellow spots. Eyes bicoloured; brownish-red on the upperside and yellow-grey on the underside.

Thorax: Prothorax black with the anterior collar yellow. Median lobe with two yellow markings. Synthorax showing a light blue pruinosity and more ventrally with less pruinosity, where it becomes yellow and black. Metepimera yellow with only little pruinosity. Legs black, with the inner side of the fore femora yellow. — Wings: venation blackish. Pt brown between blackish veins. Cells at the base of the Fw and Hw amber (up to 2 mm from body). Hw with amber patch starting at the triangle and including the anal loop. In Fw 10½-11½ Ax, in Hw 8 Ax, in Fw 13 Px, in Hw 11 Px. Fw triangle of 2, Hw triangle of 1, subtriangle of 3 cells; supratriangle uncrossed.

Abdomen: Abdomen slender, narrowest at S4 and widest at S8. S1-3 black with broad yellow streaks and ventrally with little blue pruinosity. S4-8 black with sharp yellow streaks on each side. S9 black without any yellow. Dorsum of S10 with a yellow spot in the middle. Appendages black. Anterior lamina and hamule black with pale brown bristles; secondary genitalia surrounded by white hair; for details see Figures 2a-b. Penis of holotype not examined.

Measurements [mm]: Entire length 32.4, abdomen length (excl. appendages) 20.4, Fw length 25.9, Hw length 25.5, Pt (Fw) 3.2, appendages 1.5 mm, S4 3.4 mm.

Variation in males

There is little size variation between males (n = 12): abdomen length 19.9-22.5 mm; Fw length 25.8-26.5 mm; Hw length 23.2-26.8 mm; Pt (Fw) length 3.2-3.7 mm; appendages 1.3-1.6 mm; S4 3.3-3.5 mm. The colour of Pt varied between light and dark brown, with the inner side always a slightly lighter brown. All specimens have the amber patch on Hw except for one specimen from the Zambezi River, where only a trace of amber was found. Two specimens from the Zambezi River show a small yellow spot on S9. The comma-shaped streaks on the postclypeus are absent in five Popa Falls males and in the Zambian specimens. The coloration of thorax and abdomen varied between dark brown and black. In two specimens from the Zambezi River and in five from Popa Falls the yellow is ivory.

Description of female

Described is paratype Tmor140H; 140 is locality code for Bovu Island, specimen H.

Head: Labium yellow with a broad black band in the middle, extending onto the posterior lobe and the anterior margins of the lateral lobes. Face yellow. Postclypeus without any markings. Frons and vertex metallic steel-blue/green. Antennae black. Labrum black with two elliptical lateral yellow spots. Occipital triangle black with two yellow posterior spots. Back of the head black with four yellow spots. Eyes bicoloured; brownish-red on the upperside and yellow-grey on the underside.

Thorax: Prothorax black with a little yellow. Synthorax generally has a black and yellow pattern, with black on the anterior side of mesepimera, metepisterna and metepimera and yellow on the posterior side. Mesepisterna with a central black band and metepisterna with an additional hook-shaped black streak on the ventral side. Legs black, with the inner side of the fore femora yellow. Ventral side black with three yellow spots posteriorly. — Wings: clear with blackish venation. Base of the wings amber including the first cell directly at the body in Fw and Hw. Pt brown between black veins. A trace of amber in the Hw, extends from the triangle, expanding to three cells width and up to and including the anal loop. In Fw 9½-10½ Ax, in Hw 8 Ax, in Fw 12 Px, in Hw 12 Px. Fw triangle of 2, Hw triangle of 1, subtriangle of 2 cells; supratriangle uncrossed.

Abdomen: Abdomen narrowest at S4 and widest at S7, where 2mm wide. S1-3 with yellow and black pattern like in male. S4-8 black with sharp yellow streaks on each side. S9 with a yellow spot at each side. S10 with a short yellow band in the middle.

Measurements [mm]: Entire length 31.6, abdomen length (excl. appendages) 20.5, Fw length 26.3, Hw length 25.0, Pt (Fw) 3.2, S7 2.0 mm.

Variation in females

The size of females (n = 7) varies only little: abdomen length 20.2-21.5 mm; Fw length 25.3-26.5 mm; Hw length 25.0-26.7 mm; Pt (Fw) length 3.2-3.5 mm; S7 1.8-2.1 mm broad. Two specimens have the central amber patch on the Hw, the others not. The basal amber of the wings varies between half to the whole first cell directly at the thorax. Fw with 9½-11½ Ax.

Colour plate II Male of *Trithemis palustris* sp. nov. — Okavango Delta, Third Bridge campsite in Moremi Game Reserve, Botswana (type locality), 31 January 2006. Photo by Jens Kipping.

Trithemis palustris sp. nov.

(Figures 1, 2a-c, e, Plate II, III)

Trithemis stictica (Burmeister). — Pinhey (1970: 126, 128, in part, notes on a Botswana series); — Kipping (2003); — Martens et al. (2003: in part)

Trithemis sp. nov. — Kipping (in press); — Kipping & Suhling (in press); — Suhling et al. (in press) — Suhling & Martens (2007: 233-234, in part).

Etymology

The adjective 'palustris' refers to its habitat, the swampy regions of the Okavango Delta and Kwando River.

Specimens studied

Total number of adult specimens examined: 11 ♂, 7 ♀. — **Holotype** ♂: Botswana, Okavango Delta, Moremi Game Reserve, Third Bridge (19°15´S, 23°20´E), ii 2007, leg J. Kipping; **Paratypes**: 2 ♂: Namibia, Nature Reserve Popa Falls, Okavango River at the rapids, iv 2003, leg. S. Damm; 4 ♂: Namibia, Mudumu National Park, Kwando River (18°30´S, 23°32´E), iv 2004, leg. F. Suhling. 1 ♂: Namibia, Omatako River, near Rundu (18°00´S, 20°35´E), iv 2004, leg. F. Suhling; 3 ♂, 7 ♀: same as holotype, leg J. Kipping. The holotype will be deposited in the National Museum of Namibia, Windhoek. Paratypes will stay at University of Veterinary Medicine Hannover, ITZ, Ecology & Evolution, Germany.

Description of holotype male

Head: Labium yellow with a broad black band in the middle also covering the posterior lobe and expanding onto anterior margins of lateral lobes. Face creamy yellow, postclypeus with two central, separated black streaks reaching the lower border. Labrum black with two elliptical lateral yellow spots. Frons metallic steel-blue. Antennae black. Occipital triangle black with two yellow posterior spots. Back of the head black with four yellow spots. Eyes with two colours; the upper part brownish red and the lower part grey.

Thorax: Prothorax black with anterior collar yellow. Median lobe with two yellow markings. Synthorax black and yellow dorsally, with light blue pruinosity. Metepimera yellow and black with little pruinosity. Legs black, with the inner side of the fore femora beige. — Wings: venation blackish. Pt brown between blackish veins. Base of the wings slightly yellow/amber. A light amber patch on Hw starting at the triangle covering only a few cells in the direction of the anal loop. In Fw 10 ½ Ax, in Hw 8 Ax, in Fw 14 Px, in Hw 12 Px. Fw triangle of 2, Hw triangle of 1, subtriangle of 3 cells; supratriangle uncrossed.

Abdomen: Abdomen slender, narrowest at S4 and widest at S8. S1-3 black with yellow pattern and ventrally with some blue pruinosity. S4-8 black with sharp yellow spots on each side. S9 black without any yellow. Dorsum of S10 with a yellow spot. Appendages black. Anterior lamina and hamule black with pale brown bristles and white hair around secondary genitalia. For details see Figures 2a-b. Penis of holotype not examined.

Measurements [mm]: Entire length 34.5, abdomen length (excl. appendages) 23.9, Fw length 27.0, Hw length 26.5, Pt (Fw) 3.2, appendages 1.5 mm, S4 3.8 mm.

Variation in males

Size variation in males (*n* = 11): abdomen length 22.7-23.8 mm; Fw length 26.5-27.8 mm; Hw length 25.5-27.0 mm; Pt (Fw) length 3.2-3.5 mm; appendages 1.5-1.6 mm; S4 3.7-4.0 mm. Colour of Pt is light brown in the Kwando River specimens, but dark brown in the others. The inner side is a slightly lighter brown. The amber patch on the Hw is absent in two specimens of the Okavango Delta, present in the Popa Falls males and only a trace of amber was found in the other specimens. The coloration of thorax and abdomen varied between dark brown and black.

Description of female

Described is paratype Tpal141F; 141: locality code Moremi Game Reserve, specimen F.

Head: Labium yellow with a broad black band in the middle extending onto the posterior lobe and the anterior margins of the lateral lobes. Face yellow. Postclypeus with two central comma-shaped streaks extending to the lower margins of the postclypeus. Frons and vertex metallic steel-blue/green. Antennae black. Labrum black with two elliptical lateral yellow spots. Occipital triangle black with two yellow posterior spots. Back of the head black with four yellow spots. Eyes with two colours; the upper part brown-red and the lower part grey.

Thorax: Prothorax black with yellow pattern. Synthorax yellow with black markings. Mesepisterna with a black streak in the middle; mesepimera, metepisterna and metepimera with a black streak on the anterior margin. Metepisterna additionally with a hook-shaped black streak ventrally. Legs black with the fore femora yellow on the inner side. Ventral side black with three yellow spots posteriorly. — Wings: venation blackish and Pt brown between black veins. Bases of the wings amber including half of the first cell directly at the thorax in Fw and Hw. Wing tips of Fw and Hw brownish, which also includes Pt. In Fw 10½-11½ Ax, in Hw 8 Ax, in Fw 13 Px, in Hw 13 Px. Fw triangle of 2, Hw triangle of 1, subtriangle of 3 cells; supratriangle uncrossed.

Abdomen: S4-10 thicker than in males, narrowest at S4; S7 1.7 mm broad. S1-3 with yellow and black pattern like in males. S4-8 are black with sharp yellow streaks on each side. S10 with a short yellow dorsal band in the middle.

Measurements [mm]: Entire length 33.8, abdomen length (excl. appendages) 24.5, Fw length 27.5, Hw length 26.3, Pt (Fw) 3.2, S7 1.5 mm.

Colour plate III Female of *Trithemis palustris* sp. nov. — Okavango Delta, Third Bridge campsite in Moremi Game Reserve, Botswana (type locality), 1 February 2006. Photo by Jens Kipping.

Variation in females

Size variation in females (*n* = 7): abdomen length 23.5-24.0 mm; Fw length 26.0-27.8 mm; Hw length 25.3-26.9 mm; Pt (Fw) length 3.1-3.5 mm; S7 1.4-1.7 mm broad. Most obvious is the variation in intensity and size of the infuscated area of the wing tips. The brownish coloration reaches up to the distal end of Pt in two specimens, in which the coloration is very intensive, and also three costal cells distal of the nodus are brownish. One specimen lacks darkened tips, and another has only a trace of brown at the extreme tip. Number of Fw Ax varied from 9½ to 11½ Ax.

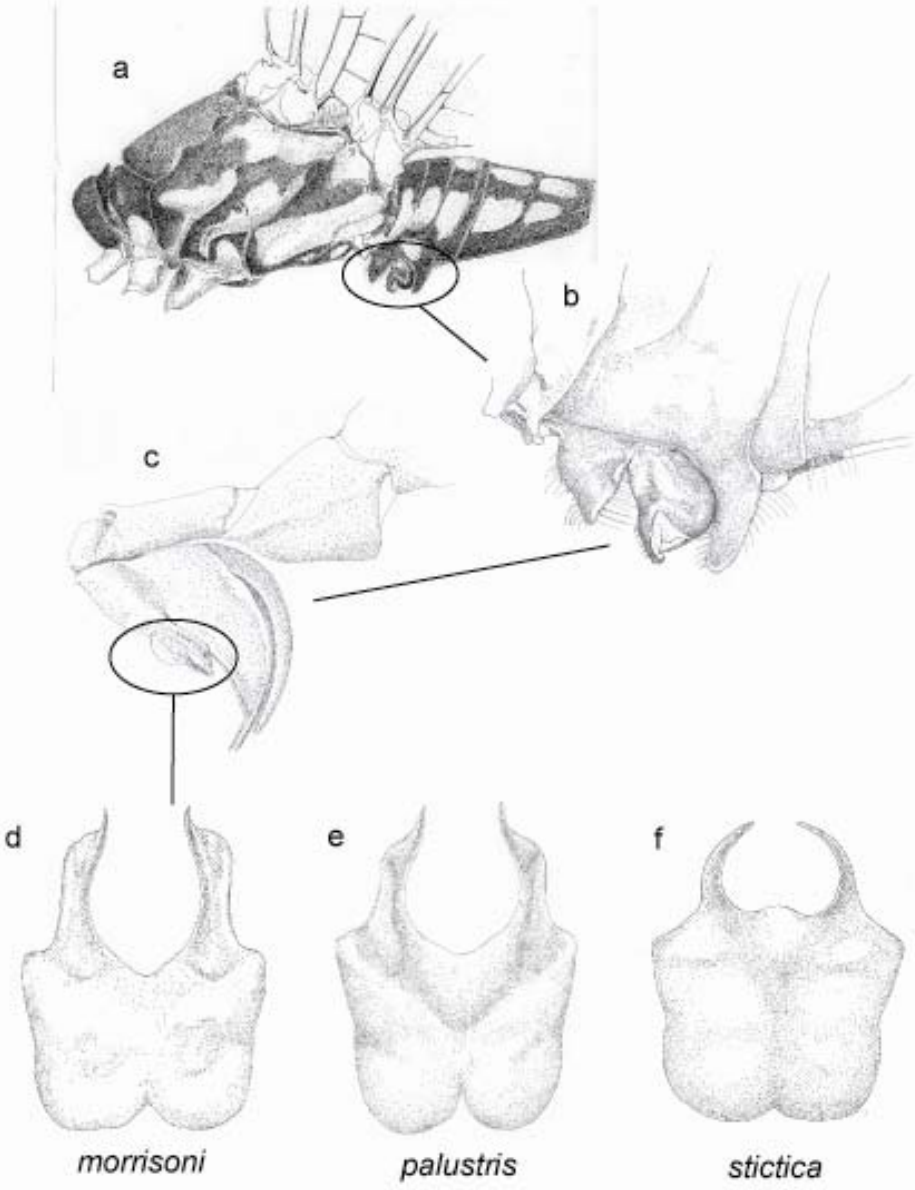

Figure 2 Male characters of *Trithemis morrisoni*, *T. palustris* and *T. stictica* — (a) thorax and S1-3, secondary genitalia only sketched, (b) secondary genitalia, (c) first two segments of the penis, including the distal segment and the lateral view of the "cornuti"; all in left lateral view of *T. palustris* but pattern and structure are the same in all three species; (d-f) comparison of the paired hook-shaped extension of the hood, the "cornuti", of *T. morrisoni* (d), *T. palustris* (e) and *T. stictica* (f).

Trithemis stictica (Burmeister, 1839)

(Figures 1, 2a-c, f)

Libellula stictica Burmeister, 1839: 850 (loc. typ. "Port natal" = Durban, RSA).

Trithemis stictica (Burmeister). — Brauer (1868).

Trithemis parasticta Pinhey, 1956: 35-37, figure 8a (loc. typ. Lake Chila, Abercorn, Zambia); — Lieftinck (1969: 52-53, "a very near ally to *T. stictica*", comparison of both species); — Pinhey (1970: 125, 129, "only a minor largish, dark variety", synonymy).

Trithemis stictica dwarfs, forms, subspecies — Pinhey (1970: 129, equatorial subspecies).

Specimens studied

Total number of adult specimens: 20 ♂. — 3 ♂: Namibia, Namib Naukluft Reserve, Tsaris Mountains, Zebra River (24°35´S, 16°20´E), iii 2003, leg. S. Damm; 2 ♂: Namibia, Namib Naukluft Reserve, Naukluft Mountains, Tsams Ost (24°15´S, 16°06´E) iv 2004, leg. F. Suhling; 4 ♂: Tanzania, East Usambara Mountains (5°05´S, 38°37´E), x 2002, leg. V. Clausnitzer; 1 ♂: Kenya, Nairobi National Park (1°25´S, 36°55´E), ix 2002, leg. V. Clausnitzer; - 5 ♂: Kenya, Kiboko River (2°15´S, 37°32´E), ix 2002, leg. V. Clausnitzer; 3 ♂: South Africa, Royal Natal Park (28°41´S, 28°48´E), 2001, leg. J. Ott; 2 ♂: RSA, Western Cape, Hawekwas Mts, Bains Kloof (33°55´S, 19°09´E), i 2006, leg. K.-D. B. Dijkstra.

Redescription of male

Described is reference male Tst 118D; 118: locality code Zebra River, Namibia, specimen D.

Head: Labium yellow with a broad black band in the middle, covering the posterior lobe and expanding to the anterior margins of lateral lobes. Labrum black with two yellow lateral spots. Frons and vertex steely blue. Face creamy yellow. Postclypeus with two central, separated, black comma-shaped streaks. Antennae black. Occipital triangle black with two yellow posterior spots. Back of the head black with four yellow spots. Upperside of eyes light red grading to light grey on the underside: the colours thus not sharply demarcated.

Thorax: Prothorax black with slight yellow markings. Synthorax except ventrally with blue pruinosity. Ventral side with yellow and black patterns. Metepimera with less pruinosity. Here yellow with the anterior side black. Legs black with the inner side of fore femora light

brown. — Wings: clear with dark brown venation. Pt brown, grading to light brown on proximal side, between dark brown veins. Light amber area starting at the triangle and covering the anal loop of Hw. In Fw 9½ - 10½ Ax, in Hw 8 Ax, in Fw 13 Px, in Hw 12 Px. Fw triangle of 2, Hw triangle of 1, subtriangle of 2 cells; supratriangle uncrossed.

Abdomen: Slender with S4 narrowest. S1 black dorsally and yellow ventrally. S2 black with two short yellow streaks. S3 black with yellow pattern. S4-8 black with a single row of yellow streaks on each side. S9 with a yellow spot on each side. S10 black with a central dorsal yellow line. Appendages dark brown. Hamule and anterior lamina black and coated on outer side with short thick setae and brown bristles (Figures 2a-b).

Measurements [mm]: Entire length 33.5, abdomen length (excl. appendages) 23.5, Fw length 28.2, Hw length 26.3, Pt (Fw) 3.3, appendages 1.5 mm, S4 3.8 mm.

Variation in males

Size variation in males (n = 20): abdomen length 22.1-24.8 mm; Fw length 27.8-30.5 mm; Hw length 26.5-29.5 mm; Pt (Fw) length 3.2-3.8 mm; appendages 1.2-1.7 mm; S4 3.6-4.0 mm. The colour of the Pt is brown in most of the specimens, but dark brown in the Tanzanian males, with the proximal side slightly lighter brown. The amber patch on Hw present in all specimens but varying in intensity. The coloration of thorax and abdomen is black and bright yellow in the South African, Tanzanian and Kenyan specimens, but brown with creamy yellow in the Namibian ones. Yellow spot on S9 is present in four of the Namibian males, but absent in the others.

Table 1 Statistical significance of Wilcoxon test (p-value) of the different morphological length parameters of males of *Trithemis morrisoni*, *T. palustris* and *T. stictica*. Bs: width of Hw base; A: length of accessory genitalia along the hamules; B: length of genital lobe; C: length of the anterior lamina; D: length of the hook of the hamule; E: width of the hamule.

	Hw	Pt Hw	Bs Hw	Abd	App	S4	A	B	C	D	E
stictica/palustris	0.01	0.15	0.01	0.76	0.14	0.69	0.15	0.35	0.21	0.16	0.91
stictica/morrisoni	0.00	0.11	0.00	0.00	0.02	0.00	0.00	0.33	0.10	0.08	0.87
morrisoni/palustris	0.21	0.68	0.75	0.01	0.36	0.01	0.09	0.94	0.76	0.06	1.0

Diagnostic characters of the three taxa

Male morphology

The most obvious character that distinguishes *Trithemis morrisoni* sp. nov. and *T. palustris* sp. nov. from *T. stictica* is the eye coloration (see Plates, III, IV). The eyes of *T. morrisoni* and *T. palustris* are red-brown on the upper- and grey-blue on the underside. In mature males of *T. palustris* the red-brown coloration can change to bluish but a brown tinge is always left (Colour plate II). In contrast, the eyes of *T. stictica* show no colour separation. A second character is the amber base of the wings that both new species have, but is absent in *T. stictica*.

The trait with the most evidence for speciation is the morphology of the penis. SEM revealed a different shape of the "cornuti" (terminology by Pinhey 1970), the paired hook-shaped extensions of the hood of the distal segment of the penis (Figures 2d-f). In *T. stictica* the "cornuti" are curved rods which are pointed at the end, as illustrated by Pinhey (1970). The "cornuti" of *T. morrisoni* and *T. palustris* are broad in the middle and only the tip is narrower. This character is readily visible with stereomicroscopy. All 23 examined males of *T. morrisoni* and *T. palustris* show this difference with *T. stictica*. Between the two new species only slight individual variation in the "cornuti" was found (Figures 2d, e).

Statistical analyses (Wilcoxon test) of the length of the hind wing, abdomen and S4 show significant differences between the three species. In *T. stictica* the hind wings are significantly longer than in the two new species. In *T. morrisoni* the length of abdomen and S4 are significantly shorter than in *T. stictica* and *T. palustris* (Table 1). These size differences between the two new species are significantly correlated with the distinct genetic patterns (SD, HH unpubl.). Together with the fact that no overlap was observed between the species, these characters are valuable morphological characters for the populations studied here. Whether other populations might show overlaps cannot be decided yet.

All analysed individuals show the same colour pattern on thorax and abdomen, and similar external secondary genitalia as described for *T. stictica* (Figures 2a, b). Nevertheless, specimens from different geographical regions show slight differences in coloration. Specimens from Kenya, Tanzania and South Africa are black with yellow markings under the blue pruinosity, while those from Namibia appear dark brown with beige-yellow markings. This is, however, not congruent with the genetic results and can be regarded as regional intraspecific colour variation. In addition, some traits were found in a few specimens of each species. The yellow spot on S9 was found in some *T. morrisoni* and *T. stictica*, but not in *T.*

palustris. The markings on the postclypeus were absent in half of the specimens of *T. morrisoni*, but present in all *T. palustris* and *T. stictica* males. In *T. palustris* these comma-shaped streaks reach to the anterior border of the postclypeus while in *T. morrisoni* and *T. stictica* only small and short commas were found. A summary of the male characters is given in Table 2.

Table 2 Comparison of morphological characters of males of *Trithemis morrisoni*, *T. palustris* and *T. stictica*. All measurements in [mm]. *p*-values are shown in Table 1.

	morrisoni (*n* = 12)	*palustris* (*n* = 11)	*stictica* (*n* = 20)
Range	Okavango River and Zambezi River (Namibia, Zambia)	Okavango River and Delta, Kwando River (Botswana, Namibia)	Eastern to southern Africa
Abd length	19.9-22.5	22.7-23.8	22.1-24.8
Hw length	23.2-26.8	25.5-27.0	26.5-29.5
Pt length	3.2-3.7	3.2-3.5	3.2-3.8
Cerci length	1.3-1.6	1.5	1.2-1.7
S4 length	3.3-3.5	3.7-4.0	3.6-4.0
Wing base width	1.4-1.7	1.3-1.6	1.3-1.8
Eyes	Bicoloured	Bicoloured	Unicoloured
Wing base	Amber	Amber	Clear
"Cornuti" of penis	Broader in the middle	Broader in the middle	As described by Pinhey (1970)

Female morphology

The size difference between *T. morrisoni* and *T. palustris* was also found in the analysed females (Table 3). *T. morrisoni* females sampled in Zambia, are very small and have a similar size to males. The females of *T. palustris* sampled in the Okavango Delta in Botswana are significantly larger (Table 3). One other character is notable: the coloration of the wings. Six of the seven *T. palustris* females from Botswana have yellow-brownish tips of the fore and hind wings, which are missing in *T. morrisoni*. Some characters were found to be species-specific in the females, but not in males. All analysed females of *T. morrisoni* have the yellow spot on S9, which is missing in *T. palustris*. However, some field-collected females of *T. palustris* do have this spot on S9 (J. Kipping pers. comm.). Therefore this difference has to be confirmed by additional sampling. The comma-shaped streaks on the postclypeus were only found in *T. palustris*, and furthermore *T. morrisoni* showed a broad S7, which is narrower in *T. palustris* (Table 3).

Table 3 Morphological characters of the analysed females of *T. morrisoni* and *T. palustris* including the *p*-values of the statistical tests. All measurements in mm.

	morrisoni (*n* = 7)	*palustris* (*n* = 7)	*p*-values
Locality	Zambezi River (Zambia)	Okavango Delta (Botswana)	
Abd length	20.2 – 21.5	23.5 – 24.0	0.02
Hw length	25.0 – 26.7	25.3 – 26.9	0.48
Pt length	3.2 – 3.5	3.1 – 3.5	0.70
Wing base width	1.4 – 1.6	1.5 – 1.6	0.23
S7	1.8 – 2.1	1.4 – 1.7	0.03
Colour eye underside	Yellow	Grey	
Postclypeus	Without black streaks	With black streaks	
Wing tip	Clear	Brownish	

Habitat and distribution

Both new species were thus far only found in the region of the Okavango and Zambezi Rivers, including the Okavango Delta, and the Omatako and Kwando Rivers. *T. morrisoni* was collected at Andara and Popa Falls (Okavango River) and at Bovu Island (Zambezi River), while *T. palustris* was found at Rundu (Omatako River), the Okavango Delta, the Kwando River and also at Popa Falls (Figure 1). *T. morrisoni* occurred at river sections with rapidly flowing water and intact gallery forest (e.g. at Popa Falls) and seemed to need at least some fast flowing side-channels of larger rivers to occur. It was absent from large and calm rivers like the Zambezi east of Lake Kariba. The main habitat of *T. palustris* appeared to be open habitats at slow flowing sections of rivers or swamps. In the Okavango Delta it was locally the most common anisopteran odonate and preferred little channels and calm rivers with swampy margins and connected floodplains. Exuviae were found at almost stagnant sections of rivers and in the nearby floodplains. It was absent from temporary flooded pans and pools. Tenerals were found in large numbers in patchy gallery forest (Kipping 2006). *T. stictica* was not found in the same region although its preference for open swamps, rivers and pools (Pinhey 1970) seems to fit. In general *T. stictica* is distributed in the whole of sub-Saharan Africa (Figure 1). The Odonata Database of Africa (ODA) (J. Kipping pers. comm.) contains 537 records of this species. The westernmost records come from Sierra Leona and Liberia; in the north it occurs in Sudan, the Ethiopian highlands and Somalia. It is scarce in the mountainous parts of Central Africa and most records come from the southern countries of Zambia, Zimbabwe and South Africa. It prefers higher elevation than other members of the genus. Mean elevation of all records of *T. stictica* is 1,052 m a.s.l. (*n* = 537).

Discussion

In his monograph on the genus *Trithemis*, Pinhey (1970) described *T. stictica* as a "variable species." He studied specimens from a wide range of localities and described several regional "forms" but none of these can be clearly assigned to either of the new species. Consequently the genetic characteristic of none of Pinhey's varieties is known. He mentioned a form, possibly a subspecies, in the Okavango region with creamy or ivory faces instead of the normal yellow. We can confirm this variation in our specimens from the Okavango region, but all other analysed specimens from Namibia also show ivory instead of yellow. We regard this variation as a phenotypic rather than a diagnostic character correlated with genealogy. However, Pinhey also noted the amber base of the wings in his Okavango specimens. This character indeed distinguishes *T. stictica* from *T. morrisoni* and *T. palustris*. Pinhey also described a "dwarf series" from Victoria Falls. These specimens are relatively small and show only two rows of cells in the fore wing discoidal field. *T. morrisoni* males and females from the Zambezi River near Victoria Falls are also smaller, but all have the normal three rows of cells. Pinhey described two females of a possible Equatorial subspecies with saffronated wings and an entirely black labrum, but these features were not found in any of the analysed specimens.

Additionally, Pinhey mentioned several other variable traits in his specimens of *T. stictica*, like the yellow spot on S9, postclypeus with or without comma-shaped streaks, amber patch centrally on the hind wing absent or present, and occasionally infuscated wing tips in the females. We found these traits in some of our specimens, but they are not species-specific. The yellow spot on S9 is absent in *T. palustris*, but was also not always present in *T. stictica* and *T. morrisoni*. The amber patch is present in most specimens of the three species, but not all. We found the darkened wing apices in six out of seven analysed females of *T. palustris*.

The status of *T. parasticta* was discussed by Pinhey (1956, 1970) and Lieftinck (1969). Pinhey (1956) described *T. parasticta* as a near ally of *T. stictica*, but larger and without the central amber patch in the hind wings. While Lieftinck (1969) confirmed its species status by comparing specimens from Lake Bangweulu with Pinhey's original description, Pinhey (1970) himself finally regarded *parasticta* merely as a larger form of *T. stictica*. We compared the diagnosis of *T. parasticta* by Lieftinck (1969) with the two new species but none of the listed traits were found. The thoracic pubescence is white as in *T. stictica* and the pterostigma has nearly the same length in all analysed individuals. Also the base of the hind wings varies only slightly in length and is smaller in *T. morrisoni* and *T.*

palustris than in *T. stictica*. The superior appendages are wholly black and the yellow or amber antenodal patch on the hind wings generally exists in all three species, but varies in intensity and is absent only in some specimens. This variation is common to all three examined groups. We conclude that none of Pinhey's forms or subspecies, including *parasticta*, is one of the new species, except his possible (but unnamed) Okavango sub-species, which may have included both new species.

The genetic and morphological results support the separation of the two new species from *T. stictica* in the Okavango and Upper Zambezi Floodplains (Rach et al. 2008; SD, HH unpubl.). There are various characters to distinguish *T. morrisoni* and *T. palustris* from *T. stictica*, like the amber base of the wings, the dichromatic eyes and the structure of the penis. The latter is clearly most important due to its potential as a reproductive barrier. In addition a high sequence divergence between *T. stictica* and the two new species in four different markers (9.0% and 8.5% in ND1, 8% and 8.3% in COI, 4.5% and 4.3% in 16S and 2.0% and 2.1% in ITS, respectively) clearly separates them at the species level (SD, HH unpubl.).

The two new species cannot be identified easily in the field. However, genetic analyses clearly separate them into distinct species. We analysed 73 specimens from different sites using four genetic markers. Each sample clearly falls into only one of the two species. The sequence divergence between the two species is clearly at the species level with 5% in ND1, 5.7% in COI, 1% in 16S and 2.1% in ITS I&II (SD, HH unpubl.). A phylogenetic analysis of the genus using 37 of 40 known species corroborates the results (SD, K.-D.B. Dijkstra, HH unpubl.). Here genetic distances between other closely related species are even lower than between *T. morrisoni* and *T. palustris*, e.g. *T. donaldsoni* and *T. dejouxi* with 3.5% in ND1 or *T. grouti* and *T. aenea* with 0.6% in 16S. These levels of genetic distances were also found between other distinct odonate species, e.g. in the genera *Pseudagrion*, *Calopteryx* and *Enallagma* with the same used markers (Misof et al. 2000; Weekers et al. 2001; Turgeon & McPeek 2002; Dijkstra et al. 2007). In comparison with the study of Samraoui et al. (2003) who describe a new “cryptic” species of *Lestes* based on ITS I sequences only, we could confirm our hypothesis with four, independently inherited sequence markers. Although both species occur in the same geographical region they show high genetic distances indicative of complete reproductive isolation. The initial examination of female morphology shows that more distinguishing features may be identified in that sex, and more female samples would complement our analyses.

Interestingly, the two new species have maintained distinct genetic patterns despite a similar morphology and geographical distribution. The range of both is the Okavango and

Upper Zambezi Floodplains. Nevertheless, within this region, the occupied sites differ: *T. morrisoni* was found near fast flowing water and rapids within intact gallery forest, e.g. Popa Falls and the Zambezi River near Victoria Falls. *T. palustris* was found in open areas in swamps and along slow-flowing river sections, e.g. Okavango Delta and Kwando River. The area around Popa Falls, where both species occur, provides both habitats. Because the habitat conditions differ especially for the larvae, morphological analyses of them may be a good next step. More data on the distribution and ecology of the two new species are necessary, but because they seem to occupy different ecological niches, speciation of *T. morrisoni* and *T. palustris* was most likely induced by a habitat shift (SD, HH unpubl.).

Acknowledgements

The study was supported by a grant from the German Ministry of Research and Education, BIOLOG Programme (BMBF 01LC0024) and was part of the Biodiversity Transect Analysis in Africa (BIOTA-South S08). We are grateful to Viola Clausnitzer, Klaas-Douwe B. Dijkstra, Vincent Kalkman, Jens Kipping, Kai Schütte, Frank Suhling and Jürgen Ott for providing specimens. We thank Klaas-Douwe B. Dijkstra, Jens Kipping, Danielle de Jong and Jessica Rach for helpful discussions and comments. Many thanks also to Ludger Wickenbrock, who prepared the drawings.

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Cryptic speciation via habitat shift:

A case study in the odonate genus *Trithemis*

Sandra Damm[1] & Heike Hadrys[1,2]

[1]*ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany*

[2]*Yale University, Department of Ecology and Evolutionary Biology, New Haven, CT, 06520-8104, USA*

This work is prepared for submission to *Proceedings of the Royal Society, Biological Science B*

Abstract

Speciation processes provide a major challenge to evolutionary biology, and understanding the underlying mechanism is of basic importance for conserving the diversity of life. The complexity of the processes behind speciation events and the different used criteria or definitions often causes problems by classifying case studies into the three major modes of speciation. A recently discovered species complex of three African dragonfly species in the genus *Trithemis* provides an interesting model system to analyse their divergence by combining biogeographical as well as population genetic parameter. The newly detected species, *T. morrisoni* and *T. palustris*, coexist in the same geographical range in the region of the Okavango River and the Zambezi River (Caprivi region), while *T. stictica,* which formerly included the two new species, is distributed throughout sub-Saharan Africa and absent in the Caprivi region. To study the underlying speciation processes we analysed different mitochondrial (ND1, COI and 16S) and nuclear markers (ITS I and II) and compared the population genetic data to morphological and ecological traits. Our results show that despite a clear geographical overlap, the two new species have been completely genetically isolated for approximately 2.4- 0.7 million years. Our data suggest that two different speciation mechanisms have driven the divergence of the three closely related species. While *T. stictica* evolved through allopatry, the other two species most likely evolved nonallopatric as a result of a habitat shift. To our knowledge this is the first example for cryptic speciation in dragonflies.

Keywords: Speciation processes, cryptic species, Odonates, sympatric speciation, *Trithemis*

Introduction

Species divergence is of great interest to evolutionary biologists and intensive research on a broad spectrum of aspects of speciation has been conducted. Theoretical and empirical studies have aimed towards an understanding of the different modes of speciation, from sympatric to parapatric and allopatric speciation (e.g. Avise et al. 1998; Barraclough & Vogler 2000; Barluenga et al. 2006). The complexity of the mechanisms and processes behind speciation events and the difficulties in diagnosing empirical studies still challenges us. Classifying individual case studies into the taxonomic system of 'modes of speciation' often causes problems and is sometimes even impossible. Part of the problem is the use of different definitions and criteria for diagnosing case studies. While allopatric speciation as the basic mode of reproductive isolation through biogeographical barriers seems to be well defined and understood, parapatric speciation and especially sympatric speciation are more difficult to prove and consequently to verify in empirical studies. Here several definitions, conceptually either biogeographical or population genetic based, are alive in the literature (e.g. see overview Fitzpatrick *et al.* 2008). The biogeographical concepts of sympatric speciation define that the new species have to evolve in the same geographical range and species must be able to move between e.g. different habitats without geographical isolation (e.g. Ridley 1996; Berlocher & Feder 2002; Coyne & Orr 2004). The population genetic definitions are more precise and require an initial panmictic population with high gene flow (i.e. m=0.5) and the mating probability of two individuals should depend on their genotypes only (e.g. Johnson & Gullberg 1998; Gavrilets 2003). In that context also the problem of the regarded geographical scale becomes apparent and terms like “microalloptry” were introduced to define the speciation processes of populations which occur allopatric on a very small biogeographical scale, like e.g. in diverging host/habitat adaptations (Berlocher & Feder 2002; Fitzpatrick *et al.* 2008).

In the biogeographical concepts excluding allopatry might be possible in studies where species occur in the same geographical range, but demonstrating continuous gene flow during the time of divergence is nearly impossible in empirical studies. Most cases which fail to satisfy the precise conditions of sympatric speciation but are clear cases of nonallopatry fall into the broad category of “divergence-with-gene-flow” (Gavrilets 2003; Bolnick & Fitzpatrick 2007; Niemiller *et al.* 2008). This model integrates all processes in which population divergences with continuous gene flow as well as alternating periods of gene flow with periods of complete isolation could occur by only strictly excluding allopatric speciation

(Bolnick & Fitzpatrick 2007; Niemiller et al. 2008). Nevertheless, a corroborative approach, combining ecology, phylogeography, population genetics and behaviour might be a way when attempting to understand the biological processes affecting divergence in nature.

The evolution of cryptic species adds an additional evolutionary arena when analysing speciation processes. Here speciation takes place without the evolution of morphological different characters. With the increasing number of population genetic studies cryptic species are found in many animal groups across nearly all biogeographical regions. However, questions concerning the evolutionary and ecologically processes leading to genetic divergence in the absence of morphological differentiation often remain unresolved (Pfenninger 2007, Bickford 2007).

Odonates – dragonflies and damselflies – are not supposed to evolve real morphological cryptic species because of its complex mating system with the 'lock and key' mechanism, where the fit of genitalia is thought to be strong evidence for distinction between species. Their complex morphology is abundantly supplied with taxonomic characters, like wing venation, thoracic patterning or colour variation. In cases of similar morphological appearance at least differences in the genital morphology were found (Pilgrim 2002). In combination with a complex life cycle (aquatic larvae and terrestrial adults), a striking diversity of different biogeographical ranges, habitat specificities, colour patterns and behaviour (Corbet 1999), speciation processes in odonates are assumed to evolve in allopatry (Stoks *et al.* 2005; Turgeon *et al.* 2005; Dijkstra & Clausnitzer 2006; Kalkman *et al.* 2008).

In this ancient group of insects the discovery of a cryptic species complex in the African libellulid genus *Trithemis* constitutes a highly interesting and special case in speciation. The species complex of three closely related *Trithemis* species was only recently discovered via population genetic analyses (Damm & Hadrys 2009). Two new species (*T. palustris* and *T. morrisoni*) were previously hidden inside a third species, *T. stictica*. While *T. stictica* can be distinguished morphologically from *T. palustris* and *T. morrisoni* by differences in genital morphology and colour patterns, the latter two stay cryptic. Both new species have thus far only been found alongside the big river systems Okavango and Zambezi, where they occur in the same geographical range.

This case study of diversification allows to analyse two different speciation processes in three closely related sister species. In addition our *Trithemis* model demonstrates an example of a cryptic speciation process in an insect order that is not expected to evolve cryptic species and might therefore provide new insights into the divergence of odonates. We analysed a set of four sequence markers with different substitution rates and origins (ND1,

16S, COI and ITSI-II) and combine biogeographical with population genetic data of the whole species complex. To reconstruct the speciation processes governing the divergence of the three species we discuss the possibility to classify our two different speciation processes into the taxonomic system of modes of speciation by regarding their various definitions.

Methods

Field sampling

A total of 108 samples of *T. stictica, T. palustris* and *T. morrisoni* were collected from 12 different localities in Namibia, Botswana (Okavango Delta), Zambia (Zambezi River), South Africa (Western Cape), Tanzania (East Usambara Mountains), Kenya (Kiboko River) and Ethiopia (Ambo) (see Table 1a and Figure 1). All samples were initially identified as *T. stictica* and cover the distributional range of this species. First genetic analyses discovered the existence of two more species (*T. palustris* and *T. morrisoni*) which are regionally restricted to the Okavango and Zambezi floodplains (Damm & Hadrys 2009; Damm et al. 2009). At all other localities *T. palustris* and *T. morrisoni* were not found. For phylogenetic analyses nine other *Trithemis* species were integrated (see Table 1b). Tissue samples were collected and stored in 70% Ethanol.

Table 1a Population sites (country and locality), used abbreviations, number (n) of individuals, number of haplotypes (No H), haplotype diversity (*h*) and nucleotide diversity (π) of ND1 and 16S for *T. stictica, T. morrisoni and T. palustris*.

Species	Country	Locality	Abbrev.	n	No H ND1 / 16S	*h* ND1 / 16S	π (10^{-3}) ND1 / 16S
T. stictica	Namibia	Naukluft	TstNauk	8	2 / 2	0.25 / 0.25	0.6 / 0.6
	Namibia	Zebra River	TstZebra	9	1 / 2	0 / 0.22	0 / 0.4
	Kenya	Kiboko River	TstKen	5	2 / 2	0.33 / 0.33	1.0 / 1.0
	Tanzania	East Usambara Mts.	TstTans	5	2 / 2	0.4 / 0.4	1.0 / 1.1
	South Africa	Western Cape	TstSA	5	2 / 3	0.67 / 0.83	8.0 / 3.36
	Ethiopia	Ambo	TstEth	1	1 / 1	-	-
T. morrisoni	Namibia	Popa Falls	TmorPopa	21	3 / 6	0.35 / 0.80	1.3 / 4.16
	Namibia	Andara	TmorAnd	3	2 / 3	0.67 / 1	2.7 / 4.03
	Zambia	Bovu Island	TmorZam	17	5 / 3	0.79 / 0.62	2.2 / 1.5
T. palustris	Namibia	Rundu	TpalRund	3	2 / 2	0.67 / 0.67	2.7 / 1.3
	Namibia	Kwando River	TpalKwan	8	4 / 2	0.64 / 0.53	3.6 / 1.1
	Namibia	Popa Falls	TpalPopa	10	5 / 3	0.72 / 0.46	2.2 / 4.8
	Botswana	Okavango Delta	TpalBot	11	8 / 4	0.93 / 0.71	3.7 / 1.8

Table 1b Population sites (country and locality), used abbreviations and number (n) of individuals for nine additionally included *Trithemis* species.

Species	Country	Locality	n
T. kirbyi	Namibia	Tsaobis/ Waterberg	5
T. arteriosa	Namibia	Tsauchab/ Waterberg	5
T. annulata	Namibia	Rehoboth/ Popa Falls	5
T. donaldsoni	Namibia	Rehoboth/ Van-Bach-Dam	5
T. hecate	Namibia	Popa Falls	5
T. furva	Ethiopia/South Africa	Nekemte/ Wakkerstrom	5
T. grouti	Liberia	Gola Forest/ Lorma Nat. Forest	5
T. nuptialis	Congo	Lingomo/ Lukomete	2
T. werneri	Namibia	Kunene	2

DNA extraction and amplification

DNA was isolated from a single leg of each individual using a modified phenol-chloroform extraction (Hadrys et al. 1992) and stored in TE-buffer at -20°C. In addition to previously amplified ND1 (all 108 individuals) and COI gene regions (five individuals of each species) (Damm et al. 2009), we isolated the mitochondrial 16S rDNA and the nuclear ITS I - II region (Internal spacer regions I and II) including the intermediate 5.8S region. For amplification of a 570 bp fragment of the 16S region, primers described in Simon et al. (1994) were used. The PCR thermal regime was as follows: 5 min initial denaturation at 93°C, followed by 35 cycles of 93°C for 20 s, 52°C for 30 s, 72°C for 40 s, and 2 min final extension at 72 °C. PCR reactions were carried out in a total volume of 25 µl, containing 1× amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 5 pmol each primer, and 0.75 U *Taq* DNA polymerase (Invitrogen). For the nuclear ITS region, primers were designed based on known insect sequences from GenBank. The forward primer (ITS-Odo fw : 5`CGT AGG TGA ACC TGC AGA AG 3`) lies within the 18S rDNA and the reverse primer (ITS-Odo rev: 5`CTC ACC TGC TCT GAG GTC G 3`) within the 28S rDNA region. Amplification was successful under following conditions: initial denaturation for 3 min at 95°C, 35 cycles of 95°C for 30 sec, 54°C for 40 sec and 30 sec at 72°C and a final extension at 72°C for 3 min. The final volume of 25 µl contained 1× amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 5 pmol of each primer, and 0.75 U *Taq* DNA polymerase (Invitrogen). Sequences of the 16S rDNA and the ITS regions (including 5.8S) are available under GenBank Accession numbers XXX (submitted and will be included).

Purified PCR-products were sequenced in both directions using the ET Terminator Mix (Amersham Bioscience). Sequencing reactions were carried out in 7.5 µl volumes

containing 7.5 pmol primer, 5-10 ng template, 1.5 µl ET Terminator mix and 0.5 µl Buffer (Amersham Bioscience). Cycle sequencing was performed according to the manufacturer's protocol. Sequencing reactions were purified and subsequently sequenced on an automated sequencer (MegaBACE 1000; Amersham Bioscience).

After sequencing, both strands were assembled and edited using Seqman II (version 5.03; DNAStar, Inc). Multiple sequence alignments were done using MUSCLE (version 3.6; (Edgar 2004)).

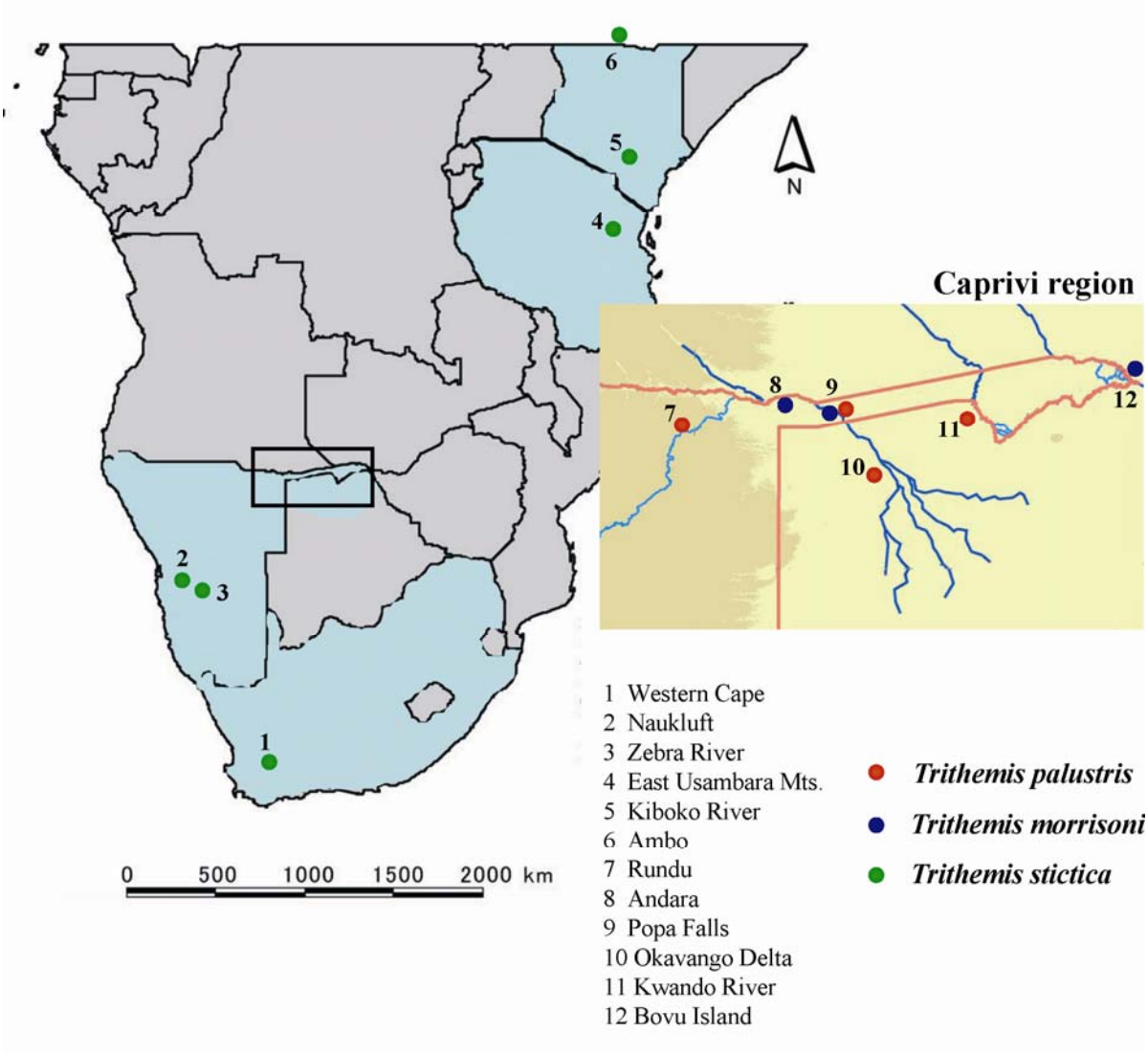

Figure 1 Overview of Southern Africa with the analysed countries displayed in blue. Shown are the samples sites of the three *Trithemis* species *T. stictica* (green dots), *T. palustris* (red dots) and *T. morrisoni* (blue dots). Area of detail: the population sites of *T. palustris* and *T. morrisoni* in the Caprivi region.

Sequence Analyses

Sequences from ND1 and COI (GenBank accession nos: FJ358442- FJ358475) of a recent study, which firstly discovered the new species *T. palustris* and *T. morrisoni* (Damm et al. 2009) were included in the analyses. Here the 108 ND1 sequences covering the three species were used for all analyses, while COI (five individuals for each species) were used to analyse sequence divergences and molecular clock analyses. This way, four genetic markers comprising different substitution rates and origins could be applied.

Sequence divergence between individuals and species were calculated using the Kimura-2-parameter substitution model via PAUP (version 4.0b10; (Swofford 2002)). Estimates of haplotype diversity (*h*) and nucleotide diversity (π) were carried out using DNASP version 4.0 (Rozas et al. 2003). Genetic differentiation (F_{st}) (Weir & Cockerham 1984) based on the average number of pairwise nucleotide differences within and between *T. stictica, T. palustris* and *T. morrisoni* was computed in ARLEQUIN version 3.0 (Excoffier et al. 2005) with significance determined by 10,000 bootstrap replicates.

Based on statistical parsimony, a mutational network for the two mitochondrial markers (ND1 and 16S) sequenced for all 108 individuals was generated using TCS version 1.21 (Clement et al. 2000) and relationships between the haplotypes of *T. stictica*, *T. palustris* and *T. morrisoni* were estimated. Individual sequences were collapsed to haplotypes and the frequency of each haplotype was incorporated into the analyses. Ancestral haplotypes were calculated by predictions of coalescent theory (Clement et al. 2000).

Phylogenetic relationships of species were inferred by Bayesian and Maximum Parsimony algorithms. For Bayesian analyses, the TrN+I model for ND1 and the HKY+I+G model for ITS and 16S were applied, which were previously selected using Modeltest version 3.7 (Posada & Crandall 1998) as the best fitting evolutionary nucleotide substitution model under the Akaike Information Criterion. The model parameters were employed in the phylogenetic analysis using MrBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001). Marcov-Chain Monte-Carlo posterior probabilities were determined for each gene partition and for a concatenated matrix. For each analysis the most appropriate parameters for among site variation, base frequencies and discrete gamma distribution were employed. The Marcov-Chain Monte-Carlo search was performed with four chains for 1,500,000 generations and trees were sampled every 750th generation. Maximum Parsimony (MP) analyses were performed as implemented in PAUP version 4.0b10 (Swofford 2002). A heuristic search for each marker and a combined dataset was performed with TBR branch swapping and random addition of taxa for 1000 replicates. Reliability of the parsimony analysis was assessed by

bootstrap sampling (Felsenstein 1985) of 1000 replicates. For detailed analyses of the species complex *T. stictica, T. palustris* and *T. morrisoni*, a combined dataset including all analysed individuals were used. *T. furva* served as an outgroup. Phylogenetic analyses of the nine *Trithemis* species and *T. stictica, T. palustris* and *T. morrisoni* were performed using ND1 and 16S sequences. *Crocothemis erythrea* (Libellulidae) served as an outgroup.

In order to test the suitability of a molecular clock to evaluate the time of divergence between the three species, ML analyses with the appropriate evolution model was performed with and without clock enforcement. The Shimodaira-Hasegawa (Shimodaira & Hasegawa 1999; Goldman et al. 2000) and the Kishino-Hasegawa (Kishino & Hasegawa 1989) tests were used to investigate whether the topologies of the two ML trees were significantly different. The genetic distances of ND1, COI and 16S were then used for comparisons and molecular divergence time estimates. The dating calculations were based on the mutation rates of 2.3% for ND1 and COI, and 1.4% for 16S as proposed for insect mitochondria (Brower 1994) and as applied in several other odonate studies (Turgeon et al. 2005; Stoks & McPeek 2006).

Results

Sequence variation

An alignment of 496 bp of the 16S fragment, containing 108 sequences from *T. stictica, T. palustris* and *T. morrisoni* exhibited 28 variable and 26 parsimony informative sites. In total, 17 different haplotypes were found with no haplotype shared by the three species. *T. stictica* is represented by five, *T. palustris* by four and *T. morrisoni* by eight haplotypes. In total 26 different haplotypes were identified for ND1, again with no shared haplotypes by the three species. *T. stictica* is represented by 5, *T. palustris* by 13 and *T. morrisoni* by 8 haplotypes. For COI nine species specific haplotyes were found.

Details of genetic diversity (number of haplotypes [NoH], haplotype diversity [*h*] and nucleotide diversity [π]) measured for each population site of the three species for 16S and ND1 are shown in Table 1. For *T. stictica* both markers show a low level of genetic diversity within all populations (except of South Africa). In the South African population, the highest number of haplotypes was found and also the highest *h* and π. In contrast to *T. stictica,* the genetic diversity in the populations of *T. palustris* and *T. morrisoni* was quite high.

For the nuclear ITS region, 98 samples were successfully sequenced and the alignment included 633 bp showing gaps at 13 positions, 26 variable and 25 parsimony informative sites. The pure ITS regions were substantially more variable than the 5.8S gene co-amplified in the sequences. ITS I (65% of the variable sites, 92 % of the gaps) and ITS II (35% variable sites, 8% of the gaps) exhibit all gaps and variable positions.

Table 2 Mean intra- and interspecific sequence divergences based on the Kimura-2-parameter (in %) of the analysed sequence markers ND1, COI, 16S and ITS of *T. stictica, T. palustris* and *T. morrisoni.*

	T. stictica				*T. palustris*				*T. morrisoni*			
	ND1	COI	16S	ITS	ND1	COI	16S	ITS	ND1	COI	16S	ITS
T. stictica	0.4	0.2	0.1	0.3								
T. palustris	9.0	7.9	4.3	1.1	0.3	0.1	0.3	0.1				
T. morrisoni	8.5	8.3	4.5	1.5	5.0	5.7	1.0	1.3	0.5	0.1	0.3	0.3

Sequence divergence

Intraspecific sequence divergence of *T. stictica* was low and varied between 0 to 1% in ND1, 0 to 0.4% in 16S and 0 to 0.7% in ITS, although the geographical distances between populations ranges from 20 to 3200 km. The highest level of sequence divergence was found between the South African and all the remaining *T. stictica* populations (1% in ND1; 0.4% in 16S and 0.7% in ITS). Between the populations of *T. palustris*, the sequence divergence ranged from 0.2 to 0.4% in ND1, 0.1 to 0.5% in 16S and 0 to 0.2% in ITS. The sequence divergence between the *T. morrisoni* populations ranged from 0.2 to 0.9% in ND1, 0.2 to 0.4% in 16S and 0.2 to 0.5% in ITS.

The two newly discovered species in the Caprivi region showed high sequence divergences when compared to populations of *T. stictica*, ranging from 7.5 to 9.2% in ND1, 4.0 to 4.7% in 16S and 0.9 to 1.6% in ITS (with geographical distances ranging 850 km to 3000 km). Although the farthest geographical distances between populations of *T. palustris* and *T. morrisoni* measures up to only 420 km, sequence divergence between them ranged between 4.8 to 5.2% in ND1, 1.0 to 1.2% in 16S and 1.1 to 1.4% in ITS. At the population site Popa Falls, where both species occur in sympatry, the sequence divergence is at the same high level, with 4.9% in ND1, 1.0% in 16S and 1.1% in ITS. Mean sequence divergences between the three species are summarized in Table 2.

Sequence divergences between all 12 *Trithemis* species included in this study varied from 2.2 to 18.4% in ND1, from 1.2 to 11.7% in 16S and from 0.9 to 10.5% in ITS. Interestingly *T. stictica* showed a lower sequence divergence to *T. nuptialis* (2.2 % in ND1 and 1.2 % in 16S) and *T. grouti* (6.5 % in ND1 and 1.9 % in 16S) than to its putative "sister" species *T. palustris* (9.0 % in ND1 and 4.3 % in 16S) and *T. morrisoni* (8.5 % in ND1 and 4.5 % in 16S). For ITS the sequence divergence between the five species is at the same level (around 1%).

Table 3 F_{st}-values calculated between species-specific groups of all individuals of *T. stictica, T. palustris* and *T. morrisoni* for ND1 and 16S. *P*-value for all comparisons are < 0.001.

	T. stictica		*T. palustris*		*T. morrisoni*	
	ND1	16S	ND1	16S	ND1	16S
T. stictica	0	0				
T. palustris	0.960	0.950	0	0		
T. morrisoni	0.944	0.944	0.906	0.691	0	0

Gene flow

Estimates of gene flow between the three species revealed an interruption of gene flow between *T. stictica* and the two new species in 16S and ND1 (F_{st}-values equal or higher than 0.944 ($p< 0.001$); see Table 3) (Cockerham & Weir 1993). Between *T. palustris* and *T. morrisoni* gene flow is also interrupted (F_{st}- values between were 0.906 ($p< 0.01$) in ND1 and 0.691 ($p< 0.01$) in 16S). Comparing the populations without considering its species origin, complete genetic isolation between each population of each species was found (see Table 4a and b). Popa Falls, the sympatric population site of *T. palustris* and *T. morrisoni*, showed F_{st}-values (0.912 in ND1 and 0.622 in 16S [p= 0.000, respectively]), which indicates interrupted gene flow although both species share the same population site. Intraspecific population comparison showed only slight sub-structuring between some populations in all three species (see Table 4a and b).

Haplotype networks

The TCS- network of ND1 revealed three separate genealogical clades representing the three species (Figure 2a). The mutational steps separating the species were 23 (*T. palustris - T. morrisoni*), 43 (*T. stictica – T. palustris*) and 39 (*T. stictica – T. morrisoni*). The majority of haplotypes within each clade are closely connected and were shared by different populations. *T. palustris* exhibited thirteen different haplotypes dominated by seven haplotypes in the

Botswana population and five at Popa Falls. In *T. stictica* only two haplotypes in the Namibian populations, one in Kenya and two in South Africa were found while *T. morrisoni* exhibited eight different haplotypes with five haplotypes at the population in Zambia (Figure 2a).

The 16S TCS-network is in concordance with the ND1 network but revealed two distinct clades, with *T. palustris* and *T. morrisoni* grouping together in one network (Figure 2b). Within this network, two subclades, one consisting of *T. palustris*, the other one of *T. morrisoni,* could clearly be identified with at least four mutational steps and no shared haplotypes between them. Here, contrary to ND1, *T. palustris* showed a lower number of haplotypes (four) as *T. morrisoni* (eight). In *T. morrisoni* the clade is dominated by six haplotypes at Popa Falls. The second network included all *T. stictica* individuals. This network is separated by at least 22 mutation steps from *T. palustris* and *T. morrisoni*.

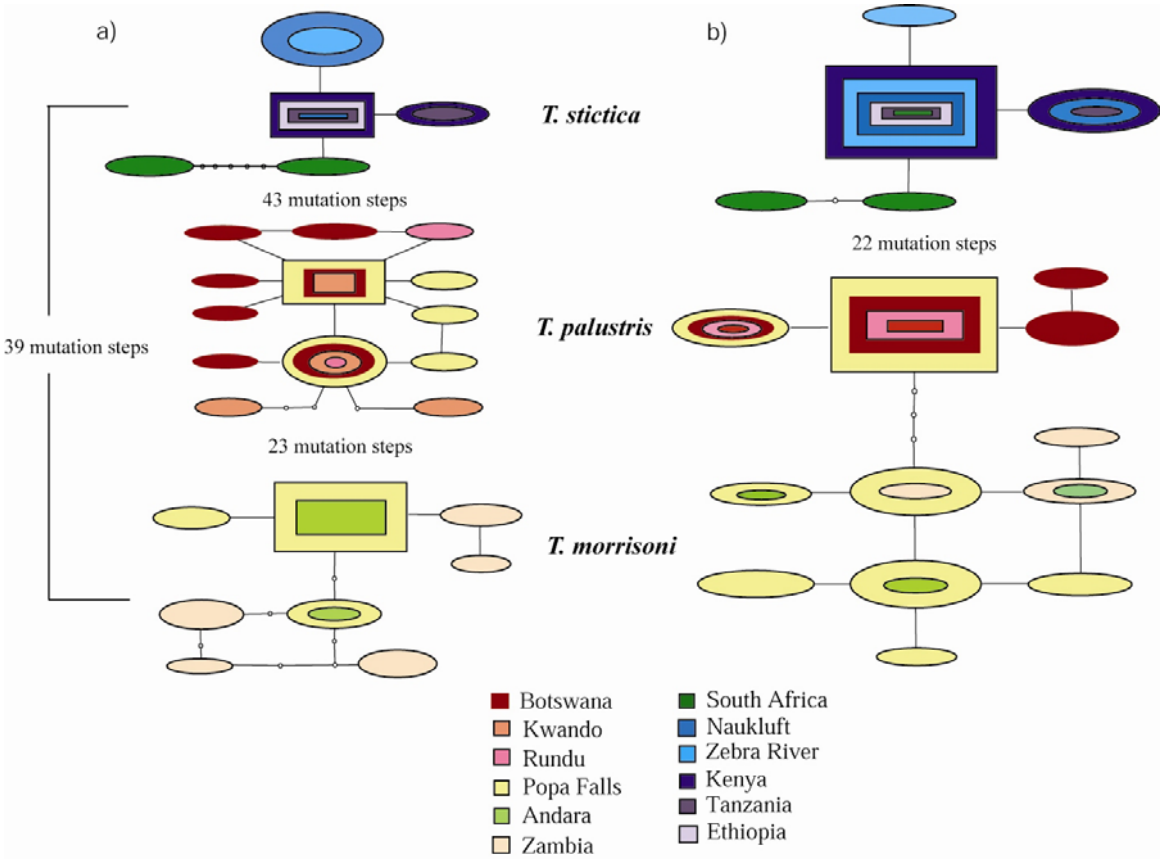

Figure 2 Haplotype networks for two mitochondrial genes. Mutational haplotype network from a) ND1 and b) 16S based on statistical parsimony displays the genealogical relationship between the different haplotypes in the analysed populations of *T. stictica, T. palustris and T. morrisoni*. Haplotypes considered to be ancestral are depicted as rectangles, all other haplotypes as circles. Missing mutational steps connecting haplotypes are represented by small non-coloured circles. Haplotypes connected by a single line differ in one mutational step. The size of the rectangle and circles correlates with haplotype frequency within each network. The different colours represent the different populations.

Table 4 Pairwise F_{st}- values analysed between the populations of *T. stictica, T. palustris* and *T. morrisoni*. (a) is based on the ND1 sequences and (b) is based on 16S sequences. Significant F_{st}- values based on 10000 permutations are displayed in bold ($p < 0.05$).

(a) ND1

	TstSA	TstTans	TstKen	TstNauk	TstZebra	TmorPopa	TmorAnd	TmorZam	TpalPopa	TpalKwan	TpalRund	TpalBot
TstSA	0.000											
TstTans	**0.449**	0.000										
TstKen	**0.426**	0.111	0.000									
TstNauk	**0.502**	0.663	0.775	0.000								
TstZebra	**0.552**	0.841	0.919	0.038	0.000							
TmorPopa	**0.926**	**0.949**	**0.946**	**0.952**	**0.957**	0.000						
TmorAnd	**0.919**	**0.981**	**0.980**	**0.987**	**0.993**	-0.166	0.000					
TmorZam	**0.898**	**0.929**	**0.924**	**0.935**	**0.942**	**0.177**	0.079	0.000				
TpalPopa	**0.956**	**0.986**	**0.986**	**0.989**	**0.993**	**0.912**	**0.965**	**0.889**	0.000			
TpalKwan	**0.935**	**0.970**	**0.968**	**0.975**	**0.981**	**0.899**	**0.929**	**0.874**	0.061	0.000		
TpalRund	**0.922**	**0.982**	**0.980**	**0.987**	**0.994**	**0.893**	0.941	**0.858**	0.351	0.076	0.000	
TpalBot	**0.950**	**0.976**	**0.975**	**0.980**	**0.984**	**0.907**	**0.946**	**0.886**	0.062	-0.033	0.068	0.000

(b) 16S

	TstSA	TstTans	TstKen	TstNauk	TstZebra	TmorPopa	TmorAnd	TmorZam	TpalPopa	TpalKwan	TpalRund	TpalBot
TstSA	0,000											
TstTans	**0,393**	0,000										
TstKen	0,381	-0,242	0,000									
TstNauk	0,250	0,348	0,455	0,000								
TstZebra	0,309	0,545	0,636	0,004	0,000							
TmorPopa	**0,907**	**0,920**	**0,916**	**0,926**	**0,931**	0,000						
TmorAnd	**0,916**	**0,956**	**0,949**	**0,967**	**0,974**	-0,020	0,000					
TmorZam	**0,956**	**0,967**	**0,966**	**0,971**	**0,973**	**0,143**	0,083	0,000				
TpalPopa	**0,894**	**0,922**	**0,913**	**0,933**	**0,942**	**0,622**	0,460	**0,662**	0,000			
TpalKwan	0,935	**0,971**	**0,971**	**0,981**	**0,985**	**0,643**	0,765	**0,860**	0,068	0,000		
TpalRund	**0,955**	**0,973**	**0,974**	**0,979**	**0,982**	**0,671**	**0,832**	**0,863**	0,116	-0,085	0,000	
TpalBot	**0,947**	**0,962**	**0,961**	**0,968**	**0,972**	**0,678**	**0,799**	**0,841**	**0,093**	0,294	0,221	0,000

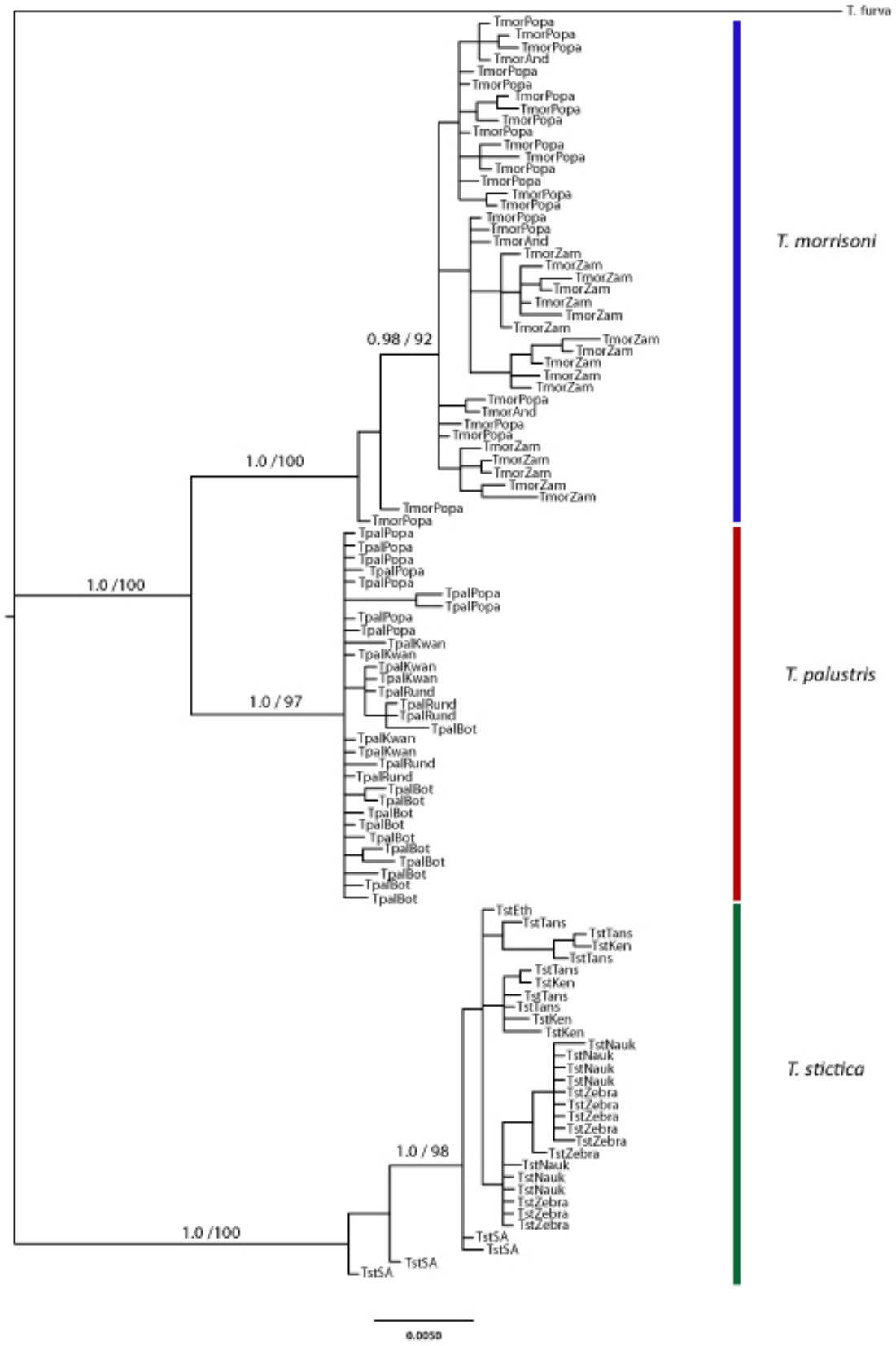

Figure 3 Bayesian tree of a concatenated matrix using ND1, 16S and ITS sequences from *T. stictica, T. palustris and T. morrisoni*. Bayesian posterior probabilities and bootstrap values of the MP analyses are included for the main nodes. *Trithemis furva* was used as an outgroup.

Phylogenetic analyses

A Bayesian phylogenetic tree of 16S, ND1 and ITS sequences including all individuals from *T. stictica, T. palustris* and *T. morrisoni* shows three main clades clearly separating the three species (supported by 100% bootstrap and a posterior probability of 1.00; see Figure 3). Within the *T. stictica* clade, the geographical regions South Africa, East Africa and Namibia formed small subclades. South African samples were separated from Tanzanian, Kenyan and the Namibian populations with high support (posterior probabilities of 1.00 and 0.98). Individuals of *T. palustris* and *T. morrisoni* formed two sister clades. In the species specific clades little sub-structuring was observed with no population specific subclade. Topology of the Maximum Parsimony tree was identical with respect to the relevant nodes (data not shown).

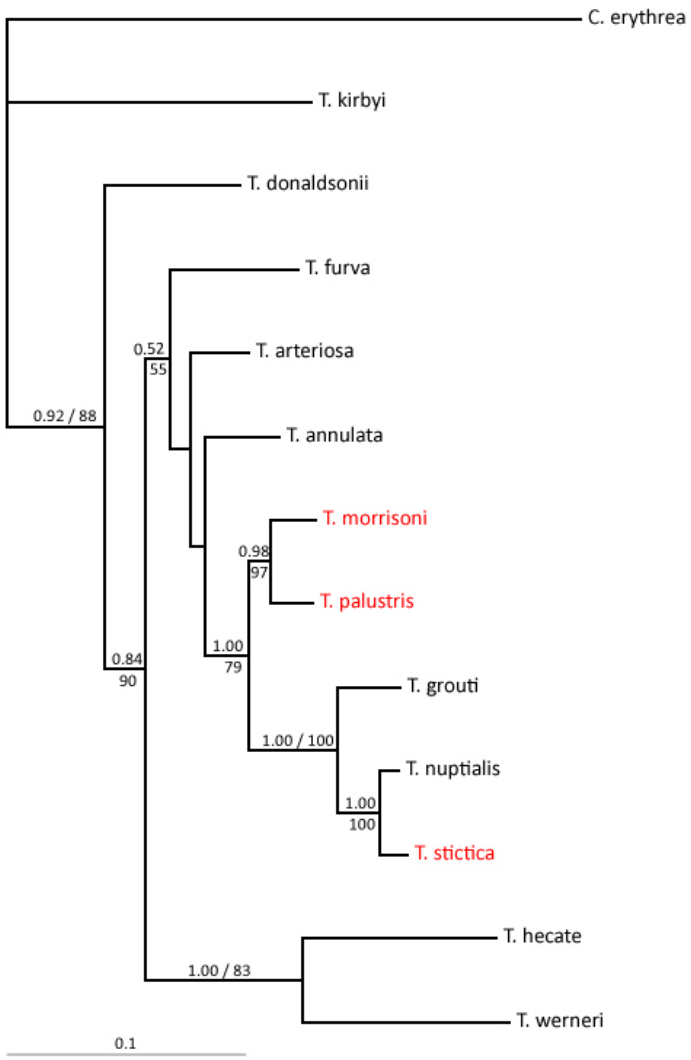

Figure 4 Bayesian tree showing the relationship of 16S and ND1 sequences from different *Trithemis* species. *C. erythrea* is included as outgroup. Bayesian posterior probabilities and MP bootstrap values are included. The three species of main interest are displayed in red.

A Bayesian tree based on 16S and ND1 sequences of all 12 *Trithemis* species showed a clear separation of *T. stictica* from *T. palustris* and *T. morrisoni* (see Figure 4). *T. stictica* turned out to be the sister species of *T. grouti* and *T. nuptialis* (supported by 1.00 posterior probabilities (PP) and 100% bootstrap) while *T. palustris* and *T. morrisoni* form a separate highly supported monophyletic clade (PP=0.98; 97%). The split of *T. palustris* and *T. morrisoni* from the clade of *T. stictica*, *T. grouti* and *T. nuptialis* is also confirmed by high support values (PP=1.00; 79%).

Table 5 Divergence time estimates between the species calculated for ND1, COI and 16S (Pliocene: 5.33-1.8 MYA; Pleistocene: 1.8 MYA – 11500 YA; new analyses dated back the beginning of Pleistocene 2.58 MYA ago [Gradstein & Ogg 2004]).

Pairs of taxa	ND1	COI	16S	Geological era
T. stictica / T. palustris	3.9	3.4	3.1	Pliocene
T. stictica / T. morrisoni	3.7	3.6	3.2	Pliocene
T. palustris / T. morrisoni	2.2	2.4	0.7	Pliocene / Pleistocene

Molecular Clock

The Shimodaira-Hasegawa and Kishino-Hasegawa tests, conducted to compare ML trees reconstructed with and without molecular clock enforced, showed no significant difference for the three mitochondrial markers (ND1: p = 0.64 and p = 0.96, respectively; 16S: p = 0.19 and p = 0.46, respectively; COI: p = 0.54 and p = 0. 87, respectively). Therefore the molecular clock was not rejected and the time since divergence was estimated. Using the mutation rate of 2.3% per million years similar estimates were obtained for ND1 and COI. Genetic distances of 9% and 7.9% between *T. stictica* and *T. palustris* could be translated to approximately 3.9 to 3.4 million years divergence time (Table 5). Genetic distances of 8.5% and 8.3% between *T. stictica* and *T. morrisoni* were translated into a divergence time of 3.7 to 3.6 million years. Thus both species diverged from *T. stictica* at nearly the same time in the geological time period Pliocene. The divergence of *T. palustris* and *T. morrisoni* was also dated in the Pliocene (2.4 to 2.2 million years ago), based on the genetic distances of 5% in ND1 and 5.7% in COI.

Calculations for the 16S region dated the divergence of *T. morrisoni* and *T. palustris* from *T. stictica* with genetic distances of 4.3% and 4.5%, respectively, 3.1 – 3.2 million years ago, which is also in the Pliocene. This is in concordance with ND1 and CO1. A younger

speciation event was calculated for *T. palustris* and *T. morrisoni* with 700,000 years ago (Pleistocene) (Table 5).

Discussion

The divergence of *T. stictica, T. palustris* and *T. morrisoni* constitutes a special case of speciation. The three closely related sister species show highly similar morphology, but the mechanisms of speciation underlying their divergence seem to be rather different. While *T. stictica* do not co-occur with the latter two and finally evolved some morphological differences, *T. palustris* and *T. morrisoni* are still cryptic species and are distributed in the same geographical area with overlapping ranges and at least one sympatric population site. In the following we discuss the mechanisms of their divergence with respect to the biogeographical history and population genetic patterns of the three species. We will critically examine the possibility to assign one of the three major modes to our speciation processes.

Species divergence in allopatry

Estimation of divergence times dates back the split of *T. stictica* and the ancestor of *T. palustris* and *T. morrisoni* to the Pliocene 3.5 mya with high genetic distances in all four markers (up to 9%). The phylogenetic tree displays *T. stictica* on a separate branch with *T. grouti* and *T. nuptialis* between *T. stictica* and the two new species. This provides evidence for a hypothetical unknown ancestor of *T. palustris* and *T. morrisoni* which form a separate monophyletic clade.

Comparisons of morphology show only slight differences in the secondary genitalia as well as in eye and wing colouration between *T. stictica* and the other two species (Damm & Hadrys 2009; Damm et al. 2009). In Odonates, the complex species-specific shape of the male and female genitalia prevents interspecific copulation. Therefore the different shape of the distal segment in *T. stictica* provides a reproductive barrier to *T. palustris* and *T. morrisoni*. Hybridization in form of interspecific reproduction and therefore gene flow between species can be ruled out, which is also supported by the high F_{st}-values (with values up to 0.96), high genetic distances and the absence of intermediate haplotypes.

The sample sites included in this study covers the whole distributional range of *T. stictica*, from South Africa to Kenya. Interestingly, *T. stictica* is widely distributed throughout sub-Saharan Africa, but absent in the Okavango and Zambezi floodplains. Its habitat

specificities (dependant on permanent waterbodies with a high degree of vegetation) seem to fit to this region, but the distributional range of *T. stictica* in Namibia or savannah regions in general is restricted. In Namibia, the Naukluft Mountains are the only region where the species have been found. Between all the population sites, with up to 3000 km geographical distances inbetween, high gene flow was estimated. With regard to its high dispersal potential colonizing also two isolated sites in Namibia *T. stictica* might be expected to occur at the Okavango and Zambezi floodplains. Nevertheless, *T. palustris* and *T. morrisoni* seem to have a selection advantage in this region resulting in a displacement of *T. stictica*.

During the mid-Pliocene the global climate changed to a cooler and drier period. Aridification and a decrease of the tropical forest belt in Africa resulted in the extinction of many tropical species worldwide (Plana 2004; Sepulchre et al. 2006). Before these changes in climate, the distribution of *T. stictica* most likely covered the area of the major drainage systems in southern Africa including the Okavango and Zambezi Rivers. Adapted to a tropical regions, the adequate habitat for *T. stictica* disappeared while aridification started, and the species distribution was restricted to areas with more optimal habitats. In these refugia, isolation promotes speciation by decreased gene flow and genetic drift (Gavrilets 2003). It seems very plausible that the recent common ancestor of *T. palustris* and *T. morrisoni* evolved by allopatry because of the island-like situation of the Okavango and Zambezi Rivers surrounded by savannah and deserts. Apparently, there was no selective pressure to evolve more differences in morphology, because the distribution of *T. stictica* did not reach the Okavango and Zambezi Rivers and the differences in the genital structure might have evolved through genetic drift.

Non-allopatric species divergence

While the above described species divergence was most likely caused through geographical or environmental induced barriers the reasons for the speciation of *T. palustris* and *T. morrisoni* are more difficult to ascertain. The two cryptic species were only recently discovered via genetic markers and molecular clock analyses dates back the split between them around 0.7 to 2.4 mya. At a broad scale both species occupy the same geographical region and sympatric speciation might be a possible mode underlying their divergence. But in contrast to allopatric speciation, the causes of sympatric speciation are often difficult to demonstrate in nature (Berlocher & Feder 2002; Gavrilets 2003; Bolnick 2004; Barluenga et al. 2006; Schliewen et al. 2006; Bolnick & Fitzpatrick 2007). Only a very limited number of studies exist, which are accepted empirical examples for sympatric speciation like in cichlid fish, birds, phytophagous

insects or palm trees (Schliewen et al. 1994; Berlocher & Feder 2002; Savolainen et al. 2006; Seehausen 2006; Friesen et al. 2007). All these examples fulfil the four biogegraphical criteria delineated by Coyne & Orr (2004) for identifying cases of sympatric speciation.

For analysing the speciation process between *T. morrisoni* and *T. palustris*, we first discuss these four criteria of Coyne & Orr (2004) to prove the possibility of a sympatric speciation. *1. Largely or complete overlapping ranges*. The recent distribution of both species is regionally restricted to the Okavango and Zambezi floodplains where their ranges overlap (Figure 1). They share the population site Popa Falls, situated in the centre of the distributional range of both species. No geographic barrier lies between the analysed population sites and although the farthest distance between population sites is 420 km, no significant intraspecific sub-structuring was found in *T. morrisoni* or *T. palustris*. This indicates high gene flow between populations within each species, supported by shared haplotypes and low genetic distances. Also the high level of π and *h* confirm the high gene flow estimates between the populations of each species (Papadopoulou et al. 2008). *2. Reproductive isolation.* Genetic structure analyses revealed complete reproductive isolation between the two species (F_{st}- values based on 16S and ND1 [0.691 and 0.906, with $p < 0.01$, respectively]), also at the shared population site Popa Falls (F_{st}-value of 0.912 ($p< 0.01$) in ND1). High genetic distances in all analysed markers and no shared or intermediate haplotypes indicate complete genetic isolation without hybridization. *3. Species should be sister species.* The phylogenetic analyses of *T. stictica*, *T. palustris* and *T. morrisoni* including (i) all analysed individuals of each species and (ii) twelve additional *Trithemis* species clearly indicate that *T. palustris* and *T. morrisoni* are sister species (supported by 100% bootstrap and 1.0 posterior probabilities; see Figure 3 & 4). Additional evidence for their close relation is based on their similar morphology. While both species are phenotypically nearly indistinguishable all other species in this genus show a great variety of distinct phenotypes. *T. palustris* and *T. morrisoni* differ only slightly in size, and share the same morphological traits distinguishing them from *T. stictica* (two coloured eyes, amber wing base, the different shape in genital morphology). *4. An historical allopatric phase is very unlikely.* Molecular clock estimates date back the split of the two species to the Pleistocene (2.4 – 0.7 mya). The genetic distances of the protein coding genes ND1 and COI between *T. palustris* and *T. morrisoni* are quite similar (5.0 and 5.7%, respectively) and lower in the more conservative 16S rDNA (1%). These estimates predict the split between the two species at a time where the great tectonic uplifting was completed (Sepulchre et al. 2006). The Palaeo-middle and upper Zambezi were already united and the big drainage systems had nearly established their present

courses (Goudie 2005). The approximate age of the Okavango Delta is 2.5 million years (Tiercelin & Lezzar 2002). Since this time no geographic barrier was formed in the Caprivi region which could have been responsible for the divergence of the species into allopatric populations.

While the first three criteria are more or less good to verify the last criterion seems to be the most difficult one to prove when trying to apply the criteria to case studies in general. Completed speciation events occurred in the past and the biogeographical situation at that time usually remains unknown. In our study ruling out an historical allopatric phase of *T. palustris* and *T. morrisoni* is difficult and highly dependent of the regarded geographical scale. Nevertheless, the four criteria relate only to the biogeographical concept of sympatric speciation (Fitzpatrick et al. 2008).

Additional important factors driving divergence in sympatry can be found in population genetic or ecological parameters. In general the ancestral population had to be panmictic, but like rejecting an allopatric phase, this condition is difficult to test for the past. Fitzpatrick et al. (2008) suggested the approach to evaluate the recent population structure of the sister species. If the sympatric sister species are still panmictic, it may be reasonable to infer that they also descended from a single panmictic population. Population structure analyses of *T. palustris* and *T. morrisoni* revealed high gene flow between the analysed populations of each species with high genetic diversity but low genetic distances which demonstrate their high dispersal potential and therefore support a nonallopatric speciation.

However, so far only one sympatric population site (Popa Falls) was found although all populations are connected with each other demonstrated by high gene flow. This highlights the most significant trait distinguishing the two species, the ecological differences. The habitat of *T. morrisoni* is characterised by fast flowing water often with rapids and a bordering gallery forest. In contrast, *T. palustris* inhabits slow flowing waters and swamp-like regions with a more or less open landscape. While Popa Falls provides both habitats, the others are only be inhabited by only one of the two species. Consequently reproductive isolation might be caused by diverging habitat requirements of *T. palustris* and *T. morrisoni* resulting in a shift in habitat specificity. Nevertheless, the sister species status, the similar morphology and the overlapping and regional restricted geographical distribution leads to the assumption of a common ancestor distributed at the Okavango and Zambezi floodplains and suggests a nonallopatric speciation caused by an adaptive radiation.

One reason for adaptation to different habitats may be the availability of new ecological niches (Gavrilets & Vose 2007) which is also described, e.g. in the odonate genus

Enallagma (Brown et al. 2000; Turgeon et al. 2005). During the severe environmental changes in the Plio/Pleistocene a variety of different habitats were developed at the Caprivi region (Andersson et al. 2003). This divers but regionally restricted freshwater environment opened up the possibility of a local adaptation to fast running waters with vegetation (*T. morrisoni*) on one hand, and slow flowing waters in an open habitat (*T. palustris*) on the other hand. Competition for various ecological resources like food or mating and oviposition sites as well as larval habitats might have driven adaptation to different habitats. In sympatry disruptive selection may act on the populations by frequency-dependent competition among ecologically heterogeneous individuals (Dieckmann & Doebeli 1999; Kirkpatrick & Ravigne 2002). Since competition among similar phenotypes is particularly strong, rare phenotypes could have gained an advantage. Due to the evolution of divergent habitat preferences, assortative mating and resulting reproductive isolation occurred as a by-product (Bolnick & Fitzpatrick 2007).

Although some criteria for sympatric speciation could be confirmed it stays difficult to assure this mode of speciation. Considering the diverse landscape of the distributional range of the two cryptic species parapatric speciation also seems to be possible. In parapatric speciation, populations share a spatial restricted border where only limited gene flow occur resulting in differentiation up to subdivided populations or even reproductively isolated species (Gavrilets et al. 2000; Gavrilets 2003). *T. palustris* and *T. morrisoni* share today the same geographically restricted area with a high diversity of different habitats which could be the cause of a secondary range expansion of formerly only bordering populations.

Cryptic speciation

Interestingly the speciation of *T. palustris* and *T. morrisoni* was not accompanied by morphological changes although their estimated time of divergence was dated at least 0.7 mya. In dragonflies species-specific habitat preferences are often closely connected with reproductive traits (Corbet 1999). At their specific habitats sexual selection has a strong influence in premating isolation which could therefore promote speciation (Svensson et al. 2006). Thus adaptation to different habitats may have played the major role in the speciation of *T. palustris* and *T. morrisoni* and because of their niche separation no constraints exist in changing morphology. Slight variations in these reproductive traits can lead to assortative mating and reproductive isolation. However, differences in habitat preferences or reproductive behaviour are assumed to be accompanied or preceded by distinct other changes in phenotypes. For example, in the genus *Calopteryx* the three European *Calopteryx* species

often occur in sympatry, but their phenotypes are clearly distinct (Misof et al. 2000). In our example no distinct phenotypes including the genital morphology in the adults occurred despite distinct genetic differences which prove reproductive isolation. Their occurrence at habitats with different flow rates of the river sections may have evolved morphological differences in the larvae. But in general most speciation studies in odonates, also in a regional restricted area like islands, revealed speciation processes which are accompanied with morphological changes (Jordan et al. 2003; Kalkman et al. 2008). We therefore could demonstrate here the first example of a cryptic speciation in two dragonfly species which are in the biogeographical context regionally sympatric.

Conclusions

In the case study presented here we find different mechanisms of speciation in three closely related dragonfly species. While one speciation event occurred most likely as a cause of allopatry and was moderately accompanied by morphological changes, the speciation of *T. palustris* and *T. morrisoni* could not be assigned easily to one of the major modes and the two species are morphologically cryptic. Our example highlights the difficulties by 'simply' mapping the traditional geographical modes onto processes of speciation which are often of higher complexity. In addition some conditions and criteria for nonallopatric speciation are often impossible to demonstrate in case studies. For the divergence of *T. morrisoni* and *T. palustris* allopatric speciation could most likely be excluded because of the high migration capacity of the two species which are found in the same regional restricted area. The speciation processes underlying their divergence might be the more promising mechanism of divergence-with-gene-flow. This mechanism may be, as suggested by Fitzpatrick et al. (2008), the most common process of divergence in nature. By integrating periods of gene flow with periods of interruption in genetic exchange this model displays the complexity of nonallopatric speciation and thereby focusing more on the reasons of speciation, in our case the diverging habitat preferences.

The adaptation of *T. palustris* and *T. morrisoni* to different habitats could be caused by internal factors like increasing food or mating competition as well as by more external factors like the opened opportunity of new ecological niches through environmental changes. In sum all factors have caused a historical habitat shift resulting in two new and cryptic dragonfly species which have most probable occurred without a clear allopatric phase.

Although we cannot clearly assign one of the three major speciation modes to our case study, the *Trithemis* example in general highlights the importance of integrating different disciplines into speciation research. The combination of molecular genetic analyses, ecological traits, and biogeographic information detected the hidden speciation processes. Additionally we could demonstrate that in odonates, despite of their high morphological diversity and their complex genital structure and mating behaviour, cryptic speciation is possible and might be more common than previously thought.

Acknowledgements

This work was supported by a German Federal Government grant given to HH (BMBF, BIOLOG Africa, BIOTA S08). We especially thank Bernd Schierwater for many helpful comments. We are grateful to K.-D. B. Dijkstra, V. Clausnitzer, F. Suhling, J. Kipping and J. Ott for providing samples.

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Supplementary material

S1 Genetic distances of the ITS region between the population of *T. stictica, T. palustris* and *T. morrisoni*

	TstSA	TstTans	TstKen	TstNauk	TstZebra	TmorPopa	TmorAnd	TmorZam	TpalPopa	TpalKwan	TpalRund	TpalBot
TstSA	0.000											
TstTans	0.002	0.003										
TstKen	0.003	0.003	0.004									
TstNauk	0.003	0.004	0.004	0.003								
TstZebra	0.003	0.004	0.004	0.002	0.003							
TmorPopa	0.012	0.013	0.014	0.015	0.015	0.005						
TmorAnd	0.012	0.015	0.015	0.016	0.016	0.004	0.002					
TmorZam	0.013	0.015	0.015	0.016	0.016	0.005	0.003	0.003				
TpalPopa	0.009	0.009	0.009	0.012	0.012	0.011	0.013	0.013	0.001			
TpalKwan	0.008	0.009	0.009	0.011	0.011	0.012	0.014	0.014	0.001	0.000		
TpalRund	0.008	0.009	0.009	0.011	0.011	0.012	0.014	0.014	0.001	0.000	0.000	
TpalBot	0.010	0.011	0.011	0.013	0.013	0.011	0.014	0.014	0.002	0.002	0.002	0.002

S2 Genetic distances of the 16S rDNA region between the population of *T. stictica, T. palustris* and *T. morrisoni*

	TstSA	TstTans	TstKen	TstNauk	TstZebra	TmorPopa	TmorAnd	TmorZam	TpalPopa	TpalKwan	TpalRund	TpalBot
TstSA	0.003											
TstTans	0.003	0.001										
TstKen	0.004	0.001	0.001									
TstNauk	0.002	0.001	0.001	0.001								
TstZebra	0.002	0.002	0.002	0.001	0.000							
TmorPopa	0.045	0.046	0.046	0.047	0.047	0.004						
TmorAnd	0.044	0.045	0.045	0.046	0.046	0.004	0.004					
TmorZam	0.043	0.044	0.043	0.045	0.045	0.004	0.003	0.002				
TpalPopa	0.042	0.043	0.043	0.044	0.044	0.008	0.009	0.008	0.005			
TpalKwan	0.040	0.041	0.041	0.042	0.042	0.010	0.012	0.011	0.004	0.001		
TpalRund	0.041	0.041	0.041	0.043	0.043	0.010	0.011	0.010	0.003	0.001	0.001	
TpalBot	0.042	0.043	0.043	0.044	0.044	0.010	0.011	0.010	0.004	0.002	0.002	0.002

S3 Genetic distances of the ND1 region between the population of *T. stictica, T. palustris* and *T. morrisoni*

	TstSA	TstTans	TstKen	TstNauk	TstZebra	TmorPopa	TmorAnd	TmorZam	TpalPopa	TpalKwan	TpalRund	TpalBot
TstSA	0.008											
TstTans	0.009	0.001										
TstKen	0.010	0.001	0.001									
TstNauk	0.010	0.002	0.003	0.001								
TstZebra	0.010	0.003	0.004	0.000	0.000							
TmorPopa	0.075	0.085	0.086	0.086	0.087	0.009						
TmorAnd	0.075	0.085	0.086	0.086	0.087	0.002	0.003					
TmorZam	0.077	0.087	0.088	0.088	0.088	0.007	0.007	0.008				
TpalPopa	0.082	0.092	0.093	0.093	0.093	0.049	0.049	0.052	0.002			
TpalKwan	0.080	0.090	0.091	0.092	0.092	0.050	0.050	0.052	0.003	0.004		
TpalRund	0.079	0.089	0.090	0.090	0.091	0.048	0.048	0.050	0.003	0.004	0.003	
TpalBot	0.080	0.090	0.091	0.091	0.092	0.049	0.049	0.051	0.003	0.004	0.004	0.004

S4 Interspecific comparison of 16S genetic distances in the genus *Trithemis*

	C. erythrea	*T. kirbyi*	*T. donald-sonii*	*T. furva*	*T. grouti*	*T. nuptialis*	*T. arteriosa*	*T. annulata*	*T. hecate*	*T. werneri*	*T. stictica*	*T. palustris*	*T. morrisoni*
C. erythrea													
T. kirbyi	0.124												
T. donaldsonii	0.107	0.090											
T. furva	0.092	0.087	0.043										
T. grouti	0.104	0.097	0.046	0.055									
T. nuptialis	0.151	0.117	0.055	0.069	0.015								
T. arteriosa	0.094	0.087	0.039	0.026	0.048	0.062							
T. annulata	0.104	0.080	0.039	0.039	0.050	0.063	0.021						
T. hecate	0.116	0.092	0.068	0.062	0.082	0.088	0.048	0.043					
T. werneri	0.099	0.083	0.055	0.041	0.057	0.072	0.046	0.052	0.078				
T. stictica	0.099	0.092	0.050	0.055	0.019	0.012	0.046	0.048	0.080	0.057			
T. palustris	0.114	0.090	0.043	0.048	0.043	0.056	0.030	0.028	0.059	0.052	0.043		
T. morrisoni	0.114	0.094	0.043	0.046	0.048	0.060	0.032	0.032	0.064	0.052	0.048	0.011	

S5 Interspecific comparison of ND1 genetic distances in the genus *Trithemis*

	C. erythrea	*T. kirbyi*	*T. donaldsonii*	*T. furva*	*T. grouti*	*T. nuptialis*	*T. arteriosa*	*T. annulata*	*T. hecate*	*T. werneri*	*T. stictica*	*T. palustris*	*T. morrisoni*
C. erythrea													
T. kirbyi	0.222												
T. donaldsonii	0.213	0.168											
T. furvaA	0.209	0.148	0.133										
T. grouti	0.245	0.165	0.139	0.102									
T. nuptialis	0.229	0.162	0.145	0.083	0.067								
T. arteriosa	0.233	0.148	0.118	0.080	0.086	0.091							
T. annulata	0.215	0.165	0.121	0.091	0.106	0.106	0.074						
T. hecate	0.222	0.169	0.136	0.122	0.139	0.145	0.114	0.127					
T. werneri	0.223	0.175	0.113	0.105	0.122	0.116	0.083	0.106	0.128				
T. stictica	0.239	0.184	0.145	0.091	0.065	0.022	0.097	0.118	0.148	0.125			
T. palustris	0.242	0.163	0.126	0.080	0.078	0.083	0.074	0.076	0.128	0.110	0.086		
T. morrisoni	0.213	0.154	0.126	0.080	0.080	0.075	0.071	0.074	0.105	0.094	0.081	0.049	

S6 Interspecific comparison of ITS genetic distances in the genus *Trithemis*

	T. kirbyi	*T. donaldsonii*	*T. furva*	*T. grouti*	*T. nuptialis*	*T. arteriosa*	*T. annulata*	*T. hecate*	*T. werneri*	*T. stictica*	*T. palustris*	*T. morrisoni*
T. kirbyi												
T.donaldsonii	0.076											
T. furva	0.082	0.050										
T. grouti	0.072	0.024	0.036									
T. nuptialis	0.067	0.024	0.036	0.012								
T. arteriosa	0.078	0.032	0.030	0.016	0.020							
T. annulata	0.091	0.051	0.057	0.038	0.042	0.032						
T. hecate	0.102	0.063	0.061	0.052	0.057	0.057	0.069					
T. werneri	0.105	0.057	0.078	0.061	0.061	0.065	0.076	0.087				
T. stictica	0.070	0.024	0.036	0.010	0.010	0.020	0.042	0.057	0.057			
T. palustris	0.067	0.020	0.032	0.011	0.009	0.016	0.038	0.052	0.057	0.010		
T. morrisoni	0.067	0.030	0.040	0.018	0.010	0.026	0.044	0.063	0.067	0.014	0.010	

Red drifters and dark residents: Africa's changing environment reflected in the phylogeny and ecology of a Plio-Pleistocene dragonfly radiation (Odonata, Libellulidae, *Trithemis*)

Sandra Damm[*], Klaas-Douwe B. Dijkstra[a] & Heike Hadrys[*†]

[*]*ITZ, Ecology & Evolution, TiHo Hannover, Bünteweg 17d, D-30559 Hannover, Germany*

[†]*Yale University, Department of Ecology and Evolutionary Biology, New Haven, CT, 06520-8104, USA*

[a]*National Museum of Natural History Naturalis, P.O. Box 9517, 2300 RA, Leiden, The Netherlands*

This work is submitted to *Molecular Phylogenetics and Evolution.*

Abstract

In the last few million years, tropical Africa has experienced pronounced climatic shifts with progressive aridification. Such changes will have a great impact on freshwater biota, such as Odonata. With about forty species, *Trithemis* dominates dragonfly communities across Africa, from rain-pools to streams, deserts to rainforests, and lowlands to highlands. Red-bodied species tend to favour exposed, standing and often temporary waters, have strong dispersal capacities, and some of the largest geographic ranges in the genus. Those in cooler habitats, like forest streams, are generally dark-bodied and more sedentary. We combined molecular analyses of ND1, 16S and ITS (ITSI, 5.8S and ITSII) with morphological, ecological and geographical data for 81% of known *Trithemis* species, including three Asian and two Madagascan endemics. Using molecular clock analyses, the genus's origin was estimated 6-9 Mya, with multiple lineages arising suddenly around 4 Mya. The basal species mostly favour open stagnant habitats: their rise coincides with savannah expansion in the late Miocene. The adaptation of red species to more ephemeral conditions leads to large ranges and limited radiation within those lineages. By contrast, three clades of dark species radiated in the Plio-Pleistocene, each within distinct ecological confines: (1) lowland streams, (2) highland streams, and (3) swampy habitats on alternating sides of the Congo-Zambezi watershed; together giving rise to the majority of species diversity in the genus. During *Trithemis* evolution, multiple shifts from open to forested habitats and from standing to running waters occurred. Allopatry by habitat fragmentation appears the dominant force in speciation, but possibly genetic divergence across habitat gradients was also involved. The study demonstrates the importance of combining ecological and phylogenetic data to understand the origin of biological diversity under great environmental change.

Keywords: Odonata, *Trithemis*, rapid radiation, Africa, molecular phylogeny, environmental changes

Introduction

Comparative phylogenetic and phylogeographic studies provide sophisticated insights into the evolutionary consequences of environmental change during the volatile Pliocene and Pleistocene periods (Avise and Walker, 1998; Avise, 2000; Hewitt, 2004). Our understanding of these processes largely relies on studies from the Northern Hemisphere. Here the recurrent formation of perennial ice over vast areas during glacial maxima caused the contraction of entire biotas into southern refugia, with subsequent expansion and recolonization at each interglacial (reviewed in Hewitt, 2000; Hewitt, 2004). These glacial cycles are expected to be promoters for high speciation rates. Molecular clock estimates dates the origin of extant species in many insect and other species group broadly back in this time period (Brower, 1994; Klicka and Zink, 1997; Avise, 2000; Knowles, 2000; Ribera et al., 2004). In tropical regions most cases of species divergence were also estimated to have taken place in the Pliocene (e.g. Hewitt, 2000; Moritz et al., 2000; Bell et al., 2007; de Paula et al., 2007).

The African continent experienced pronounced climatic shifts with the tendency to aridification especially in the last 5 million years. Alternating drier and wetter periods from the beginning of the Miocene resulted in major changes in the distribution and composition of the vegetation (Morley, 2000). The rainforest belt, which covered central Africa almost entirely 30 Mya (million years ago), decreased dramatically as savannahs expanded (Morley, 2000; Jacobs, 2004; Sepulchre et al., 2006). Different speciation models are proposed to explain the high diversification during these periods (reviewed in Moritz et al., 2000). The refugia model suggests speciation in allopatry, with forest species restricted to refuges separated by dry habitat, or vice versa. The riverine model suggests that large rivers are barriers for gene flow. In the gradient model, abrupt environmental transitions, e.g. between forest and savannah, force adaptive divergence and consequent speciation. Although the world's highest level of biodiversity resides in the tropics, especially in rainforests, we only begin to understand the evolution of this diversity in its historical complexity. While rainforest fragments and their borders have been discussed as centres of speciation (Fjeldsa and Lovett, 1997; Moritz et al., 2000; Schilthuizen, 2000), the primary direction of speciation, from forest to open habitat or vice versa, is still debated (Steppan et al., 2004).

While several studies deal with the radiation of terrestrial animals like squirrels, guenons, cobras, frogs and birds (Fjeldsa and Lovett, 1997; Steppan et al., 2004; Tosi et al., 2005; Wuester et al., 2007; Blackburn, 2008), far less is known about the consequences of the climatic shifts for the freshwater fauna. Aridification should directly affect the aquatic fauna,

leading to isolation and extinction (Daniels et al., 2006; Seehausen, 2006; Katongo et al., 2007; Koch et al., 2007). Amphibious insects like Odonata, Ephemeroptera and Plecoptera require aquatic larval and terrestrial adult habitats. Thus climatic change affects them both above and below the surface. All odonates (dragonflies and damselflies) are associated with freshwater, although their habitat requirements range from opportunistic to often highly specialized. Vulnerability to alterations of both aquatic and terrestrial habitats makes them a suitable model to study the effects of the changing environment and increasing aridity during the Plio-Pleistocene.

With about 850 extant species, the Afrotropical odonate fauna is poor compared to the American and Asian tropics (Dijkstra and Clausnitzer, 2006). Africa's unstable climatic history is suggested to have lead to the demise of much of the original fauna, with rather few relicts remaining in some isolated stable areas, but also to the recent rise of a speciose but rather homogeneous fauna (Clausnitzer, 2003; Dijkstra, 2007; Kalkman et al., 2008). Indeed, libellulid dragonflies and coenagrionid damselflies, the two odonate families best adapted to unstable habitats, are notably dominant in tropical Africa (Dijkstra and Clausnitzer, 2006). To learn more about the possible impact of climatic shifts on the evolution and diversity of freshwater organisms in Africa, we analyzed the phylogeny of the libellulid genus *Trithemis*, which dominates present-day odonate communities across Africa. Aside from about 40 continental African species (a few classified in probably synonymous genera), the genus includes five Asian and two Madagascan endemic species (Pinhey, 1970; Dijkstra, 2007). The species occupy most freshwater habitats in tropical Africa and Asia, from cool permanent streams to warm temporary pools, from desert to rainforest, and from lowlands to highlands. In association with such different habitat preferences, they differ in their dispersal capacities and coloration: species of open, often temporary, habitats are often bright red and disperse well, while those of more sheltered permanent conditions tend to be dark-bodied and probably more sedentary.

To understand the processes of speciation and coexistence that have led to this diversification we combine phylogenetic information with morphological, ecological and geographical data. By means of molecular clock analyses we intend to estimate the origin of the genus and timing of its main radiation. We investigate (1) whether speciation is associated with past environmental change, (2) what role habitat fragmentation and shifts may have had in species divergence and coexistence, and (3) if the direction of the speciation is from forest to non-forest habitats or vice versa.

Material and methods

Specimens examined

A total of 164 individuals of 38 species (81% of those thought to belong to *Trithemis*) were analyzed and 92 individuals covering all species were selected for the final alignment. *Porpacithemis trithemoides* (= *Anectothemis apicalis*) was included as ingroup taxon because it is suspected to belong in *Trithemis*. The individuals were collected in twelve different countries and at least 25 localities (Table 1). Two individuals of *Pantala flavescens* were used as outgroup, because phylogenetic studies of the Libellulidae showed that it is closely related to *Trithemis* (Ware et al., 2007; Pilgrim and Von Dohlen, 2008).

Choice of the sequence markers

To date the emergence of species, the choice of a genetic marker is crucial. The set of characters has to provide high parsimony-informative phylogenetic signals but the misleading effects of homoplasy or convergence have to be low (Collins et al., 2005). Only one possible *Trithemis* fossil, *T. pseudodistanti,* has been described (Nel, 1991), which was dated at an age of 11.2-7.1 Myr. Three molecular markers were chosen: (1, 2) Two mitochondrial genes; the NADH-dehydrogenase subunit 1 (ND1) and 16S rDNA, which show different evolutionary rates. Mitochondrial protein coding genes (like ND1) evolve up to three times faster than 12S and 16S (Knowlton and Weigt, 1998) and provide a good resolution for recently diverged species. In contrast, 16S is more appropriate for analyzing earlier speciation processes. (3) The nuclear internal transcribed spacer region I and II including the 5.8S region in between (here simply named ITS). This fragment was successfully used for phylogenetic analyses in Libellulidae before (Hovmoller and Johansson, 2004). The three regions itself have different substitution rates: ITS I is highly variable, ITS II variable and 5.8S highly conserved due to the typical proofreading mechanisms of nuclear genes. With ND1, 16S and ITS a wide range of substitution patterns was covered to overcome difficulties with resolution and polytomy.

Table 1 Localities and number of the examined individuals in this study.

Species	**Country**	**Locality**	**n**
T. aconita	Liberia	Gola & North Lorma Forests	5
T. adelpha	Philippines	Mindanao	2
T. aequalis	Botswana	Okavango Delta	3
T. aenea	Cameroon	Akonolinga	2
T. africana	Liberia	Gola Forest	2
T. annulata	Namibia	Rehoboth	10
T. arteriosa	Namibia	Tsauchab	10
T. aurora	China	Hong Kong	2
T. basitincta	Liberia	Gola Forest	2
T. bifida	Ghana	Fume	2
T. sp. nov. near *bifida*	Cameroon	Nkoélon	2
T. bredoi	Ghana	Bamboi	2
T. brydeni	Botswana	Okavango Delta	1
T. dichroa	Congo-Kinshasa / Ghana	Lokutu / Nakpanduri	5
T. donaldsoni	Namibia	Rehoboth	10
T. dejouxi	Ghana	Nakpanduri	5
T. dorsalis	South Africa	Wakkerstroom	3
T. ellenbeckii	Ethiopia	Ambo	2
T. festiva	China	Hong Kong	2
T. furva	South Africa / Ethiopia	Wakkerstroom / Nekemte	10
T. grouti	Liberia	Gola Forest	8
T. hartwigi	Cameroon	Nkoélon	2
T. hecate	Namibia	Popa Falls, Otavi	3
T. imitata	Liberia / Ghana	Gola Forest / Tamale-Kintampo	5
T. kalula	Nigeria	Afundu River	1
T. kirbyi	Namibia	Tsaobis	10
T. monardi	Botswana	Boro River	3
T. morrisoni	Namibia	Popa Falls	10
T. nuptialis	Congo-Kinshasa	Lukomete, Lingomo	3
T. palustris	Namibia	Kwando	10
T. persephone	Madagascar		3
T. pluvialis	South Africa	Western Cape	3
T. pruinata	Ghana	Agumatsa	2
T. selika	Madagascar		3
T. stictica	Kenya	Kiboko River	10
T. tropicana	Cameroon	Akonolinga	3
T. werneri	Namibia	Kunene	2
Porpacithemis trithemoides	Congo-Kinshasa	Lukomete	1
Pantala flavescens	Namibia	Tsaobis, Swakop River	2

DNA extraction, amplification and sequencing

DNA was extracted from single legs using a modified phenol-chloroform extraction (Hadrys et al., 1992) and stored at -20°C. The ND1 fragment was amplified and sequenced with the primer pair P 850 fw and P 851 rev described in Abraham *et al.* (2001). The PCR product contained 610 bp and included a 5` partial fragment of the 16S rDNA fragment, the $tRNA^{Leu}$ and a 3` partial fragment the ND1 gene region. The PCR regime consisted of 30 cycles 95°C for 30 s, 48°C for 30 s, 72°C for 1 min, an initial denaturation for 2 min at 95°C and a final extension of 6 min at 72°C. The reaction mixtures contained 2.5 mM $MgCl_2$, 1x Buffer (Invitrogen), 10 pmol of each primer, 0.1 mM dNTP, 0.75 U Taq DNA polymerase (Invitrogen) and 1-10 ng DNA template in a final volume of 25 µl. For 16S a 570 bp fragment was amplified with primers described in Simon et al. (1994). The PCR thermal regime was as follows: 5 min initial denaturation at 93°C, followed by 35 cycles of 93°C for 20 s, 52°C for 30 s, 72°C for 40 s, and 2 min extension at 72°C. PCR was carried out in a total volume of 25 µl, containing 1× amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 5 pmol each primer, and 0.75 U *Taq* DNA polymerase (Invitrogen). For the nuclear ITS region, primers were designed based on known insect sequences from GenBank. The forward primer (ITS-Odo fw : 5`CGT AGG TGA ACC TGC AGA AG 3`) is located within the 18S rDNA and the reverse primer (ITS-Odo rev: 5`CTC ACC TGC TCT GAG GTC G 3`) within the 28S rDNA region. Amplification was successful under the following conditions: Initial denaturation for 3 min by 95°C, 35 cycles of 95°C for 30 sec, 60°C for 40 sec and 30 sec at 72°C and a final extension at 72°C for 3 min. The final volume of 25 µl contained 1× amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 5 pmol each primer, and 0.75 U *Taq* DNA polymerase (Invitrogen).

The amplified products were purified by ethanol precipitation. The sequencing reactions were carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and subsequently purified using Sephadex columns (Sigma). Bidirectional sequencing was conducted with PCR primers on an ABI PRISM 310 Genetic Analyzer according to manufacturers` protocol (Applied Biosystems).

Phylogenetic analyses

Sequences were assembled and edited using Seqman II (vers. 5.03; DNAStar, Inc). Multiple sequence alignments were done with MUSCLE vers. 3.6 (Edgar, 2004) and manually edited using Quickalign (Müller and Müller, 2003). Because of its high nucleotide and length variation, the final ITS sequence alignment was obtained in two steps. First, with the help of

an interim alignment done with ClustalX (Thompson et al., 1997), the software Pfold (Knudsen and Hein, 2003) inferred a consensus secondary structure based on the KH-99 algorithm (Knudsen and Hein, 1999). Second, the consensus structure was used as input constraint for a secondary structure analysis in RNAsalsa (Stocsits et al., 2008). Here an alignment was obtained by searching for potential nucleotide interactions in the sequences while taking into account thermodynamic interactions and compensatory/consistent substitutions.

Phylogenetic reconstructions were conducted using Maximum Parsimony (MP) and Bayesian analysis (BA) for each single gene and for a combined dataset. Parsimony analyses were performed in PAUP vers. 4.0b10* (Swofford, 2002) using heuristic searches (10,000 stepwise random additions with TBR branch-swapping) and clade support was estimated via 1000 bootstrap (BS) pseudo-replicates with 10 random additions (Felsenstein, 1985). All characters were unordered and weighted equally and gaps were treated as fifth state. For BA, the best fitting nucleotide substitution model was selected for each data partition according to the Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998). BA was performed in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and was run with 3,000,000 generations each and four Marcov chains with default heating values. Two independent runs were performed and trees were sampled every 1000 generations. At completion, the runs were checked for convergence between each run and for the initial burn-in period determined by examining each of the run parameters for convergence. The initial 50,000 generations (50 trees) were discarded as burn-in. The remaining trees were used to calculate the consensus topology and the posterior probabilities (PP) for nodal support. In the combined analyses, the data of the three markers were partitioned and parameters unlinked to allow the assignment of the appropriate model for each gene partition.

Molecular clock analyses

In order to test the applicability of a molecular clock to evaluate the time of divergence between the species, Maximum Likelihood (ML) analyses with the appropriate evolution model were performed for ND1 and 16S with and without clock enforced. The Shimodaira-Hasegawa (Shimodaira and Hasegawa, 1999; Goldman et al., 2000) and the Kishino-Hasegawa tests (Kishino and Hasegawa, 1989) were used to investigate if the topologies of the two ML trees were significantly different. The genetic distances of ND1 and 16S were then used for comparisons and for molecular divergence time estimates. The dating calculations were based on the mutation rates of 2.3% for ND1 and 1.4% for 16S as proposed

for insect mitochondria (Brower, 1994) and applied in several other odonate studies (e.g.Turgeon et al., 2005; Stoks and McPeek, 2006).

In addition we performed ML for the combined dataset of ND1 and 16S with and without molecular clock enforced. The tree obtained with clock enforced including branch length was used as a fixed input tree for divergence time estimation using *r8s* vers. 1.7 (Sanderson, 2003). The absolute age of the basal *Trithemis* node was set to 10, according to the approximate mean age of 10 Mya of the only *Trithemis* fossil (Nel 1991).

Morphological, ecological and distributional data

With the purpose of investigating their development in *Trithemis* evolution, the following characters were recorded, based principally on the extensive field experience of the second author: (1) predominant body colour of mature adult male, including the development of pruinosity, a supra-cuticular layer of waxy scales that develops independently of underlying coloration, (2) permanence and flow of preferred water bodies, (3) openness and altitude of habitat surrounding these water bodies, (4) approximate distribution range. Categories, definitions and details per species are provided in Figure 4.

Results

Molecular analyses

A final alignment of 1565 bp fragment was obtained containing the following three gene regions: a 425 bp fragment of ND1, a 475 bp portion of the 16S rDNA and the ITS I and II with their intermediate 5.8S (665 bp). 93 sequences of ND1 were analyzed covering the 39 species and shows 196 variable and 186 parsimony informative sites with two gaps in the $tRNA^{Leu}$ fragment. The HKY+I+G model was chosen as the best fitting evolutionary model as suggested by Modeltest. The 16S fragment, which was analyzed for the same 93 individuals, revealed 125 variable sites with 118 parsimony informative characters. Here the TVM+I+G model was applied. The amplification of the ITS region failed for one species, *T. africana*, and thus the final alignment contained sequences of 91 individuals of 36 species. The alignment consisted of 292 bp of ITS I, 140 bp of 5.8S and 232 bp of ITS II. In total, the length of the sequences varied between 544 bp and 604 bp with maximal 121 gaps (73 gaps in ITS I, 48 in ITS II). No gap was found in the 5.8S region. The alignment showed 295 variable positions with 277 parsimony informative sites.

Pairwise genetic distances (corrected by the respective evolutionary model determined by Modeltest) were found to be highest in ND1 (ranging from 0.5% to 20.1%), followed by the ITS region (ranging from 0.7% to 14.5%) and lowest in 16S (0.4% to 9.3%).

3.2. Phylogenetic relationships

MP and BA were performed separately for ND1, 16S and the ITS regions. Phylogenetic relationships at some nodes could not be resolved clearly, because of low support values in each dataset. Therefore the single locus analyses are not presented here. Two combined datasets were used for comparative phylogenetic analyses: (1) the two mitochondrial markers, ND1 and 16S, and (2) all three markers, with ITS data lacking for *T. africana* only.

MP for the mtDNA dataset revealed 496 most parsimonious trees [length, 1105; consistency index (CI) 0.422; retention index (RI) 0.819]. For the combined dataset 64 most parsimonious trees were found [length, 2678; CI 0.438; RI 0.805]. BA of the mtDNA dataset reached a final average standard deviation of split frequencies at 0.007 after 3.000.000 generations suggesting that the chains had reached convergence. For the combined dataset a value of 0.008 was reached after the same generation time.

Tree topologies were highly similar for both datasets and for MP and BA. Slight differences were mainly found between MP and BA at a few nodes resulting from a lower resolution in MP. Figure 1 shows a BA tree for the combined dataset. Three species (*brydeni*, *kirbyi* and *hecate*) were consistently placed at the base of the tree. Four additional species were found near the base (*werneri*, *bredoi*, *persephone*, *festiva*), but the relationships of each could not be resolved because they appeared in different clades in MP and BA and support values were low. *Porpacithemis trithemoides* appeared most closely related to *T. festiva* and was placed in all analyses within the genus, suggesting it belongs to *Trithemis*.

Three monophyletic clades were found congruent in all analyses. Species of these clades are, except of one (*pluvialis*), dark coloured. The most basal of these clades was the *basitincta*-group supported by 66% BS and 1.0 PP and consisting of eight species (*aconita*, *donaldsoni*, *dejouxi*, *basitincta*, *bifida*, sp. nov. near *bifida*, *africana* and *tropicana*). Within this clade, four groups were found (*tropicana*/*africana; bifida*/sp. nov./*basitincta; aconita*; *donaldsoni*/*dejouxi*). The *dorsalis*-group formed a clade of six species (*dorsalis*, *ellenbeckii*, *pruinata*, *furva*, *pluvialis* and *dichroa*) supported by 81% BS and 1.0 PP. *Pluvialis* and *dichroa* were closely related, while the other four species formed a separate group. The *stictica*-group contained seven species (*nuptialis*, *aequalis*, *aenea*, *stictica*, *grouti*, *palustris* and *morrisoni*) supported by 99% BS and 1.0 PP in the combined dataset. Two recently

described species, *T. palustris* and *T. morrisoni* (Damm and Hadrys, 2009) formed a separate group within this clade.

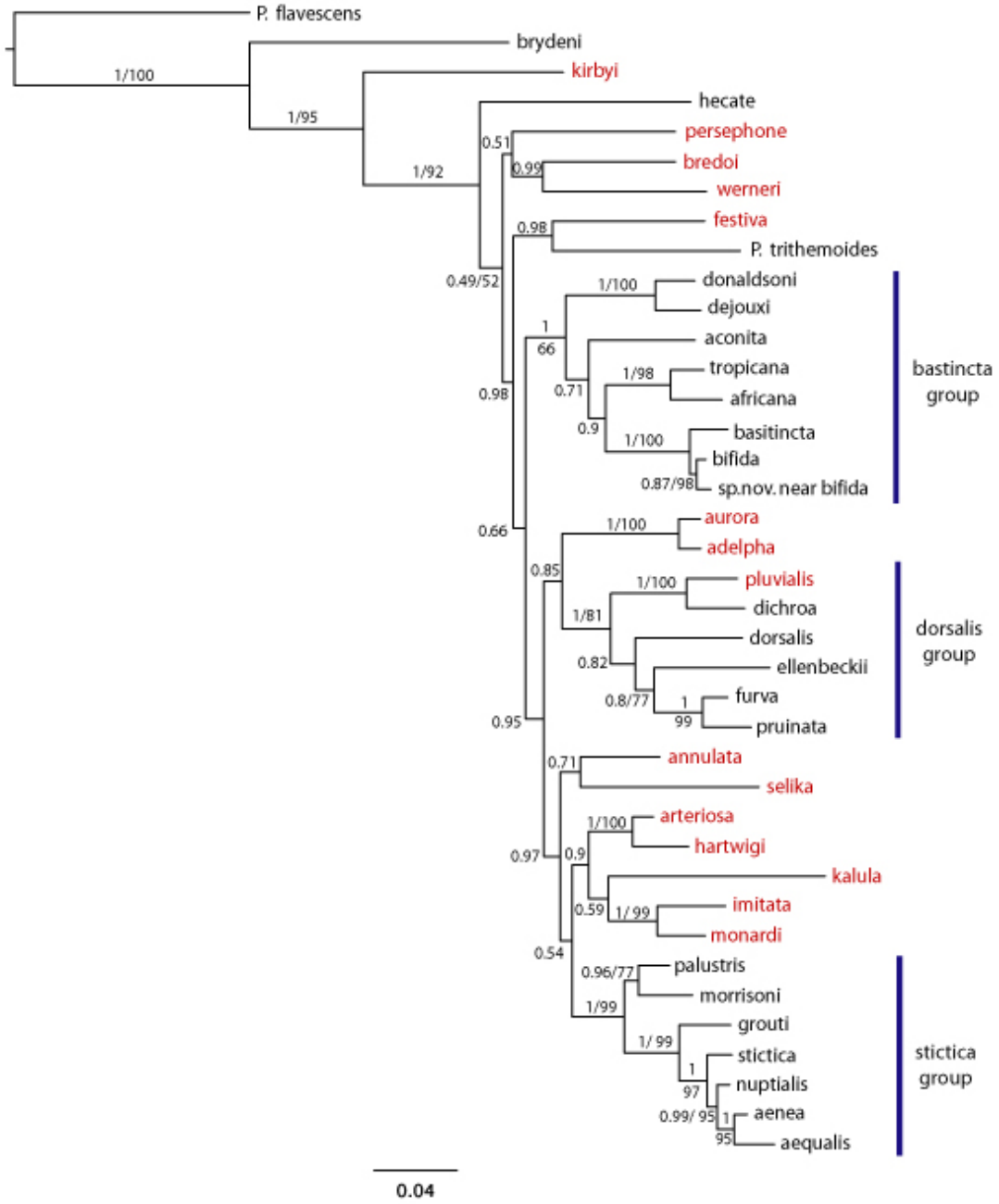

Figure 1 Bayesian tree topology obtained from the combined dataset of ND1, 16S and the ITS regions (including 5.8S). Shown is the 50% majority-rule consensus phylogram including posterior probabilities and bootstrap support (above 50) for the congruent nodes. Red species are marked red.

A fourth group of species was identified by the mtDNA and combined dataset in the BA including four red species (*imitata*, *monardi*, *arteriosa*, *hartwigi*). The species pair *annulata* and *selika*, also red coloured, are placed basal of these four species (and *kalula*) and the *stictica*-group. The exact position of the red *kalula* remained unclear, but MP and BA of the combined datasets indicated a close relation to the other red species. Neither the Asian endemics (*adelpha*, *aurora*, *festiva*) nor the Madagascan ones (*selika* and *persephone*) formed monophyletic clades, although the sister-species status of *aurora* and *adelpha* was confirmed. While *selika* was placed near the other red African species, the position of *persephone* remained unresolved.

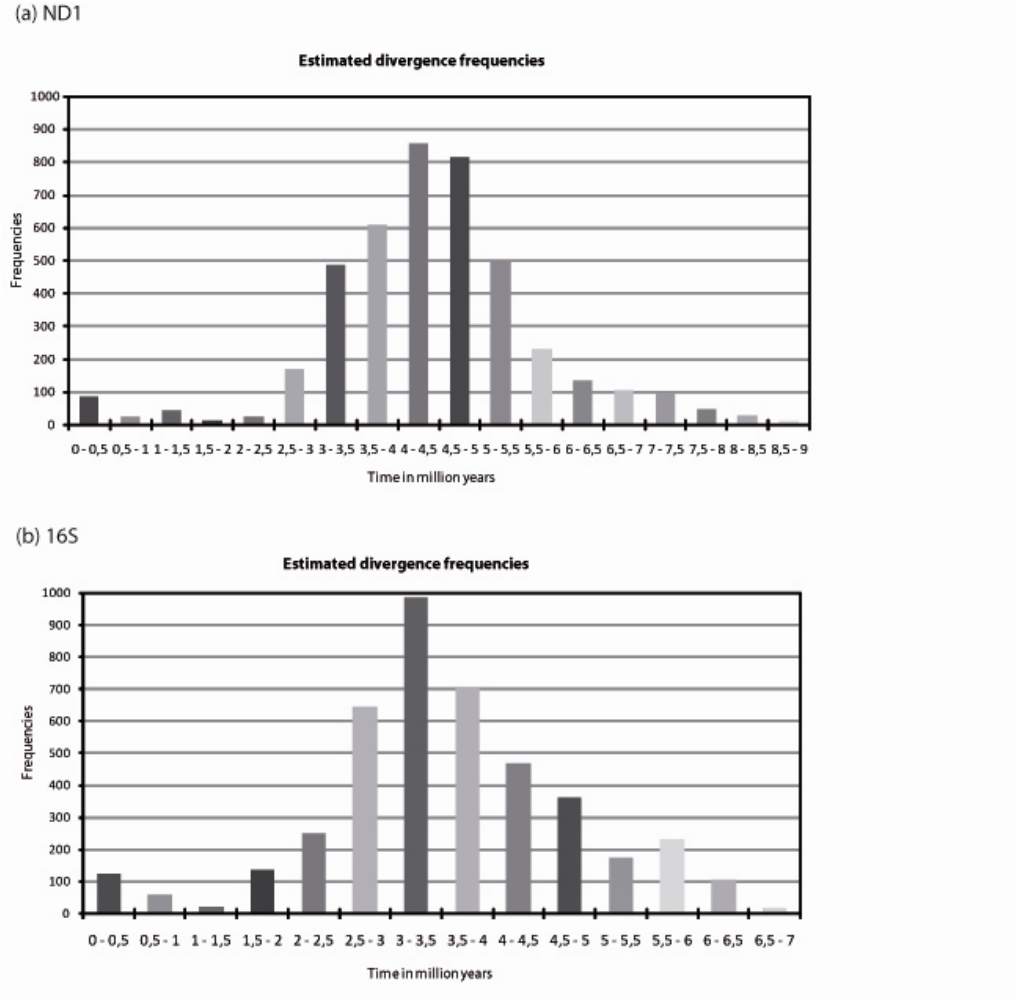

Figure 2 Frequencies of the estimated divergence time in a species pairwise comparison based on sequence distances for the two mitochondrial sequence markers (a) ND1 and (b) 16S. All 4278 comparisons were assigned to time ranges of 0.5 million years and their frequencies calculated.

Molecular clock analyses

The Kishino-Hasegawa and Shimodaira-Hasegawa tests were conducted to compare ML trees reconstructed with and without molecular clock enforced. No significant difference in each mitochondrial marker (ND1: p = 0.63 and p = 0.32, respectively; 16S: p = 0.66 and p = 0.33, respectively) or in the combined dataset (p = 0.764 and p = 0.388, respectively) was found. Therefore the molecular clock was not rejected and divergence times were estimated. Using the mutation rate of 2.3% per million years for ND1 a wide time range of speciation events was found. The lowest sequence divergence between two species, 0.5% to 1.2%, corresponds with a Pleistocene age, 0.2 to 0.5 Mya. The great majority of observed pairwise sequence divergences ranged between 7% and 13%, suggesting a concentration of speciation events in the Pliocene, 3.0 to 5.6 Mya (Figure 2a). The genus's origin might be in late Miocene 8.7 Mya, as indicated by the highest sequence divergence found, between *brydeni* and *kalula* (20.1%).

Genetic distances of 0.4% to 1.1% between nearest sister species in the 16S region corresponded with their divergence 0.28 to 0.78 Mya, which is in concordance with ND1. Also comparable were the most frequent pairwise genetic distances in 16S: these were found in the middle of the range (3.5% to 6%), i.e. with most divergences between 2.5 and 4.3 Mya (Figure 2b). The highest sequence divergence was found between *kirbyi* and *persephone* (9.3%), which again suggests an origin in the late Miocene, 6.6 million years ago.

Tree topology of ML analysis of the combined dataset of ND1 and 16S showed the same topology as the BA tree (Figure 1) and the likelihood ratio test between molecular clocks enforced vs. not enforced showed no significant differences. Therefore the tree including branch lengths was used to obtain an ultrametric tree with absolute calibration of the basal *Trithemis* node set to 10 Mya and which showed similar divergence dates between species and clades as the calculated divergence date estimates according to the mutation rates used above (Figure 3).

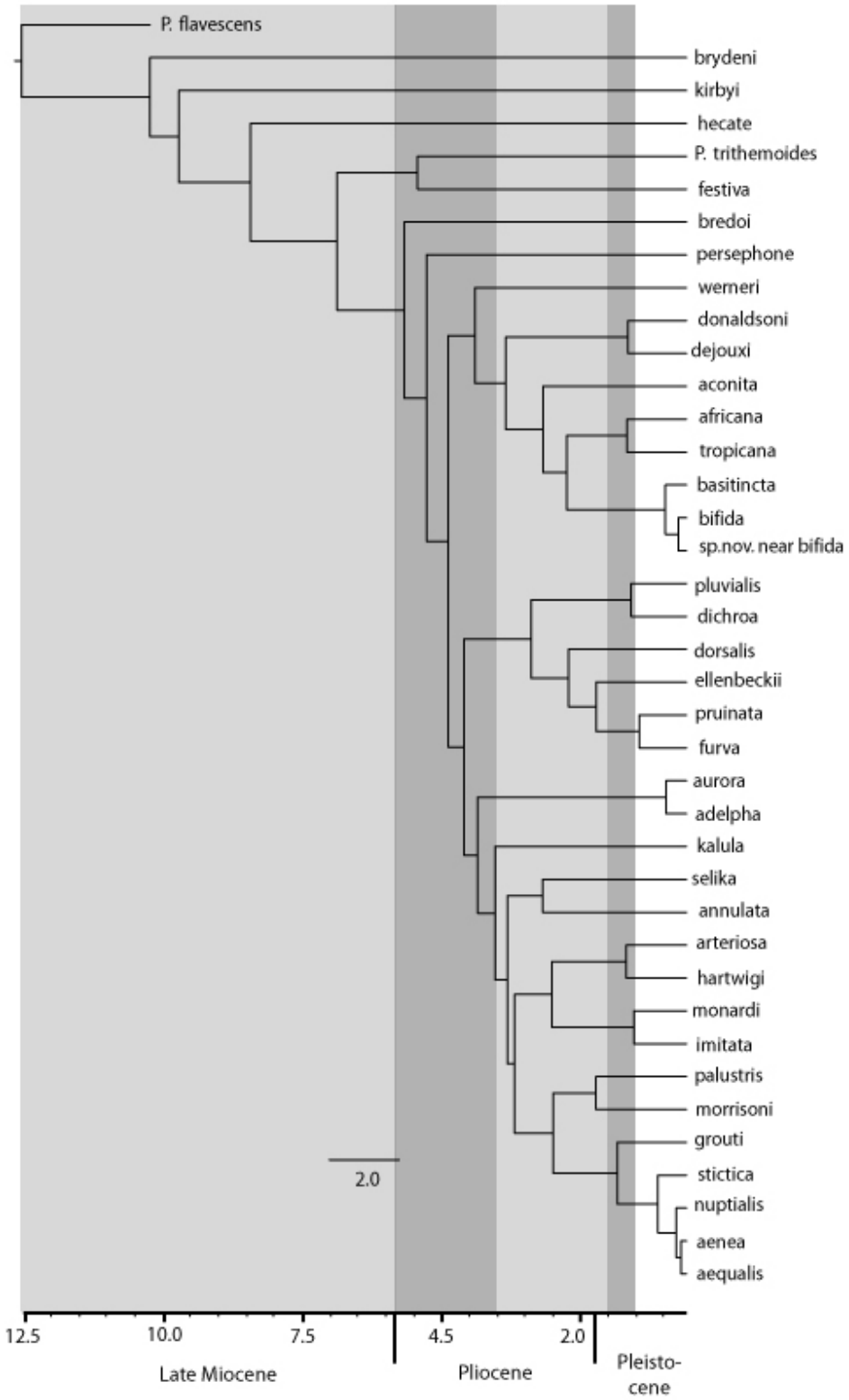

Figure 3 Ultrametric tree obtained from ND1 and 16S sequences based on Maximum likelihood branch lengths. The time was calibrated in r8s using a fixed node age of 10 Mya for the basal *Trithemis* node according to a fossil record. The grey fields indicate relatively drier (pale) and wetter (dark) periods relevant to *Trithemis* evolution (see discussion).

Morphology, ecology and distribution

Figure 4 presents a conservative estimate of the evolutionary history of the genus. The most notable features are: (1) one colour type predominates in some clades, but red and dark coloration both evolved multiple times, (2) species of open and slow-flowing to temporary habitats predominate, especially basally, but adaptation to forest and/or fast-flowing water evolved multiple times, and (3) the origin and main radiation lie in the continental Afrotropics, with multiple invasions of Eurasia and Madagascar. Full details are discussed below.

Discussion

Diversity and phylogeny of the genus Trithemis

The position of the two most basal species, *T. brydeni* and *T. kirbyi*, and the monophyly of three terminal clades were consistent in all analyses. The latter three are also well-defined morphologically (Pinhey 1970 and unpublished data) and conform to the *basitincta*-, *dorsalis*- and *stictica*-groups. They radiated in parallel within Africa, each within notably distinct ecological and geographic contexts, together giving rise to an estimated 55% of *Trithemis* species. The three not only contributed most to the genus's present diversity, but also independently invaded forest habitats. In contrast to these consistent results, the placement of three dark (rather basal) species and twelve red species was problematic, probably owing to rapid basal radiation. This possibility is discussed below, followed by separate discussions of the basal species, the red species, and the three monophyletic radiations.

Rapid radiation in the Pliocene

Molecular clock estimates calculated with insect mitochondria mutation rates (Brower, 1994) dated the origin of the genus *Trithemis* in the late Miocene, approximately 6-9 Mya. This is congruent with the *Trithemis* fossil record (Nel 1991) which was dated back 7.1-11.2 Mya. The main radiation is thought to have occurred in the Pliocene, 2.5-5.6 Mya, with ongoing speciation up to the Pleistocene. Pairwise comparisons of estimated divergence times demonstrate clear concentrations of divergences 3.0-5.6 Mya in ND1 and 2.5-4.3 Mya in 16S (Figure 2). Also in the ultrametric tree the major clades separate in a relatively short period around 4 Mya (Figure 3). The short branch lengths where the major clades diverged suggest a fast diversification. Short basal branches are a frequent problem in phylogenetic

reconstructions, especially in ancient radiations (Whitfield and Lockhart, 2007). Because 81% of the recognized *Trithemis* species were studied and the missing species are regionally restricted, insufficient sampling of extant taxa is unlikely to account for the short branches. Another explanation is the choice of genetic markers, but the three used have previously resolved the phylogenies of odonate genera successfully (Misof et al., 2000; Hovmoller and Johansson, 2004; Hadrys et al., 2006; Groeneveld et al., 2007). All three, moreover, reveal similar topologies and branch lengths. Thus we conclude that the difficulty in resolving basal relationships was caused by rapid radiation, possibly in response to sudden environmental change (see below).

Basal lineages

The two most basal species are neither close to each other nor to other *Trithemis* species. The dark *T. brydeni* is local in the open Okavango and Bangweulu swamps. Genetic distances between it and other *Trithemis* species are mostly greater than between *P. flavescens* (the outgroup) and the others. With the more distantly related libellulid *Crocothemis erythraea* as outgroup, *T. brydeni* came out more basally than *P. flavescens*, while all other *Trithemis* species stay monophyletic. Therefore a generic reassessment of this taxon is warranted. Of all *Trithemis* species, the red *T. kirbyi* is best adapted to temporary pools, with rapid larval development and strong adult dispersal (Suhling et al., 2005). Consequently, it ranges throughout Africa, also deep into deserts, and to Madagascar, southern Europe, Arabia and India.

While these two species seem to date from before the main *Trithemis* radiation, around 5.0-7.5 Mya, three dark species without close affinities are also rather basal. *T. hecate* is local in open, possibly ephemeral, swamps throughout Africa and Madagascar. *T. festiva* is restricted to open streams from Turkey to Indonesia. *P. trithemoides* was the only sampled member of a complex of three or four diminutive species found mainly in central Africa, possibly in rainforest streams, variably placed in *Anectothemis*, *Congothemis*, *Porpacithemis* and/or *Lokithemis* on account of their simplified wing venation. Finding the species firmly inside the *Trithemis* radiation offers another demonstration of the fallibility of venation to define libellulid genera (Dijkstra and Vick, 2006; Pilgrim and Von Dohlen, 2008) and all four genera must probably be subsumed in *Trithemis*.

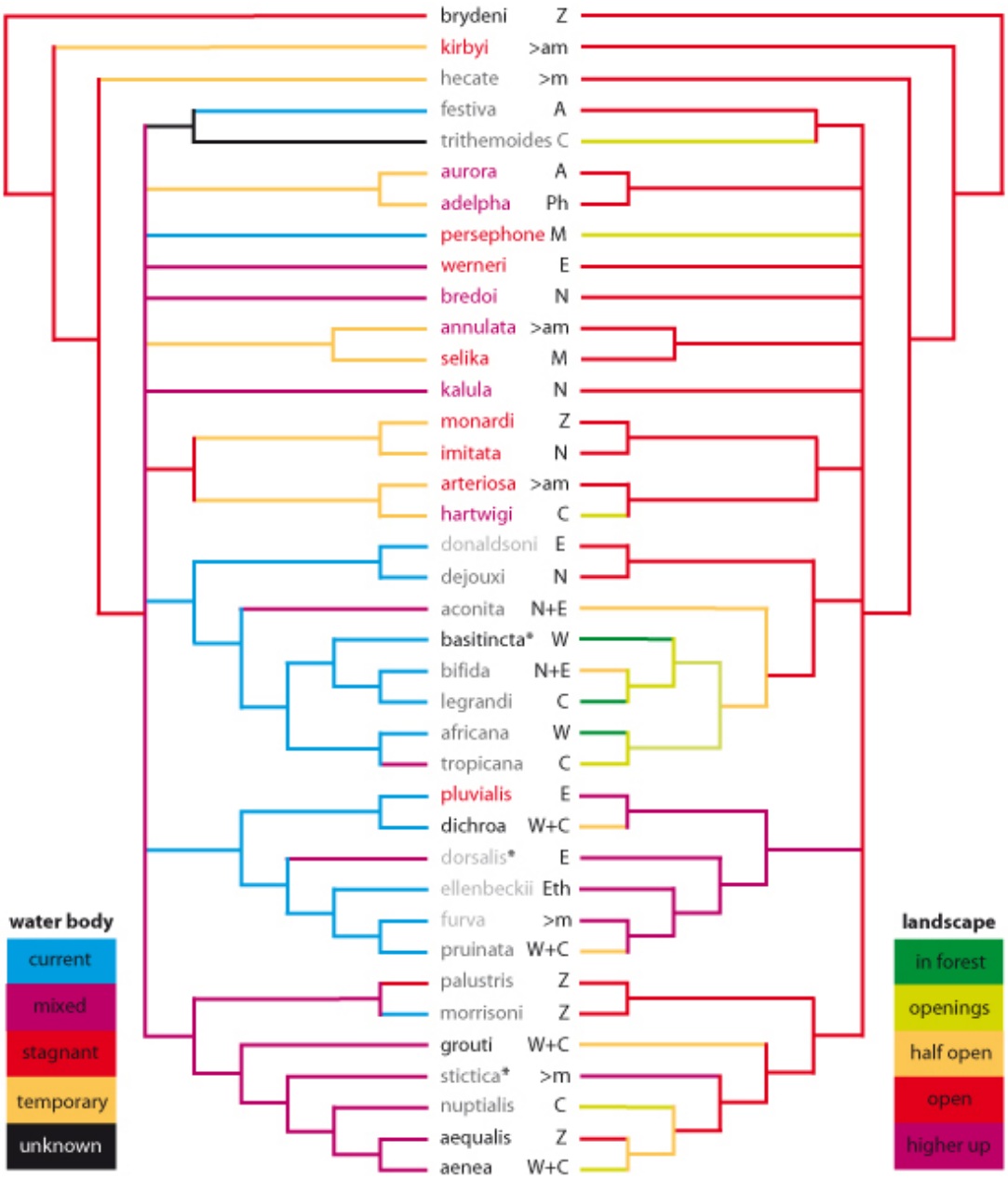

Figure 4 Strict consensus of trees in figure 1 and 3, showing ecological characters on branches (ordered, optimized manually), as well as distribution and adult coloration. Internal nodes with either low BS (<50) or PP (<0.5) support have been collapsed. Name-giver of groups are asterisked. Colour of species names indicates that of adult, being red (red), pruinose red (violet), dark with no or black pruinosity (black), dark with blue-grey pruinosity on thorax and abdomen base (dark grey), or dark and entirely pruinose (pale grey). Codes behind the name provide an approximation of range: widespread in Africa and extending to Eurasia and/or Madagascar (>am, >m); confined to Asia (A); centred on central African forest, especially Congo Basin (C); eastern and southern Africa (E); endemic to Ethiopian highlands (Eth); endemic to Madagascar (M); centred on northern savannahs (N); restricted to Philippines (Ph); endemic to Príncipe (Pr); centred on western African forest (W); centred on 'Zambezian' swamps from Katanga to Botswana (Z). Colour of branches denotes (inferred) habitat preferences, unless not known (black): Water body - preference for strong flow, especially streams and rapids (blue), weak flow like calm rivers or stagnant section in streams (violet), standing waters (red) or standing waters with tolerance for temporary conditions (orange). Landscape - main occurrence in forest shade (dark green), within forest but in sun (pale green), patchy habitats, i.e. shaded in rather open and exposed in more closed environments (orange), exposed habitats (red) and open habitats in cooler climes, like higher altitudes or Cape region (violet).

Owing to the variable support of most clades containing red species, the exact number and position of independent red lineages remain unclear, but between four and nine appear to have given rise to the twelve red species studied. In the latter extreme case, each lineage evolved just a single species, with the exception of three pairs of species well-separated by range or ecology (see below). Eight out of twelve species cope in temporary water bodies. Together with the two more basal species, *T. kirbyi* and *T. hecate*, these are all *Trithemis* species tolerant to such conditions. Among them are two of Africa's most widespread and numerous odonates, *T. annulata* and *T. arteriosa*, which dominate open freshwater in Africa, Madagascar, and adjacent Eurasia. Two sister-pairs originated within Africa 1.0-1.5 Mya: *T. imitata* and *T. monardi* inhabit open habitats north and south of the central forest belt. *T. hartwigi*, known from only five sites in central Africa, uniquely favours open pools within rainforest. Its sister-species *T. arteriosa* rarely penetrates dense forest and *T. hartwigi* may have diverged in open enclaves within the forest matrix. *T. adelpha* is the Philippine counterpart of *T. aurora*, one of Asia's most ubiquitous dragonflies, but their separation was estimated at only 0.3-0.4 Mya. The two differ little and are often treated as synonymous. The four remaining species (*T. bredoi*, *T. kalula*, *T. persephone*, and *T. werneri*) have no clear affinities within the genus and inhabit flowing water, mostly calm and open, like savannah rivers. The Madagascar endemic *T. persephone* diverged 3-4 Mya and, atypically for a red species, inhabits forested streams. Perhaps it was pushed into this habitat by the arrival of another endemic, *T. selika*, that diverged from its probable sister-species *T. annulata* 2.6-2.9 Mya.

Lowland radiation (basitincta-group)

All species inhabit running waters, mainly in lowlands, varying in degrees of exposure. Dijkstra & Clausnitzer (2006) hypothesized the group's stepwise occupation of, adaptation to, and speciation in increasingly closed habitats. Indeed the most basal lineage (represented by *T. dejouxi* and *T. donaldsoni*) inhabits exposed savannah rivers, the next (*T. aconita*) favours half-open streams on the forest-savannah transition, while the remaining lineages inhabit forest streams with varying degrees of shading. Each lineage is divided into geographically separated species, suggesting speciation in allopatry. The distribution of, and genetic distance between, *T. dejouxi* and *T. donaldsoni* is similar to those of *T. imitata* and *T. monardi* (see above). Judging from their slight genetic difference, the split of *T. africana* and *T. tropicana* in forest west and east of the Dahomey Gap respectively, only occurred in the past 0.7 Mya. By morphology, *T. congolica* from the Congo Basin and *T. nigra* from the volcanic island of

Príncipe are sister-species of *T. aconita*. Neither was sampled, but their separation would have occurred after *T. aconita* separated from the other *basitincta*-group species, about 3 Mya.

Highland radiation (dorsalis-group)

This group includes two clades, one with two species (*T. dichroa*, *T. pluvialis*) and the other with the remaining four: *T. dorsalis*, *T. ellenbeckii*, *T. furva*, and *T. pruinata*. While most species favour open streams at higher altitude, *T. dichroa* and *T. pruinata* inhabit shaded lowland streams, often in forest. The highland species show broadly overlapping ranges, mainly in the uplands from the Cape to Kenya. Most widespread, *T. furva* extends to Madagascar, Cameroon and Ethiopia; its relative *T. ellenbeckii* is restricted to the Ethiopian highlands. The habitat shift of *T. dichroa* and *T. pruinata* may result from an adaptation to cooler microhabitats in a highland area of origin, which allowed them to occupy shaded lowland streams. Both species diverged from their highland sister-species *T. pluvialis* and *T. furva* about 0.6-1.4 Mya and now occur throughout the central and western African forest. Morphologically these sister-pairs are almost identical, but *T. pluvialis* is unique within a clade of dark species to have reversed to (or very possibly retained) red coloration.

Swamp radiation (stictica-group)

The species occupy rather open habitats of 'mixed' flow, like channels in swamps and calm stretches and by-waters of streams, although they may prefer stronger current (*T. morrisoni*), a cooler microclimate (*T. stictica*) or more cover (*T. aenea*, *T. nuptialis*). With the exception of the widespread *T. stictica*, the distribution of the lineages alternates across the Congo-Zambezi watershed. While *T. aenea*, *T. grouti* and *T. nuptialis* occur mainly in the Guineo-Congolian forests, three other species are concentrated in the 'Zambezian' swamps to the south: *T. aequalis* is confined to the Okavango and Bangweulu swamps, while the species-pair *T. palustris-morrisoni* is sympatric in the Okavango and adjacent Zambezi system. The latter species were only separated from *T. stictica* after a marked genetic distance was found, and may differ subtly in habitat (Damm and Hadrys, 2009). Neither is proven to overlap with *T. stictica*, which ranges in open and often elevated habitats from the Cape to Madagascar, Ethiopia and across western Africa. Judging from their morphology, two localized species, *T. anomala* (Zambia-Katanga border region) and *T. fumosa* (Congo), belong in this group too. Although the group's radiation started around 3.3 (16S) or 3.9 Mya (ND1), the genetic distance in the most recent split (*T. aenea-aequalis*) is nil (16S) or equivalent to only 0.3 Mya (ND1).

Evolutionary implications

Ecology and coloration

Based on the above and Figure 4, *Trithemis* species of open habitats predominate and a shift towards more shaded habitats occurred on numerous occasions. The more basal species inhabit standing or slow-flowing water, with several shifts occurring to stronger currents. Depending on the phylogenetic reconstruction and the assumed ancestral state, red vs. dark coloration developed or disappeared at least three times, but probably more often. The evolution of the extent and density of pruinosity is even more complex (Figure 4). This evolutionary flexibility in ecology and coloration may be related, as the exposure of dark pigmentation to sunlight raises the body temperature, which is counteracted by reflective pruinosity (Corbet, 1999). Indeed of fourteen studied red species, nine favour standing (often temporary) water and twelve inhabit open habitats. In contrast, only three of 24 dark species favour such waters, while thirteen dark species prefer half-open or closed habitats, such as forest. Moreover, while the three dark lineages each produced between six and eleven species, the red lineages each gave rise to only one or two, indicating different 'evolutionary potentials' (see below).

Distribution and speciation

The mode and location of speciation events can only be inferred from current distributions. However, most *Trithemis* species have large ranges and presumably good dispersal capacities. For example, eight species invaded Madagascar independently, while there were between five and seven dispersal events to Asia (two species not sampled). Nonetheless, of the well-supported sister-species relationships found, (1) five involve pairs of allopatric species (*aenea-aequalis*, *africana-tropicana*, *aurora-adelpha*, *donaldsoni-dejouxi*, and *monardi-imitata*), (2) three show narrow geographic overlap, but distinct habitat preferences (*arteriosa-hartwigi*, *dichroa-pluvialis*, and *furva-pruinata*), and (3) only one pair (*morrisoni-palustris*) is broadly sympatric within different habitats. Allopatry in regions of suitable habitat, separated by uninhabitable regions, may be the primary mode of speciation in these examples (followed by some secondary overlap) and the genus in general. Nonetheless, divergent selection across ecological gradients (e.g. on the forest-savannah transition) is also a potential force for speciation (Smith et al., 1997; Moritz et al., 2000; Schilthuizen, 2000). This gradient model may have operated in the invasion of increasingly shaded habitats in the

basitincta-group, the two shifts from high- to lowland in the *furva*-group, and the alternation between open swamp and forest in the *stictica*-group.

Biogeographical hypothesis

The African Neogene (<23 Mya) was characterized by climatic vicissitudes with a trend towards increasing aridity. During the early Miocene rainforest stretched between coasts and to northern Ethiopia, but savannah began expanding 16 Mya and became widespread 8 Mya (Lovett, 1993; Vbra, 1993; Morley, 2000; Jacobs, 2004; Sepulchre et al., 2006). At the end of the Miocene (5 Mya) rainforest was limited and much of Africa's Paleogene diversity was eliminated (Plana, 2004). While the evolution of *Trithemis* appears to have begun in this period of savannah bloom, the major lineages originated afterwards, in the relatively wet early Pliocene (3.5-5 Mya). While aridification generally disadvantages water-dependent species, it favours adaptations to exposed and temporary conditions, as seen in most red and basal *Trithemis* species. While the genus may have arisen in the savannah-expansion of the late Miocene, populations in open areas possibly became isolated by forest-expansion in the early Pliocene, with subsequent allopatric speciation giving rise to the many poorly-resolved lineages. Adaptation to temporary conditions dictates good dispersal ability and as forests shrank again and open habitats coalesced after 3.5 Mya, the species expanded to establish largely overlapping ranges (e.g. *T. annulata* and *T. arteriosa*). Without isolating mechanisms, these lineages did not radiate further, with the exception of a few allopatric species-pairs (see below).

By contrast, the three dark lineages were ecologically more constrained and therefore could radiate excessively under pressure of the changes in the next 3.5 Mya. Pronounced drying occurred 3.5, 3.2 and 3.0 Mya and especially 2.5-2.8 Mya with the onset of the first northern hemisphere glaciation (Morley, 2000), with further step-like increases in aridity 1.7-1.8 and 1.0 Mya (deMenocal, 1995). The highland radiation (*dorsalis*-group) coincided with the major Pliocene and early Pleistocene uplift that created the Great Rift Valley and the Congo Basin (Plana, 2004). The lowland shift of *T. dichroa* and *T. pruinata* may have been triggered by the expansion of forest in a wetter interlude 1.0-1.5 Mya, offering access to suitable new habitat in the form of shaded streams. At the same time the retreat of open habitats could separate the pairs *T. arteriosa-hartwigi*, *T. monardi-imitata* and *T. donaldsoni-dejouxi*. There was a strong increase of climatic variability 0.8 Mya and perhaps the separation of forest species like *T. africana* and *T. tropicana* occurred at this time.

Conclusions and outlook

The present-day diversity and dominance of *Trithemis* result from its species' flexible responses to the climatic fluctuations in Africa since the late Miocene. Today the genus occupies a great variety of habitats, displaying its high adaptation potential. Its success seems to be related to the origin of extensive savannah, which favoured opportunistic species and their dispersal ability. Less mobile species of more stable habitats (e.g. permanent water, rainforest) either became extinct under these conditions or were restricted to pockets of optimal habitat (e.g. Fjeldsa and Lovett, 1997; Hadrys et al., 2006; Burgess et al., 2007; Fjeldsa and Bowie, 2008). It has been suggested that the ecological constraints of ancestral adaptations dictate the direction of radiations (McPeek, 1995; Richardson, 2001). In this genus too, groups with more restrictive adaptations radiated within distinct ecological confines. Nonetheless, *Trithemis* straddled ecological barriers in different directions multiple times. Most shifts occurred from open to forested habitats and from standing to running waters. Phylogenetic analysis of related genera must provide further insight into the ancestral habitat, but in general this is thought to be forest streams in Odonata (Kalkman et al., 2008). Thus the repeated re-invasion of these habitats via different ecological routes in *Trithemis* is exemplary of the rise of a 'modern' freshwater fauna in, and under influence of, Africa's changing environment (Dijkstra, 2007). This is one of several recent studies revealing explosive African radiations in the Plio-Pleistocene (e.g. Gaubert and Begg, 2007; Van Daele et al., 2007; Dubey et al., 2008; Koblmueller et al., 2008). It demonstrates the importance of combining ecological and phylogenetic data to understand the origin of biological diversity under great environmental change. Such studies will be crucial to guide conservation efforts by anticipating ecological and evolutionary responses to future change.

Acknowledgements

This work was supported by German Federal Government grants given (BMBF, BIOLOG Program 01LC0024 und 01LC0025). We thank Bernd Schierwater and Sabrina Simon for many helpful comments. We are grateful to Viola Clausnitzer, Jens Kipping, Mike Parr, Kai Schütte, Frank Suhling and Reagan Villanueva for providing samples and ecological information.

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7. Acknowledgements

This section is to pay tribute to those people who have contributed to this work by sharing resources, knowledge, ideas, sympathy, passion or money. Without them this work would not have been possible.

First I would like to thank my supervisor Dr. habil. Heike Hadrys for giving me the opportunity to work on such an interesting subject. Her academic and moral support has guided me along the different phases of this work. I am thankful for her valuable suggestions and advice. I also want to express my gratitude to Prof. Dr. Bernd Schierwater, who has always accompanied my work. Over the years and during my time in his institute he provided encouragement and inspiration as well as an excellent working environment and an outstanding team.

The help, hospitality and spirit of the entire ITZ team at the Tierärztliche Hochschule Hannover, its former as well as its present members, were essential for me and my work. Thanks to you all. I am especially grateful to Jessica Rach and Sabrina Simon for many interesting discussions and all kinds of support. Many thanks to Jutta Bunnenberg and Karina Zimmer for their help during the daily lab work and the time in between. I would also like to thank Olalla Carballa Lorenzo and Danielle DeJong for scientific discussions and for reading my manuscripts.

Many people participated in the field work and I am grateful to all of them. Special thanks go to K.-D. B. Dijkstra who supported me with ideas and samples during the whole period of my thesis. Many thanks also to Frank Suhling for introducing me to the African Odonates and – together with Viola Clausnitzer, Jens Kipping and Kai Schütte – for providing samples from all over Africa.

I am also grateful for three years of financial support of the German Federal Ministry of Education and Research for the biodiversity research project BIOTA South. With the financial support of the Gesellschaft der Freunde der Tierärztlichen Hochschule Hannover I was able to present my work to the Odonate community on the 4th WDA Symposium of Odonatology in Pontevedra, Spain.

Many thanks also to Janne Timm for the great time in Namibia and for sharing very special moments of our lifes.

Life in general would not have been the same without close friends. I am deeply grateful especially to Andrea Wolf and Oliver Lahr, Marc and Silvia Zimmer, Thomas Pust, Jan-Hajo Wössner and Birsen Yesik for their friendship. To talk, walk, hike, dance, run, travel, discuss and laugh with you is irreplaceable.

Ein besonders herzlicher Dank gilt meinen Eltern, Schwiegereltern und Julia. Ihre liebevolle Unterstützung hat einen großen Teil zum Entstehen dieser Arbeit beigetragen. Sie um mich zu wissen, erfüllt mich mit großer Dankbarkeit und Freude.

My last and deepest thanks go to my husband Sven Mirko. Without his love, none of this would have been possible.

8. Curriculum vitae

Curriculum vitae

Sandra Damm
(geb. Giere)

Stiftung Tierärztliche Hochschule Hannover
ITZ, Ecology & Evolution
Bünteweg 17d
D-30559 Hannover
sandra.damm@ecolevol.de

Auf dem Emmerberge 11
D-30169 Hannover

PERSONAL DATA

Date and Place of Birth	7. April 1974, Bremen
Family Status	married

EDUCATON

1986 – 1993	Graduation from Gymnasium Achim with "Allgemeine Hochschulreife"

PROFESSIONAL EDUCATION

1993 – 1995	Completed professional education as medical-laboratory assistant at the Medizinische Hochschule Hannover with state examination
1995 – 1996	Full-time position at the Landesgesundheitsamt, Hannover

UNIVERSITY EDUCATION

1996 – 1998	Undergraduate studies of Biological Science at the Technische Universität Braunschweig
1998 – 2002	Undergraduate studies of Biological Science at the Leibniz Universität Hannover
	Priority: Ecology, Zoology, Evolution and Molecular Biology
2002	Diploma thesis at the Stiftung Tierärztliche Hochschule Hannover, ITZ Ecology & Evolution; Title: Genetic variation and isolation patterns in African libellulid dragonflies of the genus *Trithemis*; Advisor: Prof. Dr. Bernd Schierwater Grade: "sehr gut" (best grade)
2003 – present	Doctoral thesis in Biological Science at the Stiftung Tierärztliche Hochschule Hannover, ITZ Ecology & Evolution; Title: Conservation genetics, Speciation and Biogeography in African dragonflies; Advisor: Dr. habil. Heike Hadrys

EXPERIENCE

1998 – 2003	Employment as Technician at the Medizinische Hochschule Hannover during the university education
2001	Undergraduate research at the Stiftung Tierärztliche Hochschule Hannover, ITZ Ecology & Evolution (Genexpression studies of the Heat shock protein HSP70 in *Aurelia aurita*; Molecular studies to analyse the basal metazoan evolution in *Trichoplax adherens*)

TEACHING EXPERIENCE

since 2003	Teaching Assistant: Intensive Course in Molecular Ecology and Evolution, Tierärztliche Hochschule Hannover (Laboratory methods and computational analyses of molecular evolution, population and conservation genetics)

FIELD WORK

2002 & 2003	Field work in Namibia for sample and data collection for the diploma and doctoral thesis. Projects are part of the biodiversity research program BIOTA (Biodiversity Monitoring Transect Analysis in Africa, BIOTA South S08) of the German Federal Ministry of Education and Research

9. List of Publications

Articles

Giere S. & H. Hadrys (2006). Polymorphic microsatellite loci to study population dynamics in a dragonfly, the libellulid *Trithemis arteriosa* (Burmeister, 1839). *Molecular Ecology Notes* **6**, 933-935.

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Damm, S. & H. Hadrys (2008). Odonates in the Desert – Dispersal Strategies of the Libellulid Dragonfly *Trithemis arteriosa.* 2nd North German Evolution and Development Symposium in Kiel.