Global change genomics

- comparative genomic analyses on environmental associated speciation and adaptation processes in Odonata

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Dedicated to Mia Aimée.

Zusammenfassung

Globale und lokale Umweltveränderungen erfordern innovative Schutzkonzepte und geeignete Tiermodellsysteme, die empirische und genomische Daten integrieren. Libellen (Odonaten) sind weltweit bereits prominente Modelorganismen zur Evaluation von Ökosystemen und stellen eine evolutive Schlüsselposition an der Basis der geflügelten Insekten dar. Die rasante Entwicklung genetischer Arbeitsmethoden in den letzten zwei Dekaden erlaubte es, Modellsysteme des Freilandes die, entgegen den prominenten genetischen Modellsystemen (z.B. *Drosophila*) nicht oder nur schwer im Labor gehalten werden können,nun zu Modellsystemen integrativer Forschung zu machen. Hiermit ergibt sich die Möglichkeit nun die Lücke zwischen ökologischen Faktoren und deren genetischen Folgen und / oder Ursachen zu schließen. Eben hierbei spielt die Ordnung der Odonata (Klein- und Großlibellen) eine herausragende Rolle. Aufbauend auf dem soliden Fundament umfangreicher ökologischer Daten, gibt es für Libellen mittlerweile zahlreiche genetische Forschungsansätze. Konsequenterweise hat die vorliegende Dissertation zwei Libellenarten mit sehr unterschiedlichen ökologischen Nischen, fast schon gegensätzlichen Anpassungsstrategien und Verbreitungsdynamiken, zum Inhalt. Es handelt sich zum einen um *Lestes macrostigma,* eine stenöke, an seltene Brackwasserökosysteme angepasste Kleinlibelle mit räumlich sehr begrenzter Verbreitung. Das andere Untersuchungsobjekt ist die kosmopolitisch verbreitete und migrierende Großlibelle *Pantala flavescens* deren Habitat alle Arten von temporären Gewässern ist, die durch lokale Regenfälle entstehen. Beide Habitate sind durch ihre temporäre Natur besonders anfällig für Klimaveränderungen, unterscheiden sich jedoch deutlich in ihrem räumlich-zeitlichem Vorkommen. Die daraus resultierenden unterschiedlichen Anpassungsstrategien und Verbreitungsdynamiken beider Arten wurden daher in dieser Arbeit genetisch auf (i) zeitlicher, (ii) lokaler und (iii) globaler Ebene charakterisiert um potentielle Gewinner und Verlierer in Bezug auf das aktuelle Insektensterben und sich rasant ändernde Umweltbedingungen zu vergleichen:

In einem lokalen Langzeitmonitoring wurden für *L. macrostigma* stark schwankende Populationsgrößen und daraus resultierende genetische Bottlenecks entdeckt, die zur genetischen Verarmung dieser Population in Südfrankreich führen. Die Ergebnisse lassen die Vermutung zu, dass die schwankenden Niederschlags- und Dürreperioden einen großen Einfluss auf diese Entwicklung haben. In einer weiteren Studie auf Populationsebene innerhalb des kompletten Verbreitungsgebiets dieser gefährdeten Art, wurden diese lokalen Daten in geografischen Kontext gesetzt. Hierbei konnte gezeigt werden, dass sich die fragmentarische Verbreitung der Brackwasserhabitate auch in der genetischen Zusammensetzung der Populationsstrukturen der Art widerspiegelt. Dabei wurden zwei signifikante Management Units in Spanien und Frankreich entdeckt, deren Schutzstatus von den zuständigen Autoritäten neu evaluiert werden muss. Zusätzlich lassen die Daten vermuten, dass der Ursprung dieser Art in Asien lag, da hier ihre ökologische Nische auch Süßwasserökosysteme umfasst.

Ganz im Gegensatz zu der engen ökologischen Nische des Habitatspezialisten *L. macrostigma* und den daraus resultierenden genetischen Mustern auf Populations- und

Artebene ist die Großlibelle *P. flavescens* ein Kosmopolit und Generalist. Die Ergebnisse der ersten globalen Populationsstudie dieser Art zeigen auf mitochondrialer und nukleärer Ebene eine widersprüchliche geografische Strukturierung. Trotz ihres extraordinären Migrationspotentials zeigt sich, dass Inselpopulationen lokale Anpassungsprozesse durchlaufen. Insbesondere die Osterinselpopulation stellt sich genetisch isoliert und verarmt dar, wohingegen alle anderen Populationen eine bemerkenswert hohe intraspezifische Diversität aufweisen. Die ungewöhnlich hohe Nukleotiddiversität deutet jedoch auf eine Sättigung des CO1 Genfragments hin, womit dieser Marker für populationsgenetische Studien ungeeignet wäre um lokale Anpassungsprozesse zu detektieren. Auf nukleären Genen basierende Ergebnisse unterstützen diese Hypothese und zeigen, dass diese Art trotz ihres hohes Migrationspotentials räumlichen Mustern unterliegt und keine panmiktische Population darstellt.

Um den Weg für adäquate Markersysteme und genomischen Studien zur Migration zu ebnen, wurde in einer weiteren Studie das komplette mitochondriale Genom eines Osterinselindivuums charakterisiert. Migration fliegender Insekten wurde bislang lediglich an zwei phylogenetisch sehr abgeleiteten Modellsystemen (*Danaus plexippus* und *Locustra migratoria*) eingehender untersucht. Da Libellen phylogenetisch an der Basis der geflügelten Insekten stehen eröffnet sich mit *P. flavescens* die Möglichkeit die Frage nach der Evolution von Migration und deren genetischer „Ursachen“ anzugehen. Zusätzlich wurden drei weitere Mitogenome wichtiger Schlüsselarten (*Anax imperator, Ischnura elegans* und *Megaloprepus caerulatus*) für weiterführende Studien erstellt.

Stichworte: Odonata, Naturschutzgenetik, Bioindikatoren, Insektensterben, Populationsgenetik, Mitochondriale Genome, Migration

Abstract

Proceeding global and local environmental changes require innovative conservation concepts and animal model systems which integrate empirical and genomic data. Odonata (dragonflies and damselflies) are a highly suitable model system for the evaluation of ecosystems and on top hold an evolutionary key position at the base of winged insects. The rapid (r)evolution of genetic methods in the last two decades has allowed the transition of traditional ecological field model systems (which cannot or only with difficulty be kept in the laboratory in contrast to traditional genetic model organisms) to model systems of integrative research. This raises the possibility of closing the gap between ecological factors and their genetic consequences and / or causes. Here, the order Odonata plays a prominent role based on the solid foundation of extensive ecological data and now numerous genetic research approaches for dragonflies. Consequently, the presented dissertation deals with two odonate species with very different ecological niches, almost opposing adaptation strategies and distributional dynamics. These are on the one hand *Lestes macrostigma*, an endangerd, stenotopic damselfly adapted to temporary, brackish ecosystems with spatially very limited distribution. The other target species is the cosmopolitan, abundant and migratory *Pantala flavescens*, inhabiting all kinds of temporary waters created by local rains as an ecological generalist. Due to their temporary nature, both habitats are particularly prone to climate change, but they differ significantly in their spatio-temporal occurrence. The resulting different adaptation strategies and distributional dynamics of both species were therefore genetically characterized in this work on (i) temporal, (ii) local and (iii) global levels to compare potential “winners” and “losers” in terms of the current insect extinction and rapidly changing environmental conditions:

A long-term local monitoring of a population of *L. macrostigma* in southern France revealed highly fluctuating population sizes resulting in genetic bottlenecks. These results were associated with fluctuating precipitation and drought periods and were compared to populations covering the entire distribution range of this species and are discussed in a geographic context. The global comparison approach reveals that the fragmented population structure is reflected in the genetics of this species and that populations are even genetically isolated. Furthermore, two conservation units were discovered in Spain and France requiring the re-evaluation of the conservation status of these populations by the relevant authorities. Finally, a new dispersal model of this species was proposed, suggesting that this species originated in Asia with a subsequent westward expansion.

A first global population study on the cosmopolitan dragonfly species *P. flavescens* surprisingly revealed contradicting geographic structuring based on mitochondrial and nuclear data. Despite their extraordinary migratory potential, island populations of *P. flavescens* show genetic signals of local adaptation processes, going along with phenotypic differences and behavioral adaptations. In particular, the investigated population on Easter Island is genetically isolated and depleted whereas all other populations show remarkably high intraspecific diversity. However, nucleotide and amino acid diversity indicate saturation of the CO1 barcode

gene fragment, highlighting the limited suitability of marker for population genetic studies as local adaptations could not be detected.

To complement single marker gene analyses and to pave the way for genomic studies on insect migration, the complete mitochondrial genome of an Easter Island indivdual of *P. flavescens* was characterized and compared to mitogenomes of the key odonate species *Anax imperator*, *Ischnura elegans*, and *Megaloprepus caerulatus*. These new mitogenome data are a highly valuable resource for future comparative mitogenome studies on the population level and for insect migration. Migration of flying insects has so far only been studied in detail on two phylogenetically very derived model systems (*Danaus plexippus* and *Locustra migratoria*). Since dragonflies are phylogenetically placed on the base of winged insects, studies on *P. flavescens* open up the possibility of tackling the question of the evolution of migration and its genetic mechanisms.

Keywords: Odonata, Conservation genetics, Bioindicators, Insect extinction, Population genetics, Mitochondrial genomes, Migration

Content

Abbreviation

µL	Microliter
/C	China
/E	Equador
%	Per cent
°C	Degree celsius
A	Adenine
Ala / A	Alanine
AR/K	Arabuko, Kenya
Arg / R	Arginine
Asn / N	Asparagine
Asp / D	Aspartic acid
atp6	ATP-Synthase, Fo subunit 6
atp8	ATP-Synthase, Fo subunit 8
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of life data system
bp	Base pair
C	Cytosine
CA	character attributes
CO1	Cytochrome c oxidase subunit 1
cob	Cytochrom b (complex III)
cox1	Cytochrom-c-Oxidase, subunit 1
cox2	Cytochrom-c-Oxidase, subunit 2
cox3	Cytochrom-c-Oxidase, subunit 3
CRN	Cape Range Nationalpark, Northern Australia
Cys / C	Cysteine
D	Tajima's D test
DNA	Deoxyribonucleic acid
DnaSP	DNA Sequence Polymorphism
DTT	Dithiothreitol
e.g.	Exempli gratia
EDTA	Ethylenediaminetetraacetic acid
EI	Easter Island
et al.	et alii

EtBr	Ethidium bromide
etc.	Et cetera
EtOh	Ethanol
FBL	Flight boundary layer
Fig.	Figure
fw	Forward
G	Guanine
Gln / Q	Glutamine
Glu / E	Glutamic acid
Gly / G	Glycine
GPMM	Grand Port Maritime de Marseille
Hap	Haplotype
H_2O	Water
HCl	Hydrochloric acid
HD	Haplotype diversity
His / H	Histidine
ibid	Ibīdem, "in the same place"
Ile / I	Isoleucine
ITCZ	Inter-tropical convergence zone
ITS	Internal transcribed spacer
ITZ	Institut für Tierökologie und Zellbiologie/ Ecology and Evolution
IUCN	International Union for Conservation of Nature and Natural Resources
kb	Kilo bases
KCl	Potassium chloride
km	Kilometer
KR/K	Karacha Pools, Kenya
Leu / L	Leucine
Lys / K	Lysine
Met / M	Methionine
$MgCl_2$	Magnesium dichloride
ML	Maximum Likelyhood
MP/K	Marich Pass, Kenya
mtDNA	Mitochondrial DNA
Mya	Million years ago
n	Number

NaCl	Natrium chloride
nad1	NADH-Dehydrogenase, subunit 1
nad2	NADH-Dehydrogenase, subunit 2
nad3	NADH-Dehydrogenase, subunit 3
nad4	NADH-Dehydrogenase, subunit 4
nad4l	NADH-Dehydrogenase, subunit 4l
nad5	NADH-Dehydrogenase, subunit 5
nad6	NADH-Dehydrogenase, subunit 6
NCBI	National Center for Biotechnology Information
ND1	NADH dehydrogenase subunit 1
ND3	NADH dehydrogenase subunit 3
nDNA	Nuclear ribosomal DNA
NJ	Neighbor joining
N_m	Migrants
ns	Not specified
NY	New York
PBL	Planet boundary layer
PCR	Polymerase chain reaction
PEG	Polyethylenglycol
pH	Potentia hydrogenii
Phe / F	Phenylalanine
prep.	Preparation
Pro / P	Proline
RADtags	Restriction site associated DNA tags
rev	Reverse
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
S	Segment
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Ser / S	Serine
SNP	Single nucleotide polymorphism
SP	Sequence polymorphism
T	Thymine

Tab.	Table
TAE	Triacetate-EDTA-Buffer
Taq	Thermus aquaticus
TdV	Tour du Valat
Thr / T	Threonine
TN/N	Tsaobis, Namibia
TO/N	Tsaobis, Namibia
Tris	Tris(hydromethyl)aminomethane
tRNA	Transfer RNA
Trp / W	Tryptophan
Tyr / Y	Tyrosine
Val / V	Valine
vs.	Versus

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"Die Natur versteht gar keinen Spaß, sie ist immer wahr, immer ernst, immer strenge, sie hat immer recht, und die Fehler und Irrtümer sind immer des Menschen."
(Johann Wolfgang von Goethe)

I. 1 Introduction

Motivated by the ever increasing human impact on natural ecosystems the studies in the scope of this thesis aim to deal with conservation genetic aspects on different spatial levels using dragonflies and damselflies as bioindicators. In this context a variety of genetic and genomic approaches were used, ranging from traditional sequence markers such as the CO1 barcode fragment, over newly developed sequence markers providing greater resolution at the population level, to next generation sequencing approaches and whole mitochondrial genome analyses. These marker systems were applied to ecological, evolutionary as well as biogeographical questions, dealing with the increased anthropogenic selection pressure, species on this planet are facing.

Source: http://www.anthropocene.info/

A. Biodiversity conservation: Welcome to the Anthropocene

The impact of anthropogenic influence on our planet´s biodiversity increased exponentially over the last century and is out-competing more and more natural processes (DeSalle & Amato 2004, Crutzen 2006). The scientific community established the term “anthropocene” for the current geological epoche (e.g. Crutzen 2006, Lewis & Maslin 2015, Ruddiman 2013). The civil awareness for this topic is rising, mainly due to anthropogenic climate change. The list of major drivers for environmental change is long: climate change, destruction and overuse of natural areas and resources, environmental pollution, intensive agriculture/nitrification, invasive species, and many more (e.g. Crutzen 2006, Sala et al. 2010). For instance, the global forest area shrank from 4128 M ha in 1990 to 3999 M ha in 2015 (Keenan et al. 2015). The Amazon rainforest is burning. The media announced the death of the Great Barrier Reef, even if this is still debated among scientists (e.g. Conroy 2019, Morison et al. 2019, Pendleton et al. 2019) and not yet confirmed by the Great Barrier Reef Marine Park Authority. The number of species on the red list is increasing steadily (IUCN 2019, see Fig. 1.A.1).

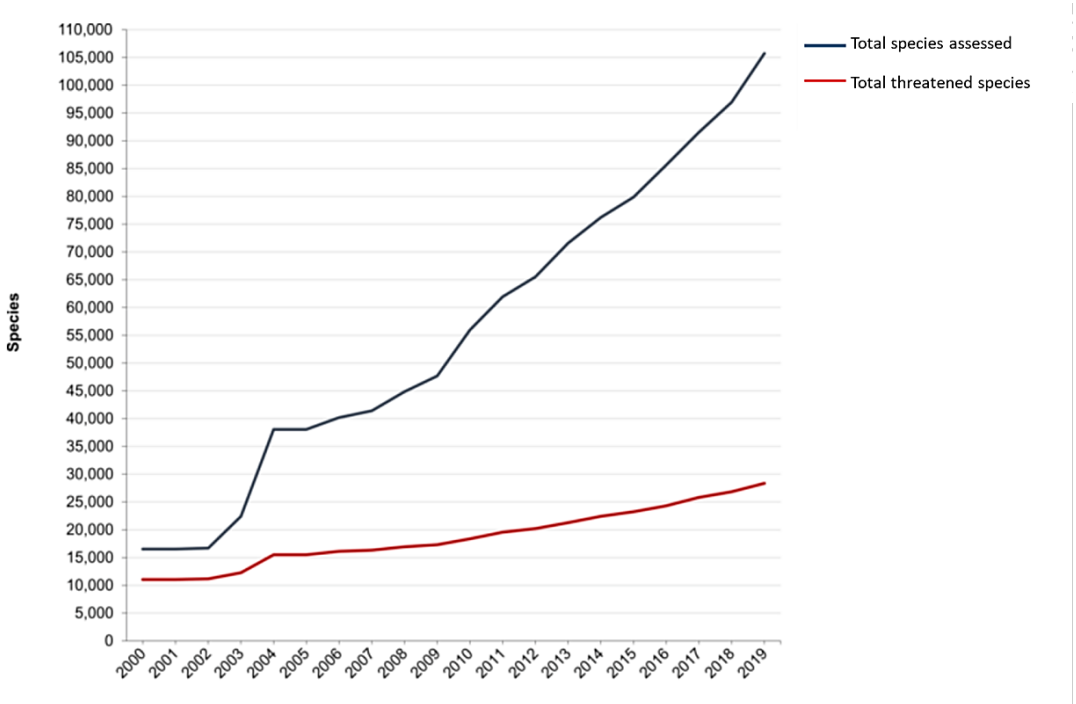

Figure 1.A.1: Summary statistics of the total number of species covered by IUCN risk assessments since 2000, including the total number of thereby detected species which are threatened (IUCN 2019).

In order to conserve nature and biodiversity, it is essential to identify species and define their endangerment status. The most diverse group of animals are insects: estimations regarding the number of species ranges from two to 50 million (Stork 1993). Approximately 70 % of the described species are insects, affecting all types of habitats and ecosystem services (Samways 2018). Nevertheless this class is usually underrepresented in conservational studies, risk assessments and on red lists (see Fig. 1.A.2). Current research shows that insects, especially flying insects, are on the verge of mass extinction and disappearing at a rate that cannot simply be explained by current scientific means (Seiboldt et al. 2019, Regnier et al. 2015, Conrad et al. 2005, Dirzo et al. 2003). Consequently not only vast monitoring programs, but studies explaining the causal mechanisms behind the scene, are urgently needed to shed light on the observed diversity loss. The current (r)evolution in genomic technologies opens up new remedies to bridge the gap between laboratory genetics, largely focused on understanding basic cellular and developmental processes, and systems-level analyses of genetic adaptations and interactions among organisms (shaped by their natural environment) in their natural setting. A multitude of new approaches are becoming available to begin addressing the genetics of adaptations and ecological interactions in natural populations (e.g. Nadeau et al. 2014, Bierne et al. 2012, Jones et al. 2012, Davey et al. 2011, DePristo et al. 2011, Hohenlohe et al. 2010, Nadeau & Jiggins 2010, Stapley et al. 2010). Nowadays, it is much easier to develop genetic approaches for ecological model systems than trying to understand the ecology of established genetic model organisms.

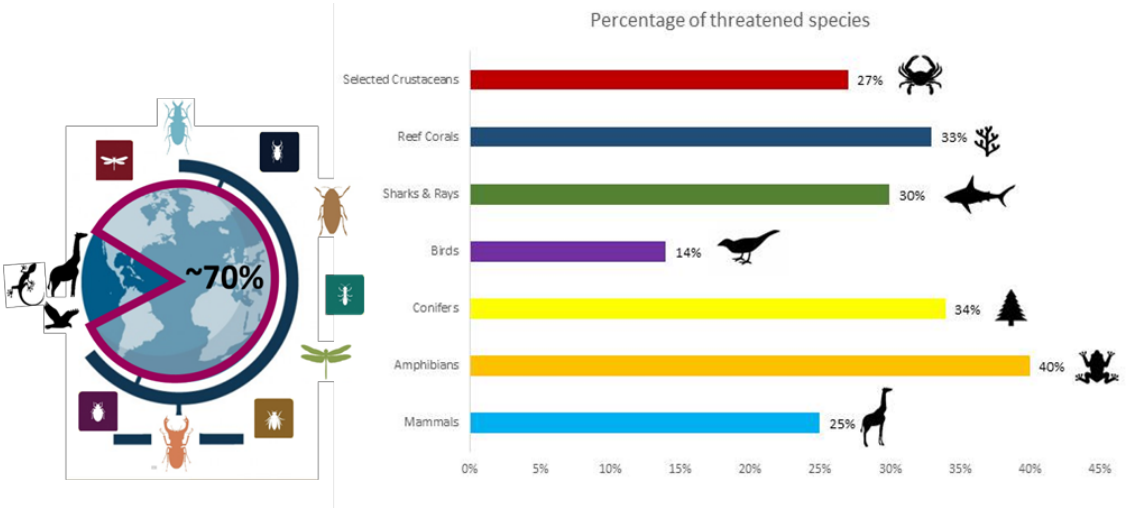

Figure 1.A.2: Circa 70 % of known species are Insects (left). The percentage of threatened species on the right, as shown on the IUCN Redlist webpage (https://www.iucnredlist.org/). Insects were not even displayed.

Levels of biodiversity

The protection of all levels of biodiversity is no longer an exclusive scientific interest, but subject to political debates and discussed in the media. The reason for this increasing awareness is, of course, the loss of biodiversity that proceeds at an unprecedented pace (Meadows 2016). Biodiversity most commonly refers to the number of species. However, biological diversity is composed at the level of ecological diversity, species diversity and genetic diversity (Heywood 1995). Ecological diversity can be assessed in terms of the variety of biomes, landscapes, ecosystems and habitats (Heywood 1995). Species diversity is often considered equivalent to species richness (Daly et al. 2018) but can also be translated into organismal diversity as a measure for the number of kingdoms, phyla, families, genera, species, subspecies and populations (Heywood 1995). Genetic diversity can be measured at the population, individual, chromosome, gene and nucleotide level (Frankham 2003). Frankham et al. (2002) described consequences of anthropogenic influences on genetic diversity, highlighting that human-related influences can reduce a given species to population sizes where they are especially prone to stochastic effects (e.g. environmental, demographic and genetic). The correlation between heterozygosity and population fitness is described by Watts et al. (2006). To study and maintain genetic diversity is of conservational interest, as the loss of genetic variation is correlated with the capability to adapt to environmental changes (Antao et al. 2010, Reed & Frankham 2003).

Conservation genetics

Conservation biology aims to study and protect biodiversity on all described levels. The exploration of genomic approaches in conservation biology enables the assessment of diversity at the genetic level and leads to the establishment of conservation genetics as a research field, which greatly improves decision-making (e.g. Schwartz et al. 2007, DeSalle & Amato 2004). To speed up the generation of data, regarding the increasing amount of endangered species, the “crisis-disciple” conservation genetics has taken more and more advantage of genetic approaches since the beginning of this century. In Figure 1.A.3 the various interests in conservation genetics are illustrated. Conservation genetic surveys can reliably detect population structure and processes as

well as inbreeding in small populations. Furthermore, they add fundamental knowledge to the understanding of species biology, their taxonomic classification and their evolutionary history (Schwartz et al. 2007, Hartl 2000).

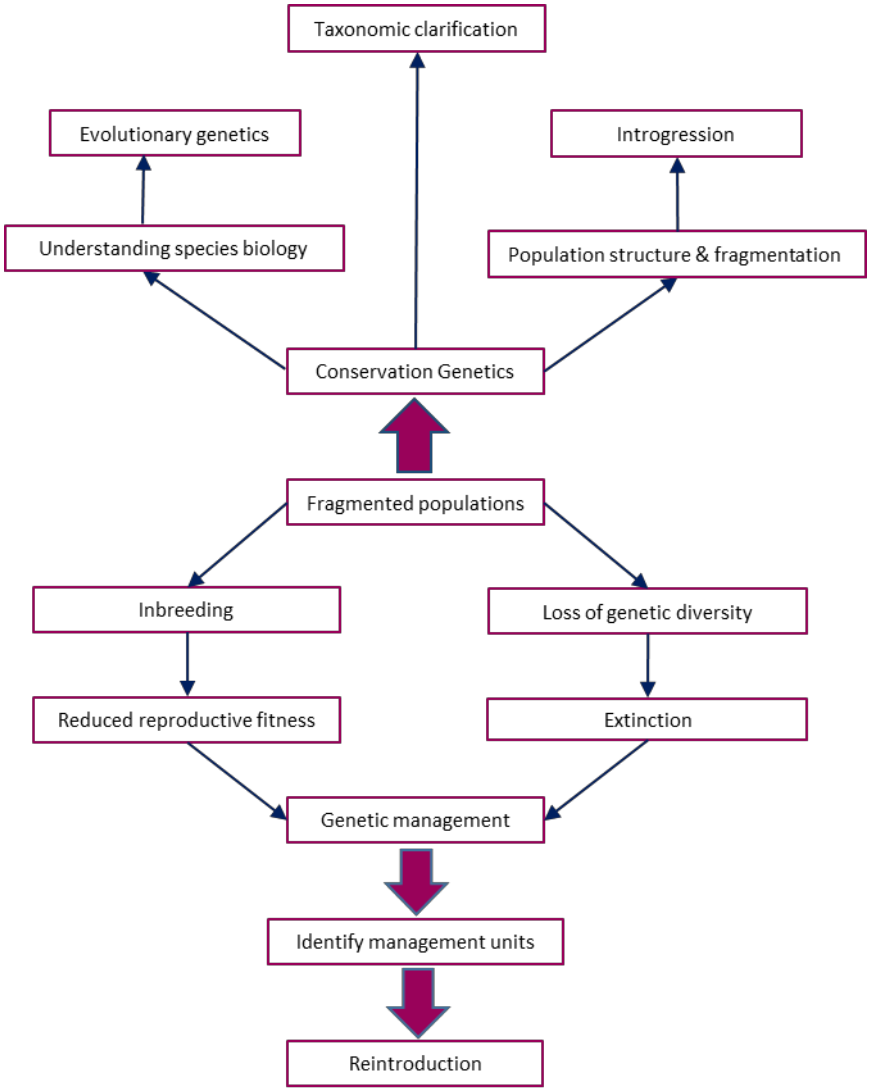

Figure 1.A.3: Interests in conservation genetics, potential threats to fragmented populations and workflow to manage fragmented populations (modified after Souty-Grosset et al. 2003).

As genetic diversity is the basis for individuals, populations and species to be able to adapt to environmental changes (Frankham et al. 2002, Hartl 2000). The "vortex of extinction" fragmented populations are likely to enter, can be accelerated when genetic diversity is reduced. Additional adverse events associated with isolated populations are inbreeding, which again adds up to fitness reduction and increases the risk of extinction again (Fig. 1.A.3). Conservation genetic studies aim to detect and evaluate genetic diversity in order to preserve it (Avise 2008). This research field

combines methodologies ranging from ecology, molecular biology, population genetics as well as systematics, and provides important information on genetic diversity about species or populations of conservational interest (*e.g.* Amato et al. 2013, Schwartz et al. 2007, DeSalle & Amato 2004). Thereby special management units (MUs) can be identified. The detection of MUs is crucial to develop short-term management plans for the conservation of natural populations (Palsbøll et al. 2007). This term is used to delineate entities with special management needs which require monitoring in an effort to detect and regulate the anthropogenic activity these entities experience (Schwartz et al 2007). For genetic monitoring approaches non-invasive DNA sampling techniques can be applied to rare or dangerous species by using hair, scat, feathers or other materials as tissue samples (Avise 2004) to investigate population size and structure, dispersal, sex ratio, hybridization or inbreeding (Proctor et al. 2005, Boulanger et al. 2004, Banks et al. 2003, Lucchini et al. 2002, Mowat & Strobeck 2000). Thereby it is possible to obtain data of threatened species without the necessity to capture or disturb them directly (Wolinsky et al. 2012, Rudnick et al. 2005).

Genetic markers and Mitogenomics

Over the last 50 years several different genetic markers (e.g. allozymes, restriction fragment length polymorphisms, microsatellite markers, other DNA-based markers) were applied to identify levels of genetic variation in conservation genetic studies (Ellegren 2014). The most successful method might be DNA-barcoding, using a standardized mitochondrial DNA region e.g. the cytochrome c oxidase 1 (CO1). An international initiative of the Consortium for the Barcode of Life (CBOL) created a worldwide database (BOLD, www. http://boldsystems.org), were researchers can deposit CO1 sequences of the proposed standard for species identification and taxon assignment. As to date (November 2019), 3,618,175 sequences from 213,376 species and 93,154 interim species are available on this website as barcode records. Therefore, CO1 as a genetic marker became a “must-have” in conservation genetic studies used to compare data on a spatial or timely level to available data. This minimized the individual sampling efforts (Taylor & Harris 2012). The latter displays an advantage especially for species with a large distribution range. However, the barcoding region CO1 shows limitations and problems have occurred regarding undescribed or cryptic

species as well as in species groups which show only low variability or saturation within this gene fragment (Hickerson et al. 2006, Rubinoff 2006, DeSalle et al. 2005).

The development of next-generation sequencing technologies (NGS) enabled the generation of vast amounts of sequence data in a shorter time and at much lower cost, compared to traditional sequencing methods (Hendricks et al. 2018). This now allows large-scale genome or transcriptome analyses even for non-model organisms and opens up the possibility to use genomic data to also address evolutionary questions in wild populations (Ellegren & Sheldon 2008). Therefore, an increasing demand in using large sets of single nucleotide polymorphisms (SNPs) in the field of conservation genetics was noticed (Eckblom et al. 2018). Several studies already benefitted from technical developments by recent state-of-the-art whole genome sequencing (e.g. Chattopadhyay et al. 2019, Çilingir et al. 2019, Hendricks et al. 2019, Meek & Larson 2019). Nevertheless, traditional sequence markers are still important and a frequently used tool in conservation genetic studies because the modern NGS methods accompanied by these massive data sets present new challenges regarding study design, data management, bioinformatic expertise, data interpretation and visualization (Hendricks 2018).

The use of genome-scale data in conservation related studies is still limited, most likely due to the following reasons: (I) the novel challenges accompanying these data sets, (ii) the cost of sequencing is indeed decreasing, but especially conservation relevant projects have to deal with limited budgets, which only allows NGS sequencing of restricted sample sizes. The cost effectiveness regarding the sample size and number of loci to sequence is a critical consideration and has to be discussed indivually for each study design and specific research question. And (iii) the availability of a reference genome for data analyses, which is often not the case for non-model organisms (Hendicks 2018).

Therefore, sequencing whole mitochondrial genomes (mitogenomes) displays an alternative as the amount of data is higher but still manageable at a fraction of costs compared to whole genome studies (Qin et al. 2019). Mitogenomes have been commonly used in phylogenetic, population genetics and conservation studies (e.g. Qin et al. 2015, Cameron 2014, Salvato et al. 2008, Simon et al. 2006, Wilson et al. 2000). Odonate mitogenomes are relatively conserved, comprising 37 genes including 13 protein-coding genes (PCGs) (Simon & Hadrys 2013). Because of their maternal

inheritance, mitogenomes are evolving quickly, which is particularly useful for phylogenetic analysis (Hebert et al. 2003).

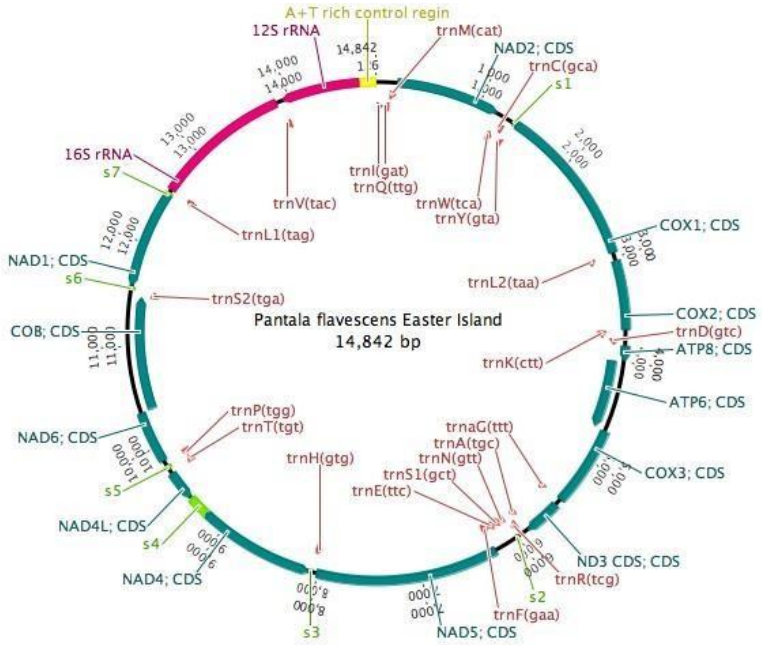

Figure 1.A.4: Organization and gene content of the mitogenome of *Pantala flavescens* from Easter Island.

In order to maximize the possible benefits of these rapidly developing genomic technologies, however, a solid foundation of ecological, geographical and morphological studies paired with solid phylogenies is *sine qua non*. Combining detailed ecological information with new genetic and genomic insights will bring a deeper understanding of the status quo of conservation relevant species and will therefore add to the quality of future management plans.

B. Odonates as bioindicators

Dragonflies and damselflies (Odonata) are currently on their way to emerging as an ecological model (Bybee et al. 2016). They are key to insect evolution and represent one of the most ancient groups of winged insects, having an unmatched fossil record (Simon et al. 2009, Bybee et al. 2008, Grimaldi & Engel 2005). This makes this group particularly amendable to phylogenetic and evolutionary studies. A unique set of morphological traits, diverse natural histories and a staggering diversity of behaviors complete the list of attractive features for broad-based research of this group (Bybee

et al. 2016, Corbet 1999). Odonata is a comparatively small but evolutionary important insect order with approximately 7500 extant species (e.g. Deijksta et al. 2015) that can be reliably dated back to the Permian (Kalkman 2008). They are, according to recent research, at the base of the most species-rich animal group on earth - the winged insects and thus at the base of wing development and the explosion of biodiversity (Simon et al. 2009, Grimaldi & Engel 2005). They developed a unique flying mode and exceptional reproduction and life cycle strategies that allowed an unrivalled variety of adaptation patterns to life in the air and in the water (Corbet 1999). The genital morphology of odonates is unique in the animal kingdom. The females have sperm-storage organs and the males have 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 first studies analyzing the evolution of the reproductive system in the context of sexual selection, sperm competition and female choice were done in odonates and have changed our understanding of mating systems in general (e.g. Cordero Rivera et al. 2004, Cordoba-Aguilar et al. 2003, Fincke & Hadrys 2001, Waage 1979). In odonates, natural as well as sexual selection are strongly involved in reproductive behaviour as well as mate recognition and could therefore promote speciation in a variety of ways (McPeek & Gavrilets 2006, Svensson et al. 2006). With the introduction of molecular methods, odonate paternity studies gave additional insights into mating strategies and provided, through the combination of behaviour, population genetics and speciation processes, crucial information for conservation and evolution (e.g. Damm & Hadrys 2009, Hadrys et al. 2007, Cordero Rivera et al. 2004, Hadrys et al. 1993).

Odonates are already prime indicators for the evaluation of freshwater ecosystems (Bried & Samways 2015, Kalkmann 2008). They are associated with freshwater habitats by their complex life cycle composed of an aquatic larval and terrestrial adult stage. 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. 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. Bried & Samways 2015, Cordoba-Aguilar 2008, Samways 2007, Clausnitzer 2003, Corbet 1999, Samways 1993). This is particularly important because freshwater

ecosystems and resources are increasingly affected by anthropogenic influences and / or global changes (Sala et al. 2000). In addition, the complex life cycle of Odonata offers the opportunity to evaluate both terrestrial and aquatic effects of environmental changes (Bried & Samways 2015, Kalkmann 2008).

Adaptation and speciation research in odonates concentrates on past radiation processes, using phylogenetic analyses at different taxonomic levels (from genera to suborders). While some studies exclusively focus on phylogeny, others attempted to analyse the history of odonate diversification. Here, combined data sets concerning biogeography, habitat specificities, color patterns and behavior lead to a more sophisticated view of the speciation processes (Corbet 1999). The overall conclusion resulting from the studies is that the most prominent geographical mode of speciation in odonates is likely to be allopatric speciation (Kalkman et al. 2008, Dijkstra & Clausnitzer 2006, Stoks et al. 2005, Turgeon et al. 2005). However, a disruption of gene flow as a result of geographical separation is most likely not the only reason for speciation in such powerful flying insects as odonates, which could cover even long geographic distances. In addition fine-scale local adaptations through environmental or behavioral changes might take place. The combination of their unique reproductive system, their complex life cycle and habitat sensitivity make odonates a perfect system to study the impact of natural selection on speciation and potential adaptation processes.

Genetic mechanisms of adaptation

As already discussed, many species are confronted with dramatically altered environments due to anthropogenic impact. Therefore, the scientific interest to understand the principles and the pace of adapation increased over the last years (Franks et al. 2007, Palumbi 2001, Bradshaw & McNeilly 1991). Because of the rapidly progressing advances in genomics technologies (e.g. NGS) studies in this research field are increasing. The adaptation to new environmental conditions can take place by selection on pre-existing genetic variation (standing variation) or selection on new mutations (Barrett & Schluter 2008). The latter was for a long time believed to be the only source of adaptation (Orr 2005). These two mechanisms can result in different evolutionary histories and varying genetic outcomes. If adaptation can be realized from standing genetic variation, then evolution is possible in a shorter amount of time

(Barrett & Schluter 2008). This also includes the fixation of a higher amount of alleles with smaller effects leading to more recessive alleles (Hermisson & Pennings 2005). Alleles with a potential benefit are maintained in the ancestral gene pool by a combination of mutation, selection and drift (Przeworski et al. 2005). Prior to a new selection pressurese these alleles are neutral or deleterious (Hermisson & Pennings 2005, Orr & Betancourt 2001). This stresses the necessity to maintain and manage genetic variation in natural populations in order to preserve the adaptive potential to altered environmental conditions.

Natural species differ in their adaptive potential based on their adaptive strategy: Eurytopic species are less specialized to a certain environment, capable of utilizing a wider range of resources and found in various habitat types. In contrast, stenotopic species are adapted to a narrow ecological niche and tolerate only in narrow range of environmental conditions (Futuyama & Moreno 1988). Comparative studies have shown that specialization is linked to the potential of extinction making stenotopic species more vulnerable (Purvis et al. 2000). When confronted with environmental changes, species with broad tolerances are more likely to persist than species specialized to narrow or fragile niches (Pulvis et al. 2000).

Habitat specialists and generalists

Not only that stenotopic and eurytopic species differ in their viability and therefore require different management strategies, they also vary in their usability as bioindicators for certain types of habitats (Samways 1994). Further, their distribution and abundance can be affected by the grade of specialization, as habitat specialists are restricted to rare and limited habitats; in contrast generalists that tolerate a whole range of environmental conditions are often cosmopolitan and abundant (ibid.). However, the basic principle behind assigning bioindicator species is that these reflect parameters of interest about their habitat. Regarding the type of information desired, abundant generalists can also serve as surrogate to measure abiotic parameters or other biota. Bioindicator studies can be based on presence / absence data, abundance, relative abundance, reproductive success and viability, community structure or combinations of these terms (Landres et al. 1988, Hellawell 1986). This thesis mainly focuses on two odonate species with contrasting adaptational mechanisms to identify valuable strategies within this period of insect mass extinction: The cosmopolitan

eurytopic species *Pantala flavescens* and the endangered stenotopic *Lestes macrostigma*.

Study species portraits: Lestes macrostrigma

Lestes macrostigma, commonly named the Dark Spreadwing, can be easily identified by its dark body: the thorax and the top of the head as well as the segments S1+2 and S8-10 as are covered with an intensive, purple to bluish pruinosity while the abdominal segments S3-7 are mostly bronzy green. This coloration in combination with the large Pterostigma (= macro stigma), which spans three adjacent underlying cells, are distinctive features for *L. macrostigma* (Bellmann, 2010). The total length of this robust damselfly is 39 – 48 mm, with the abdomen being the largest part (31 – 38 mm) (Dijkstra & Lewington2006). The hindwing length is 24 – 27 mm (*ibid.*). *L. macrostigma* is a stenoecic species adapted to brackish water (Jödicke 1997, Plattner 1967, Robert 1958). The natural habitat of *L. macrostigma* covers a regions of the South Palaearctic from the Mediterranean area to Siberia and the Central Asia (Boudot et al. 2009, Dijkstra & Lewington 2006). Given the exclusive habitat preference, the regional occurence of this species is patchily distributed over ist geographical range (Sahlen et al. 2004, Jödicke 1997). An important ecological aspect of *L. macrostigma* is its association with brackish waters and *Bolboschoenus maritimus*, a characteristic coastal plant prefered for oviposition. Jödicke (1997) suggested that adults of *L. macrostigma* also use this plant species to identify suitable habitats for larval development. Many studies describe this species laying eggs into *Bolboschoenus maritimus* (L.) Palla (Cyperaceae) (e.g. Schweighofer et al. 2010, Lambret et al. 2009, Stark 1980) but also within other plants species, such as *Carex stenophylla*, *Schoenoplectus lacustris*, *Juncus maritimus, Juncus acutus, Phragmites australis* and *Tamarix gallica* (Lambret 2011, Lambret et al. 2009, Martynov & Martynov 2007, Stark 1980).

Adults of *L. macrostigma* can be observed during spring and early summer, while the specific flight season can depend on location and local climate (Dijkstra & Lewington 2006, Jödicke 1997). For instance in the Camargue, typically during the first weeks of May until late June or early July, adults can be observed (Lambret 2010, Lambret et al., *in prep*.). The embryos develop in the plant tissues and the larvae hatch in the spring of the following year (Schiel 2013, Lambret 2012).

Figure 1.B.1: Tandem of *L. macrostigma*, a typical habitat in the Camargue area, and a single individual of this study species (from left to right).

Study species portraits: *Pantala flavescens*

The Wandering Glider, *Pantala flavescens* (Fabricius 1798) belongs to the family Libellulidae (Odonata : Anisoptera). Together with *Pantala hymenaea* (Say 1839*)* it forms the genus *Pantala.*

Its wing span ranges between 72 mm and 84 mm with an abdominal length of up to 45 mm. The abdomen and thorax are similarly colored in a yellow to golden color with a dark brown to black line. The wings generally show a yellow to brown colored pterostigma and, and a broad wing base (Ross 2000). The eye color varies between red and brown (Dijkstra & Lewington 2006). Within its worldwide distribution phenotypical differences were noticed (*ibid.*). Individuals with olive to brown or even black colored wings were described (Ross 2000) and especially island populations show shorter wings and a darker colored abdomen (Samways & Osborn 1998). *P. flavescens* is globally distributed on every continent, except for the Antarctica and major parts of Europe. Here, only on Cyprus, in Greece and the European parts of Turkey this species can be found (Dijkstra & Lewingston 2006). Generally *P. flavescens* occurs within the 20 °C isotherms (Samways & Osborn 1998). The wandering glider is the only known dragonfly on the Easter Island, with 3,833 km distance to mainland, the world´s most remote inhabitat island (Samways & Osborn 1998, Dumont & Verschuren 1991). *P. flavescens* gained scientific attention, after Hobson et al. (2012) discovered its 14,000 – 18,000 km long, multigenerational migration route, which is by far the longest known distance compared to other migratory insects. As an obligatory migrant, *P. flavescens* follows rainfalls transported by the inter-tropical convergence zone (ITCZ) or monsoon rains, by using the arising winds in massive swarms up to several million individuals. Swarm sizes of up to 34 km² are documented (Hobson et al. 2012, Corbet 1999). Due

to its complex life cycle with aquatic larval development, it reproduces in ephemeral water bodies emerging by these rainfalls. As adaptation to its fugacious breeding habitat, the larval stadium is remarkably short (~43 days) and already immature individuals start to migrate (Corbet 1999).

In contrast to other odonates, *P. flavescens* migrates not only intercontinental distances but also over large open water bodies. One such known example is from India to Africa, thereby covering distances of up to 3500 km (Corbet 1999). Furthermore, this species flies higher than any other odonate. Even trans-Himalayan migrations with altitudes up to 6300 m are presumed (Hobson et al. 2012). Due to its huge distributional range, representative sampling of genetic material from all continents is challenging. Though a first genetic study proclaimed a panmictic global population structure (Troast et al. 2017), the used sampling set of 49 individuals from three continents does not reflect this species remarkable abundance and occurrence.

Figure 1.B.2: *Pantala flavescens,* with larvea, and a swarm of the study species (from left to right by Rod Miller, Gary McClellan, Philip Steinhoff).

C. This thesis' aims

Current research shows that insects, especially flying insects as the world most diverse animal group, are on the verge of mass extinction and are disappearing at a rate that cannot simply be explained by current scientific means (Regnier et al. 2015, Conrad et al. 2005, Dirzo et al. 2003). Consequently not only vast monitoring but studies explaining the causal mechanisms behind the scene are urgently needed to shed light on this observed adverse event of biodiversity loss. While the causal mechanisms of species extinction and their determinants are the focus of a majority of studies, the influence of environmental changes on adaptation and speciation processes had yet been difficult to determine. This however would be crucial to estimate the adaptation

potential of (endangered) species and consequently evaluate the global and local anthropogenic impacts on changing natural ecosystems. The increasing destruction of habitats cannot be halted nor reversed despite all efforts of conservation management and environmental policy. However, to ensure a sustainable crisis management the genetic plasticity and "potential" of a species to adapt to (fast) changing conditions needs to be explored and compared among species with different adaptation strategies.

Consequently, the presented dissertation deals with two odonate species with very different ecological niches, almost opposing adaptation strategies and distributional dynamics. These are on the one hand *Lestes macrostigma*, an endangerd, stenotopic damselfly adapted to temporary, brackish ecosystems with spatially very limited distribution. The other target species is the cosmopolitan, abundant and migratory *Pantala flavescens*, inhabiting all kinds of temporary waters created by local rains as an ecological generalist. Due to their temporary nature, both habitats are particularly prone to climate change, but they differ significantly in their spatio-temporal occurrence. The resulting different adaptation strategies and distributional dynamics of both species were therefore genetically characterized in this work to identify and evaluate successful strategies of the persistence of a species facing climate change.

In this context a variety of genetic and genomic approaches were used, ranging from traditional sequence markers like the CO1 barcode fragment, over newly developed sequence markers providing greater resolution at the population level, to next generation sequencing approaches and whole mitogenome analyses. These marker systems were applied on different spatio-temporal levels:

(i) on the scale of a single nature reserve, over a ten years period,

(ii) on an Eurasian level covering the distribution area of the endangered and highly stenotypic damselfly *Lestes macrostigma* as the first genetic risk assessment of this species to identify management units and

(iii) on a global level to study the most successful dragonfly on earth, *Pantala flavescens,* in terms of abundance, distribution area, generalistic adaptation and migratory capacity.

This target species choice and comparison enables, at least among odonates, an evaluation of potential “winners” and “losers” with their respective life-history traits and different adaptation strategies within this period of (insect) mass extinction. By using dragonflies and damselflies as bioindicators the conducted studies add valuable data to the in risk assessments underrepresented class of Insecta, the world´s most diverse animal group, which is disappearing at a rate that cannot simply be explained by current scientific means. Further, these model organisms help to elucidate ecological, evolutionary as well as biogeographical questions dealing with increased anthropogenic selection pressure.

Odonate mitogenomes and new genetic markers

One aim of this thesis was to use Next Generation Sequencing approaches to unravel whole mitochondrial genomes of selected odonate species as the fundament for further phylogenetic, evolutional or conservational studies.

In order to faciliate studies on range shift, expansion, and the adaptive potential related to global warming, the mitogenome of *Anax imperator* was analysed (see Chapter 1.A). This species was among the first dragonflies for which a recent range shift, northwards (e.g. Parr 2010) and towards higher altitudes (Hunger et al. 2006, Westermann 2003) due to global climate change, was detected.

Ischnura elegans is a promsing model for bridging the gaps between developmental, environmental and evolutionary studies as its complete life can be cultured in the lab (Simon & Hadrys 2014, 2013). Because of ist high value for eco-evo-devo studies it was the first odonate species for which a transcriptome and ESTs were available (Chauhan et al. 2014, Simon et al. 2009). A complete mitogenome of this species paves the way for future studies in this advancing research field (see Chapter 1.B).

The world largest damselfly, *Megaloprepus caerulatus,* emerged as a valuable bioindicator for intact tropical rainforest ecosystems. Therefore, it was already included in ecological studies (e.g. Fincke & Hedström 2008). A first population genetic study gave evidence for speciation processes within this species (Feindt et al. 2014) and future studies in this subject will highly benefit from the availabilty of its mitogenome (see Chapter 1.C).

One of the main target species of this thesis is *Pantala flavescens,* for which a primer set from available transcriptomic data was developed, in order to sequence the whole mitochondrial genome for future studies on insect migration (see Chapter 2.A). Furthermore, additional sequence markers, despite the barcode CO1, were highly desired for population genetic studies. In this thesis the suitability of the NADH dehydrogenase subunit 1 and 3 gene, for global population studies on this cosmopolitan dragonfly, was tested.

The genetics of the cosmopolitan, migratory odonate Pantala flavescens

The cosmopolitan migratory odonate *Pantala flavescens* displays a promising candidate for investigating the phylogeography and genetic mechanisms of a global panmictic population. This species gained scientific attention due to its remarkable migratory capacities and a first population genetic study postulated a panmictic population structure with gene flow all around the globe. In the scope of this thesis a first large-scale study with 27 populations, from every continent of *P. flavescens´* distribution range, was conducted, to enhance the understanding of the population structure of this unique migratory insect species. The most remote population, from the Easter Island, was included in the data set to detect potential isolation or speciation processes. These island individuals show an altered phenotype and no migratory behavior as a local adaptation (Osborn 1998) and this thesis aims to further elucidate the special status of this population.

Conservation genetic assessments of the endangered damselfly *Lestes macrostigma*

As described before, this species is restricted to brackish ecosystems with a special plant composition needed for reproduction. However, a unique population was recently detected in Siberia, Russia that is adapted to freshwater ecosystems instead. The exclusive habitats of *L. macrostigma* are patchily scattered across its distribution range (Europe to Mongolia), probably affected by anthropogenic habitat fragmentation and especially vulnerable to climate change effects.

Within a local 10 years monitoring program for *Lestes macrostigma*, the population genetic dynamics within the Camargue area in Southern France, based on mitochondrial gene fragments (the barcode CO1 and OdoCO1 fragment) is studied.

Understanding this species dynamics regarding abundance and genetic metrics displays a pivotal background for future management efforts and for classifications within risk assessments. The high potential of habitat destruction (due to tourism and land use) and adverse climate change effects threatening this region (e.g. Cramer et al. 2018, Sala et al. 2010, Giorgia & Lionello 2008), makes it mandatory to investigate this stenotopic species ability to deal with population bottlenecks and their genetic consequences.

The results obtained during the local study will be put into geographical context in a large-scale population genetic study covering major parts of *L. macrostigmas* distribution range. This thesis focusses on the conservation genetics of this threatened damselfly to assess (1) the overall genetic diversity of this stenoecious species, especially in order to contribute to future assessments of the species´ extinction risk;
(2) the impact of habitat fragmentation across the distribution range and potential migration routes, especially to fine-tune the implementation of future habitat restoration programs and to consider possible reintroduction actions; and (3) whether the habitat differences between Siberia and the rest of the species distribution range is associated with the status as subspecies, thereby possibly redefining the species conservation strategy.

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2 Chapter 1: Mitogenomics in Odonates

A. The complete mitochondrial genome of the emperor dragonfly *Anax imperator* LEACH, 1815 (Odonata : Aeshnidae) via NGS sequencing

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MITO COMMUNICATION

OPEN ACCESS

The complete mitochondrial genome of the emperor dragonfly *Anax imperator* LEACH, 1815 (Odonata : Aeshnidae) via NGS sequencing

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Abstract:

Here we report the complete mitochondrial genome of the emperor dragonfly, *Anax imperato*r (Odonata: Aeshnidae) as the first of its genus. Data were generated via next generation sequencing (NGS) and assembled using an iterative approach. The typical metazoan set of 37 genes (13 protein-coding genes, 22 tRNA genes, and 2 rRNA genes) was detected in the same gene order as in other odonate mitogenomes. However, only three intergenic spacer regions are present in *A. imperator* lacking the distinct s5 spacer, which was regarded as informative feature of the odonate suborder Anisoptera (dragonflies) but absent in Zygoptera (damselflies). With 16,087 bp, it is the longest anisopteran mitogenome to date, mainly due to the long A+T-rich control region of 1291 bp.

B. Short read sequencing assembly revealed the complete mitochondrial genome ofIschnura elegansVander Linden, 1820 (Odonata: Zygoptera)

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MITOGENOME ANNOUNCEMENT

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Short read sequencing assembly revealed the complete mitochondrial genome of *Ischnura elegans* Vander Linden, 1820 (Odonata: Zygoptera)

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Abstract:

Damselflies of the genus *Ischnura* emerge as organisms with high potential in ecological, evolutionary and developmental research at the base of flying insects. *Ischnura elegans* and *Ischnura hastate* are for example one of the few odonate species where a complete life cycle over generations can be reared under laboratory conditions. We here report the complete mitochondrial genome of *Ischnura elegans* as a valuable genomic resource for future eco-evo-devo studies at the base of flying insects. The genome has a total length of 15,962 bp and displays all typical features of Odonata (dragonflies and damselflies) mitochondrial genomes in gene content and order as well as A+T content. Start and stop codons of all protein-coding genes are consistent. Most interestingly, we found four intergenic spacer regions and along A+T rich (control) region of 1196 bp, which is almost double the size of the close relative *Ischnura pumilio*. We assume that the adequate insert size and iterative mapping may be more efficient in assembling this duplicated and repetitive region.

C. The complete mitochondrial genome of the neotropical helicopter damselfly *Megaloprepus caerulatus* (Odonata: Zygoptera) assembled from next generation sequencing data

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MITOCOMMUNICATION

OPEN ACCESS

The complete mitochondrial genome of the neotropical helicopter damselfly *Megaloprepus caerulatus* (Odonata: Zygoptera) assembled from next generation sequencing data

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Abstract:

Odonata (dragonflies and damselflies) is a small order at the base of flying insects (Pterygota). Resolving family-level phylogenetic relationships within this order receives great attention. Hereby, genetic data already resulted in various changes, which are however still under discussion. Mitochondrial genomes may further enhance such phylogenies. This study presents the complete mitochondrial genome of the Neotropical damselfly *Megaloprepus caerulatus* based on next generation sequencing (NGS) data on total genomic DNA. The total length comprises 16,094 bp and includes the standard metazoan set of 37 genes together with a 1376 bp long A+T rich (control) region. Gene content, gene arrangement and base frequency are consistent with other odonate mitochondrial genomes. It further contains four inter-genic spacer regions, indicating a possible family specific feature for the *Coenagrionidae* and its close relatives.

3 Chapter 2: The genetics of the cosmopolitan, migratory odonate *Pantala flavescens*

The complete mitochondrial genome of *Pantala flavescens* Fabricius, 1798 (Libellulidae : Odonata) from an isolated population on one of the world's most remote islands, Easter Island

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Key words: Mitochondrial genome, Odonata, *Pantala flavescens*, A+T rich control region

Manuscript prepared for submission to Conservation Genetic Resources

Abstract

Pantala flavescens is not only the world's most abundant and widely distributed dragonfly, but with its outstanding migratory capacity a perfectly suited model organism to study insect migration at the evolutionary base of winged insects. We here report on the first complete mitochondrial genome (mitogenome) of *P. flavescens* from a geographically isolated population from Easter Island. In contrast to the overall global population, the *P. flavescens* population from Easter Island does not show migratory behavior and is therefore of special interest for comparative population genomic approaches as well as for future mt gene expression analyses. .In this context we found that the complete mitogenome of *P. flavescens* (14,842 bp) is the most compact anisopteran mitogenome characterized until today. It encodes a typical set of 13 protein-coding genes, 22 tRNAs and two rRNAs, which are arranged in the same gene order as observed in other odonate mitogenomes. Remarkable features are the exceptionally short control region (160 bp) and a truncated *nad4* gene leading to a strong genome size reduction.

Keywords: Mitochondrial genome, Odonata, *Pantala flavescens,* A+T rich control region

The migratory odonate *Pantala flavescens* is described as the most successful odonate species in terms of abundance and geographic occurrence (Dijkstra & Clausnitzer 2014). This species global dominance is likely achieved by its remarkable migratory capacity with long, multigenerational migration routes of up to 18,000 km. This is the longest known distance compared to other migratory insects (Hobson et al. 2012). Consequently, this species constitutes a promising model organism for investigating the genetic patterning processes related to global migration. To date, only a nuclear microsatellite panel and partial mitochondrial genes (*cox1, nad1* and *nad3*) were used in phylogenetic studies of *P. flavescens* (e.g. Alvial et al. 2019, Troast et al. 2016). These studies revealed the genetic isolation of the population from Easter Island, the only known population to be obligatory non-migrant (Dumont & Verschuren 1991). The comparison of whole mitogenomes from migratory and resident populations is a

powerful approach to reconstruct the mitogenetic patterning linked to migratory behavior.

A standard Phenol-Chloroform protocol by Hadrys et al. (1992) was used to extract total genomic DNA from a mid-leg of an individual collected on Rapa Nui, Easter Island. PCR primers were designed based on a draft mitogenome assembled from *P. flavescens* transcriptomic data (PRJNA239794). Primers were designed to cover the whole mitogenome by overlapping PCR fragments. Sanger sequencing was conducted at DNA Analysis Facility at Yale University, New Haven, CT and Geneious version 8.1.8 (https://www.geneious.com) was used to assemble the resulting overlapping mitogenome sequences. For mitochondrial genome annotation the MITOS WebServer (mitos.bioinf.uni-leipzig.de/index.py) was used and results were manually checked using BLAST (Altschul et al. 1990) as well as available odonate mitochondrial genomes (e.g. Herzog et al. 2016, Yu et al. 2016). Transfer RNA genes were predicted using tRNAscan-SE v.1.21 Search Server (Lowe & Eddy 1997) and ARWEN v.1.2 (Laslett & Canbäck 2008), respectively. The phylogenetic position of *P. flavescens* was assessed in the context of all available anisopteran mitogenomes to date (28.10.2019) mined from GenBank. Therefore, protein-coding genes (PCGs) were aligned using MAFFT v.7.017 (Katoh et al. 2002) and concatenated. Bayesian inference (BI) analyses were performed using MrBayes ver. 3.2.6. Maximum likelihood (ML) analyses were performed using the best-fitting model GTR+F+I+G4 under the Bayesian information criterion detected via ModelFinder (Kalyaanamoorthy et al. 2017) with IQ-tree keeping default settings (Hoang et al. 2017, Nguyen et al. 2015). *Ischnura elegans* (NC_031824) as zygopteran species served as outgroup.

The complete circular mitochondrial genome sequence of *Pantala flavescens* (GenBank accession number will be provided upon manuscript acceptance) with the length of 14,482 bp is the most compact known mitogenome among Anisoptera. The standard metazoan gene content of 37 genes (i.e. 13 protein-coding genes, 22 tRNA genes and two rRNA genes) is identically arranged as in other odonate mitochondrial genomes (e.g. Simon et al. 2013) (Tab. 3.A.1). However, a 207 bp long non-coding spacer region is located between *nad4* and *nad4l*, due to a premature stop codon in the *nad4* gene. Overall base frequency is 72.8 % AT and therefore AT-biased. Regarding mitochondrial invertebrate start codons, with ATT (*nad2*, *atp8),* ATA (*cox1*, *nad3, nad4, atp6*), ATC

(*nad5, nad6*), ATG (*cob*, *cox2, cox3, nad4l*) and TTG (*nad1*) standard codons are used. All protein coding genes use TAA as complete stop codon except *cox2,* which possess a single T completed by post-transcriptional polyadenylation. The AT rich control region (CR) is 160 bp long and therefore the shortest observed CR among all anisopterans. For the other observed species, that were used for the phylogenetic analyses (see supplementary data S1), the CR length ranges from 190 bp in the *Hydrobasileus croceus* mitogenome to 1291 bp in *Anax imperator*. This indicates decreasing length of the control region proportional to evolutional divergence, as the family Libellulidae is believed to be the youngest among Anisoptera (Nel & Paicheler 1993).

The phylogeny of all available Anisoptera displays an identical topology for the BI and ML methods (Fig. 3.A.1). All families within Anisoptera were consistently supported as monophyletic groups if represented by more than one species (Fig. 3.A.1). *Pantala flavescens* is placed as a sister to *N. pygmaea* within the Libellulidae. However, some nodes in this family are only poorly supported. The Libellulidae radiated into the most diverse anisopteran family and increasing taxon sampling will be necessary to resolve the evolutionary history of this diverse group (Jeong et al. 2018, Kalkmann et al. 2008). However, the mitochondrial genome of *Pantala flavescens* is a valuable contribution to this still underrepresented family and an important resource for future genomic studies on migration patterns in Odonata. Furthermore, it paves the way to comparative approaches between the only known resident population on Easter Island of this key species for insect migration.

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Display items:

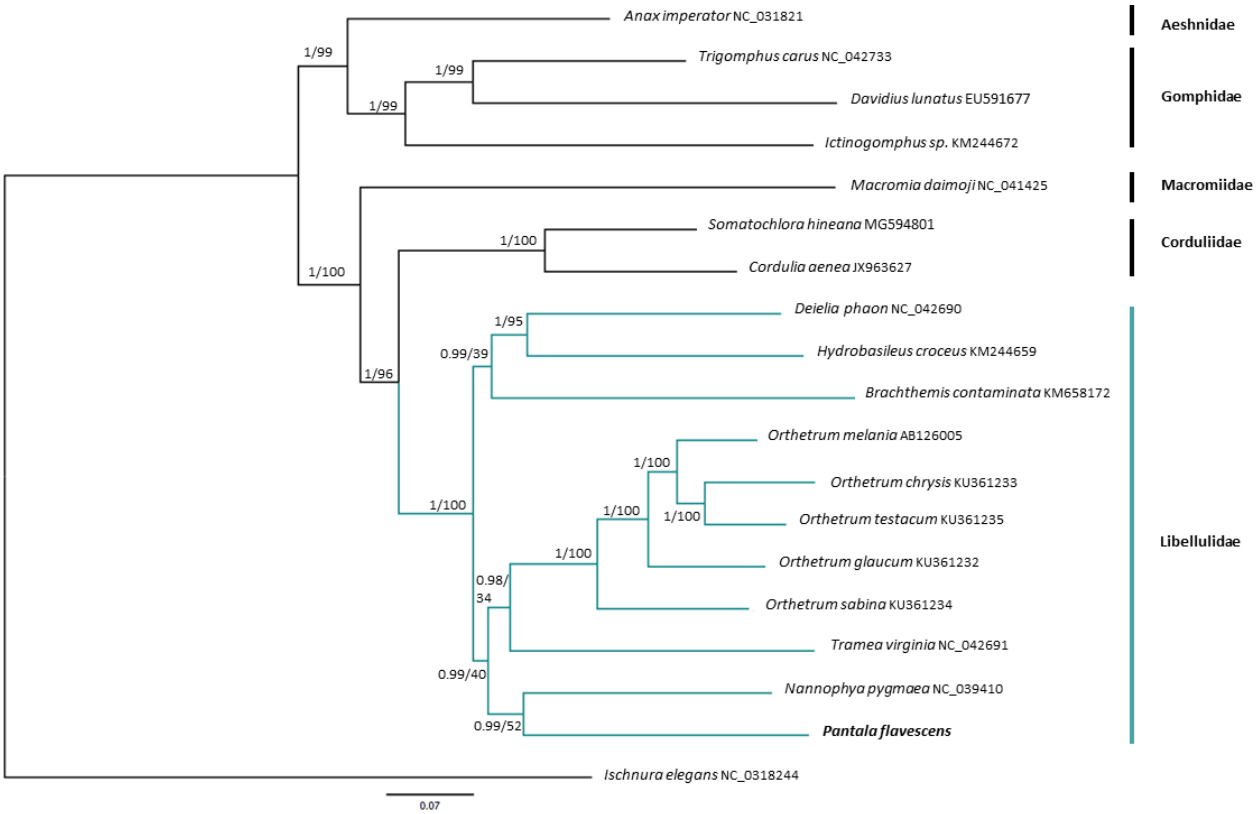

Figure 3.A.1: Maximum Likelihood (ML) tree for Anisoptera using 12 concatenated mitochondrial protein-coding genes (9,601 bp). GenBank Accession numbers are provided. Bayesian posterior probabilities and Maximum Likelihood bootstrap support values are given at each node, respectively. The Zygoptera *Ischnura elegans* served as outgroup and the here presented mitogenome of *Pantala flavescens* is given in bold.

Table 3.A.1: Organization of the mitochondrial genome of *Pantala flavescens*.

Gene/region	Strand	Start	Stop	Length	Anti/start codon	Stop codon
trnI	+	1	64	64	GAT	/
trnQ	-	65	130	66	TTG	/
trnM	+	130	198	69	CAT	/
nad2	+	199	1194	996	ATT	TAA
trnW	+	1195	1259	65	TCA	/
trnC	-	1253	1321	69	GCA	/
trnY	-	1321	1386	66	GTA	/
s1	NA	1387	1408	22	/	/
cox1	+	1409	2962	1554	ATA	TAA
trnL2	+	2958	3023	66	TAA	/
cox2	+	3024	3711	688	ATG	T(AA)
trnK	+	3712	3782	71	CTT	/
trnD	+	3782	3847	66	GTC	/
atp8	+	3848	4009	162	ATT	TAA
atp6	+	4006	4679	674	ATA	TAA
cox3	+	4680	5467	788	ATG	TAA
trnaG	+	5468	5532	65	TTT	/
nad3	+	5530	5886	357	ATA	TAG
trnA	+	5885	5950	66	TGC	/
trnR	+	5950	6015	66	TCG	/
s2	NA	6016	6017	2	/	/
trnN	+	6018	6084	67	GTT	/
trnS1	+	6085	6151	67	GCT	/
trnE	+	6152	6216	65	TTC	/
trnF	-	6217	6286	70	GAA	/
nad5	-	6240	8012	1773	ATC	TAA
trnH	-	8013	8072	60	GTG	/
s3	NA	8073	8074	2	/	/
nad4	-	8075	9208	1134	ATA	TAA
s4	NA	9209	9415	207	/	/
nad4l	-	9416	9706	291	ATG	TAA
trnT	+	9707	9773	67	TGT	/
s5	-	9774	9782	9	/	/
trnP	-	9783	9850	68	TGG	/
nad6	+	9851	10,354	504	ATC	TAA
cob	+	10,354	11,487	1134	ATG	TAA
trnS2	+	11,486	11,552	67	TGA	/
s6	NA	11,553	11,568	16	/	/
nad1	-	11,569	12,519	951	TTG	TAA
s7	NA	12,520	12,521	2	/	/
trnL1	-	12,522	12,587	66	TAG	/
l-rRNA	-	12,564	13,870	1307	/	/
trnV	-	13,871	13,940	70	TAC	/
s-rRNA	-	13,935	14,682	748	/	/
A + T-rich (control) region	NA	14,683	14,842	160	/	/

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A global player? Large-scale population genetic analyses on the cosmopolitan migratory dragonfly *Pantala flavescens* (Fabricius, 1798) reject a panmictic population structure

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Abstract

The cosmopolitan migratory odonate *Pantala flavescens* displays a promising candidate for getting deeper insights into the phylogeography and genetic mechanisms of a global, potential panmictic population. This dragonfly gained scientific attention due to its remarkable migratory capacities. A first population genetic study postulated a panmictic population structure with gene flow around its global distribution range.

We here present the first large-scale study with populations covering every continent of *P. flavescens´* distributional range. An understanding of the population structure of this unique migratory insect species (including its most remote population on Easter Island) may shed light onto the causal mechanisms and evolutionary history of its migratory behavior. Based on three mitochondrial gene fragments (cytochrome c oxidase subunit I, NADH dehydrogenase subunits I and III) and one nuclear marker (Internal Transcribed Spacer, ITS), 420 individuals of *P. flavescens* from 27 countries were analysed. In general, populations of *P. flavescens* show high genetic diversity measures, for at least one marker gene, each. Comparative analyses between mitochondrial and nuclear markers are conflicting. A high amount of private haplotypes indicates demographic events e.g. range expansions. High levels of gene flow alone cannot explain genetic similarity of geographically distant populations, as we observed genetically distinguishable populations also on shorter distances. Phylogenetic analyses give evidence that this species may originated in Africa, from where range expansion took place. Consequently, we assume the relatedness between African populations to other populations to be a phylogenetic signal rather than evidence for global gene flow.

The complex genealogical pattern we observed in this study probably resulted from a combination of population demographic events, evolutionary history and phylogenetic niche conservatism instead of real panmixia.

Introduction

In times of increasing anthropogenic impact on natural ecosystems, organisms with distinct life-history traits that favor persistence, adaptation and flexibility in their distribution, have enormous advantages during this period of mass extinction. One of the most important traits herein is dispersal, as it not only delivers the opportunity to physically leave adverse environmental conditions (and thereby enables range expansion), but also provides the foundation for gene flow between populations within a species and thus reduces their extinction risk (e.g. López-Uribe et al. 2019, Broquet & Petit 2009, Korkeamäki et al. 2002, Bohonak 1999). Gene flow, as one of the four forces of evolution, is a crucial mechanism for the exchange and preservation of genetic diversity between populations and their potential to adapt to changing environmental conditions (Tonteri et al. 2006). Migration is one powerful source to ensure gene flow. Consequently, the interest of mechanisms and patterns of migration is lately increasing - especially in vertebrates like birds, whales or antelopes (Merlin & Liedvogel 2019, Druskat et al. 2019, Berg & Jodi et al. 2019). Considerably less is known about migration and dispersal in insects, though winged insects display the largest and fastest declining animal group on earth (Fonseca 2009, Engel & Grimaldi 2004). Their migration is mainly air-borne using either weak winds in the flight boundary layer (FBL) near the ground or strong winds in the planetary boundary layer (PBL) in high altitudes (Holland et al. 2006, Dingle 1986). Prominent model systems for insect migration are the Monarch Butterfly (*Daunus plexippus)* in North America, flying about 4000 km en route (Calvert & Brower 1986) and – for Odonata – the dragonfly *Anax junius,* also migrating on the North American continent (May 2008).

The wandering glider or globe skimmer (*Pantala flavescens* (1798, Fabricius)) recently gained scientific attention, after Hobson et al. (2012) discovered its 14,000-18,000 km long, multigenerational migration route, which is by far the longest known distance compared to any other migratory insect. *P. flavescens* is the world's most abundant and distributed dragonfly – only absent in Antarctica and major parts of Europe (Dijkstra & Clausnitzer 2014). As an obligatory migrant, *P. flavescens* follows rains transported by the inter-tropical convergence zone (ITCZ) or monsoon rains, by using arising winds in massive swarms that consist of up to several million individuals. Swarm sizes up to 34 km² are documented (Hobson et al. 2012, Corbet 1999). Due to

its complex life cycle with aquatic larval development, it reproduces in ephemeral water bodies emerging by these rainfalls. As an adaptation to its fugacious breeding habitat, the larval stadium is remarkably short (~43 days) and already the immature adults start migrating (Corbet 1999).

In contrast to other odonates, *P. flavescens* migrates not only intercontinental distances but also over large open water bodies, as from India to Africa, covering distances up to 3500 km (Corbet 1999). Furthermore, this species flies higher than any other odonate. Even trans-Himalayan migration with altitudes up to 6300 m is presumed (Hobson et al. 2012). Moreover, it is the only dragonfly that can be found on the world's most remote inhabited island Easter Island (Dumont & Verschuren 1991), with 3833 km distance to mainland (David 2017). In a recent study, Alvial et al. (2018) demonstrated that this island population is genetically isolated and differs morphologically compared to the southern American mainland population, which was already described by Osborn in 1998. Due to its huge distributional range, representative sampling of genetic material from all continents is challenging. Although a first genetic study proclaimed a panmictic global population structure (Troast et al. 2017), the sampling set of 49 individuals from three continents can not reflect this species' remarkable abundance and occurrence.

Consequently, one aim of this study is to enlarge the sample set on a local scale, as the species is known to occur in large swarms, to gain deeper insights into their genetic relationship. The second aim is to cover this species´ whole distribution range for the first time. Hence, we performed genetic analyses on the cytochrome c oxidase subunit I fragment (CO1), in which we combined available data from NCBI and BOLD to conduct a real global analysis meeting this species' geographical occurrence. Further, we employed three different genetic markers (mitochondrial NADH dehydrogenase subunits I and III and ITS as nuclear marker) for a subset of individuals, to get a better resolution of the genealogical relationships of this putative model organism for insect migration.

Material & Methods

Sample collection and study sites

Tissue samples from Africa were captured during a BMBF BIOTA project. Table 3.B.1 shows tissue samples collected by the authors and collaborators, while Table 3.B.2 contains information about the minded sequences from GenBank and NCBI. The total data set is visualized in Figure 3.B.1. For tissue sampling, a single middle leg of each individual from the different populations was used. This sampling method, as described by Fincke & Hadrys (2001), is considered non-invasive and individuals were released afterwards. The legs were stored in 98 % alcohol at -20 °C until analysis.

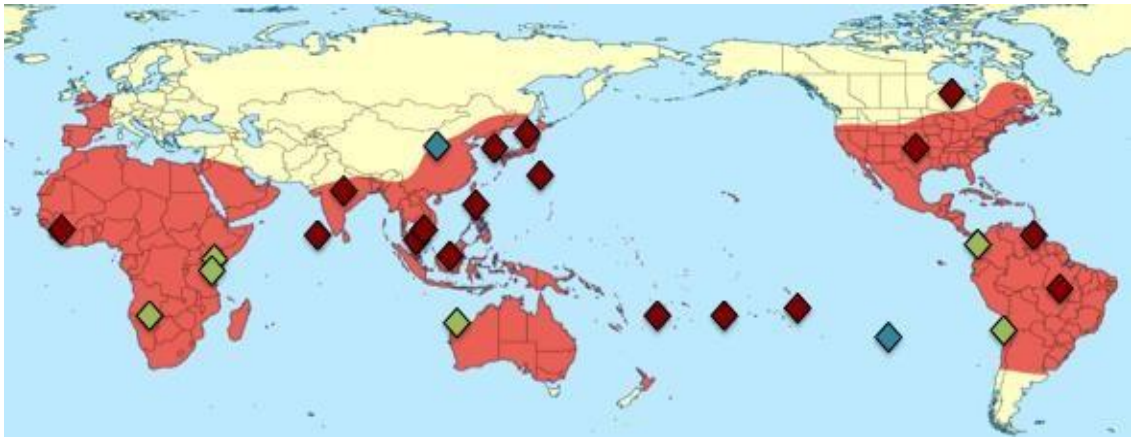

Figure 3.B.1: Current known occurrence of *Pantala flavescens* (orange shaded area) and distribution of sampling sites. Green symbols indicate samples collected by the authors and collaborators. Red symbols indicate sequence data from the respective sample sites that was mined from GenBank. Blue symbols indicate both sites sampled from the authors and additional samples mined from GenBank (modified after John Tann [CC BY 4.0 (https://creativecommons.org/licenses/by/4.0)]).

Table 3.B.1: Genetically analyzed tissue samples including information on country, location, abbreviation used, total sample size (n) and sample size per marker (CO1/ND1/ND3/ITS) of *Pantala flavescens.*

Country	Location	Abbreviation	(n)	CO1 (n)	ND1 (n)	ND3 (n)	IST (n)
Total:			**84**	**77**	**60**	**78**	**46**
Australia	ns	P.fl.CRN	3*	3	3	3	1
China	Peking	P.fl./C	4	4	4	4	3
Chile	Easter Island	P.fl.Ei	9	9	8	9	9
Ecuador	Montanita	P.fl./E	1	1	1	1	1
Kenya	Arabuko	P.fl.AR/K	1	1	1	1	1
	Karacha Pools	P.fl.KR/K	1	1	1	1	/
	Marich Pass	P.fl.MP/K	2	2	/	2	1
Namibia	Nauklauft/Tsauchab	P.fl.TN/N	3	3	2	3	2
	Tsaobis	P.fl.TO/N	51*	45	36	50	22
Tanzania	Ruifji River	P.fl.RR/T	9	8	4	7	6

* Individuals from one large swarm

Additional sequences used in this global analysis were mined from NCBI GenBank and BOLD Systems (see Table 3.B.2); each sequence was verified with BLAST for correct species identification upon analysis. The total dataset consists of samples from North America (Canada, USA), South America (Brazil, Chile (including Easter Island), Costa Rica, Ecuador, Guyana), Africa (Kenya, Liberia, Namibia, Tanzania), Asia (Cambodia, China, French Polynesia, India, Indonesia, Japan, South Korea, Malaysia, Northern Mariana Islands, Philippines) and Australia (see Fig. 3.B.1).

Table 3.B.2: CO1 sequences for *P. flavescens* mined from NCBI and BOLD including the Country of origin, location, accession numbers and sample size (n) for each country. The X symbolizes the accession numbers KY200583 - KY200609 and KY934249 - KY934261, which were uploaded as sequences from 40 haplotypes. Original sequences were provided by the authors upon request.

Country (n)	Location	Accsession Numbers	(n)
Brazil (8)	Mato Grosso Du Sol	BNBTO088	1
		BNBTO169 - BNBTO170	2
		BNBTO309	1
	ns	KY947425	1
		KY947462 - KY947463	2
		KY947481	1
Cambodia (7)	ns	LC326557	1
	ns	LC326575 - LC326577	3
	ns	LC326876 - LC326878	3
Canada (6)	New Brunswick	BBEOD030	1
		BBEOD168	1
	Ontario	ODRMA332	1
	ns	HM413487	1
	ns	HM413599	1
	ns	JF839425	1
China (7)	Sichuan	LC326565 - LC326571	7
Chile (109)	Arica	X	29
	Easter Island	X	80
Costa Rica (40)	Alajuela	ASORT712	1
	Guanacaste	X	17
	Heredia	X	22
Fiji (3)	ns	X	3
F. Polynesia (9)	Huahine Island	KX054309	1
	Moore Island	KX054305 - KX054306	2
		KX054310 - KX054311	2
		KX054313	1
	Tahiti Island	KX054307 - KX054308	2
		KX054314	1
Guyana (20)	ns	KU641567 - KU641587	20
India (1)	Kerala	KR011198	1
Indonesia (2)	Borneo	LC326572 - LC326573	2
Japan (24)	Hokkaido	LC326560 LC326586 LC326559	1
		KU641598 - KU641602	1
	Kagawa	KU641604	1
	Miyagi	LC326581	5
		LC326583 - LC326585	1
	Okinawa	LC326590 - LC326592	1
		KU641595 - KU641597	3
		KU641590 - KU641594	3
	Saitama		3
	Tokio		5
Tonga (40)	ns	X	40
South Korea (2)	ns	LC326578	1
		LC366670	1
Liberia (1)	Nimba	KU566246	1
Malaysia (25)	ns	KR080109 - KR080133	25
Maledives (10)	ns	X	10
N. Mariana I. (1)	Saipan	LC326558	1
Philippines (4)	ns	LC326561 - LC326564	4
Peru (19)	Tacna	X	19
USA (5)	Arizona	ODNBB580	1
	Florida	USODO011	1
	Texas	KU641588 - KU641589	2
		ODNBB587	1
Total:			**343**

Genetic methods and analyses

Genomic DNA was extracted from each single midleg of all individuals as described in Hadrys et al. (1992). Three mitochondrial and one nuclear gene fragment were amplified. The CO1 (cytochrome c oxidase subunit 1) fragment was amplified according to Folmer et al. (1999). For the ND1 (NADH dehydrogenase subunit 1) fragment, including partial 16S rRNA and tRNALeu, primers and conditions were used as in Abraham et al. (2001). The ND3 primer pair fw: 5' -TTA TGG CCA CTT TCA T- 3' and rev: 5' -AGT TAG CAG CTT TTG A- 3' was designed using the assembled and annotated mitochondrial genome (Herzog, *in prep*.) based on available *P. flavescens* transcriptomic data (PRJNA239794).

Polymerase chain reactions (PCRs) were as follows for ND3: 1x amplification buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl; Invitrogen), 2.5 mM $MgCl_2$, 0.05 mM dNTPs, 0.5 pmol/l of each primer, and 0.03 U/l *Taq* DNA polymerase under a thermal regime with 3 min initial denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 50 °C for 40 s, 72 °C for 40 s, and 2 min extension at 72 °C.

For the internal transcribed spacer (ITS) region the primer pair OdoITS 1+2 5' -CGT AGG TGA ACC TGC AGA AG- 3' and OdoITS 1+2 5' -CTC ACC TGC TCT GAG GTC G- 3' was used and the temperature regime according to Damm et al. (2010) was applied. Amplification success was checked on an 1.2 % agarose gel. Sanger sequencing of forward and reverse sequences was performed at DNA Analysis Facility at Yale University, New Haven, CT.

Geneiuos version 8.1.8 (Leigh, ns) was used to assemble forward and reverse sequences,which then were aligned with the Geneious plug-in Muscle (Edgar 2004). Tajima´s D and both nucleotide and haplotype diversities were calculated in DnaSP version 5.10.01 (Librado & Rozas, 2009). Haplotype networks were constructed in PopART (version 1.7) using the TCS algorithm (Clement et al., 2002) that is based on statistical parsimony. Sequence divergence within and between populations was calculated in Mega 7 (Tamura et al. 2013) according to the Kimura2-parameter model as described in Kimura (1980). Genetic differentiation in terms of fixation indices (F_{ST}) was computed using Arlequin (version 3.5.1.2). Migrants (N_m) were calculated based on F_{ST}-values after Wright´s formula (1931).

A Neighbour Joining phylogeny based on the concatenated mitochondrial alignment was constructed using Geneious TreeBuilder. 1000 permutations were performed and

Orthetrum melania triangulare served as an outgroup. For this purpose, the respective genes were mined from the complete mitochondrial genome available on NCBI (AB126005, Yamauchi et al. 2004).

Results

A total of 420 individuals of *P. flavescens* from 27 different countries (see Tab. 3.B.1 and Tab. 3.B.2) were compared based on the CO1 gene fragment. The global CO1 alignment is 394 bp long and includes 67 parsimony informative sites. The translated alignment contains 131 amino acids (aa), including five sites with amino acid substitutions in one to five individuals. For a subset of individuals, additional ND1 (n =77, 488 bp), ND3 (n = 73, 635 bp) and ITS (n = 47, 311 bp) alignments were analyzed. The most parsimony informative sites (73) were detected by using the ND3 marker.

Genetic diversity parameters and haplotype distribution

The global dataset based on CO1 only (n=420) clusters in 117 haplotypes containing 94 private haplotypes (Tab. 3.B.3). The overall global haplotype and nucleotide diversity is 0.834 and 0.650 %, respectively. The haplotype diversity is ranging from 0.022 on Easter Island with no private haplotypes to 1.0 in Australia, Kenya, Cambodia, Fiji, Indonesia, South Korea, the Philippines and the USA, while the nucleotide diversity ranges from 0.006 % (Easter Island) to 1.269 % (Indonesia). Haplotype diversities below average are only found in populations from Canada (0.533), Chile (0.791), French Polynesia (0.556), Tonga (0.790) and Easter Island (0.022), which also includes the largest sample size (n=89). Herein, Chile and Tonga also have large sample sizes (both n = 40).

Table 3.B.4 displays haplotype and nucleotide diversity for the data subset analyzed with additional genetic markers (ND1, ND3, ITS). In this dataset, private haplotypes are overrepresented as well: 30/33 for ND1, 48/52 for ND3 and 11/14 for the ITS marker gene. Haplotype diversity (HD) in total is high in all additional markers ranging from 0.711 for ITS up to 0.963 for ND3. Between populations, results vary from 0.0 HD for ND1 in Kenya (while the other markers show a haplotype diversity of 1.0 with only private haplotypes), to several population sites with only private haplotypes leading to HD values of 1.0 (e.g. for ND1, ND3 and ITS in China, as well as ND1 and ND3 in

Tanzania). The Easter Island subset again exhibits the lowest diversity measures for the different marker genes: 0.25 (ND1), 0.222 (ND3) and 0.583 (ITS). However, for the more conserved ITS region, four haplotypes out of nine individuals can be reported.

As a measure of population demography, Tajima´s D was calculated, which considers the mean number of pairwise differences and the number of segregating sites, which are expected to be equal in a neutrally evolving population of constant size. For the overall global dataset, Tajima´s D is -2.257 with a p-value < 0.01, indicating a recent selective sweep or a population expansion after a genetic bottleneck (Tab. 3.B.3). Positive values were observed for Brazil (0.504), Canada (1.031) and the Philippines (0.673), but they showed no statistical significance. The lowest value observed is in the Namibian population, with a value of -2.021 and a significant p-value (< 0.05). These results are consistent with the Tajima´s D values based on the additional markers (Tab. 3.B.4).

Table 3.B.3: Genetic diversity parameters based on CO1. Sampling country, location, the total sample size and genetic diversity parameters (number of haplotypes (H), haplotype diversity (HD), nucleotide diversity in % (π) and standard deviation and Tajima's D test and p-value) of 420 individuals of *Pantala flavescens.*

Country	(n)	H total/private	HD (±SD)	π % (±SD)	Tajima's D (P)
Total	**420**	**117/94**	**0.834 (±0.018)**	**0.650 (±0.0003)**	**-2.257 (<0.01)**
Australia	3	3/0	1.000 (±0.272)	0.508 (±0.0017)	n/c
China	11	10/4	0.982 (±0.046)	0.812 (±0.0012)	-1.221 (>0.10)
Easter Island	89	2/0	0.022 (±0.022)	0.006 (±0.0006)	-1.040 (>0.10)
Ecuador	1	1/0	n/c	n/c	n/c
Kenya	4	3/0	1.000 (±0.177)	0.761 (±0.0025)	-0.808 (>0.10)
Namibia	48	33/23	0.976 (±0.011)	0.973 (±0.0007)	-2.021 (<0.05)
Tanzania	8	6/3	0.929 (±0.084)	0.934 (±0.0020)	-1.032 (>0.10)
Brazil	8	4/2	0.857 (±0.082)	0.544 (±0.0006)	0.504 (>0.10)
Cambodia	7	7/2	1.000 (±0.126)	0.660 (±0.0017)	-0.668 (>0.10)
Canada	6	2/1	0.533 (±0.172)	0.271 (±0.0008)	1.031 (>0.10)
Chile	29	8/0	0.791 (±0.051)	0.640 (±0.0096)	-0.791 (>0.10)
Costa Rica	40	19/7	0.922 (±0.027)	0.797 (±0.0008)	-1.508 (>0.10)
Fiji	3	3/0	1.000 (±0.272)	0.846 (±0.0028)	n/c
French Polynesia	9	3/2	0.556 (±0.165)	0.268 (±0.0012)	-1.149 (>0.10)
Guyana	20	11/4	0.842 (±0.077)	0.589 (±0.0011)	-1.329 (>0.10)
India	1	1/1	n/c	n/c	n/c
Indonesia	2	2/1	1.000 (±0.500)	1.269 (±0.0063)	n/c
Japan	24	21/11	0.975 (±0.024	0.869 (±0.0010)	-1.315 (>0.10)
Tonga	40	12/6	0.790 (±0.051)	0.633 (±0.0007)	-0.912 (>0.10)
South Korea	2	2/0	1.000 (±0.500)	0.508 (±0.0025)	n/c
Liberia	1	1/0	n/c	n/c	n/c
Malaysia	25	23/14	0.990 (±0.016)	0.964 (±0.0011)	-1.659 (>0.10)
Maledives	10	8/4	0.933 (±0.077)	0.931 (±0.0018)	-0.612 (>0.10)
Northern Mariana I.	1	1/0	n/c	n/c	n/c
Philippines	14	4/2	1.000 (±0.177)	0.888 (±0.0021)	0.673 (>0.10)
Peru	19	10/3	0.877 (±0.056)	0.850 (±0.0016)	-1.178 (>0.10)
USA	5	5/2	1.000 (±0.126)	0.711 (±0.0017)	-1.161 (>0.10)

Table 3.B.4: Genetic diversity parameters based on ND1, ND3 and ITS. Location, the total sample size (n) and the sample size of each marker and genetic diversity parameters (number of haplotypes (H), haplotype diversity (HD), nucleotide Diversity in % (π) with standard deviation and Tajima's D test with significance) of in total 84 individuals of *Pantala flavescens*.

Country	Genetic marker	(n)	H total/private	HD (±SD)	π % (±SD)	Tajima's D (P)
Total:	**ND1**	**60**	**33/30**	**0.909 (±0.030)**	**0.653 (±0.0010)**	**-1.933 (>0.10)**
	ND3	**73**	**52/47**	**0.962 (±0.013)**	**0.691 (±0.0006)**	**-2.393 (<0.01)**
	ITS	**47**	**14/11**	**0.711 (±0.062)**	**0.674 (±0.0014)**	**-2.318 (<0.01)**
Australia	ND1	3	2/1	0.667 (±0.314)	0.137 (±0.0006)	n/c
	ND3	3	2/1	0.667 (±0.314)	0.210 (±0.0009)	n/c
	ITS	1	1/1	n/c	n/c	n/c
China	ND1	4	4/3	1.000 (±0.177)	0.649 (±0.0015)	-0.314 (>0.10)
	ND3	4	4/4	1.000 (±0.177)	0.868 (±0.0020)	-0.837 (>0.10)
	ITS	3	3/1	1.000 (±0.272)	0.434 (±0.0014)	n/c
Easter Island	ND1	8	2/1	0.250 (±0.180)	0.051 (±0.0003)	-1.054 (>0.10)
	ND3	9	2/1	0.222 (±0.166)	0.035 (±0.0002)	-1.088 (>0.10)
	ITS	9	4/2	0.583 (±0.183)	0.561 (±0.0025)	-1.477 (>0.10)
Equador	ND1	1	1/0	n/c	n/c	n/c
	ND3	1	1/1	n/c	n/c	n/c
	ITS	1	1/1	n/c	n/c	n/c
Kenya	ND1	2	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c
	ND3	4	4/1	1.000 (±0.177)	0.866 (±0.0017)	-0.837 (>0.10)
	ITS	2	2/2	1.000 (±0.500)	3.571 (±0.0178)	n/c
Namibia	ND1	38	25/22	0.957 (±0.019)	0.812 (±0.0014)	-1.787 (>0.10)
	ND3	50	36/32	0.975 (±0.021)	0.659 (±0.0007)	-2.289 (<0.01)
	ITS	24	5/3	0.594 (±0.083)	0.491 (±0.0017)	-1.967 (<0.05)
Tanzania	ND1	4	4/3	1.000 (±0.177)	0.854 (±0.0028)	-0.446 (>0.10)
	ND3	7	7/7	1.000 (±0.076)	1.410 (±0.0028)	-1.233 (>0.10)
	ITS	6	3/1	0.733 (±0.155)	0.413 (±0.0014)	-0.185 (>0.10)

Genealogical relationships

The haplotype networks for each marker, based on statistical parsimony, are shown in Figures 3.B.2 – 3.B.5. The global CO1 network (Fig. 3.B.2) shows 117 haplotypes, of which 93 are private (80.3 %). It depicts one most common haplotype (Hap_1) including 166/420 individuals from 17/27 populations, originating from Easter Island (n= 88), Costa Rica, Ecuador, French Polynesia, Guyana, Japan, Kenya, Maldives, Malaysia, Peru, Philippines, Namibia, Tanzania, Tonga, USA, Canada and Chile. 23 further haplotypes are shared, though containing less individuals. Hap_3 (n = 15) represents individuals from Australia, China, Costa Rica, Fiji, Indonesia, Japan, Liberia, Malaysia, Maldives, Namibia and South Korea. Another haplotype, Hap_4, is shared by 17 individuals, with individuals coming from Brazil, Chile, Costa Rica, Guyana, Maldives, Namibia, Peru, Tonga and one individual from Easter Island (the only additional haplotype in this group). Hap_16 is a South American haplotype with individuals from Chile, Peru and Costa Rica (n = 16). Hap_2 is the second largest shared haplotype with 20 individuals that differs from the major haplotype in a minimum of three mutational steps and is represented by individuals from Australia, Chile, Costa Rica, Fiji, Guyana, Japan, Maldives, Tonga and the USA. The population of Tonga further shows a private haplotype including nine individuals. The vast majority of private haplotypes are represented by single individuals.

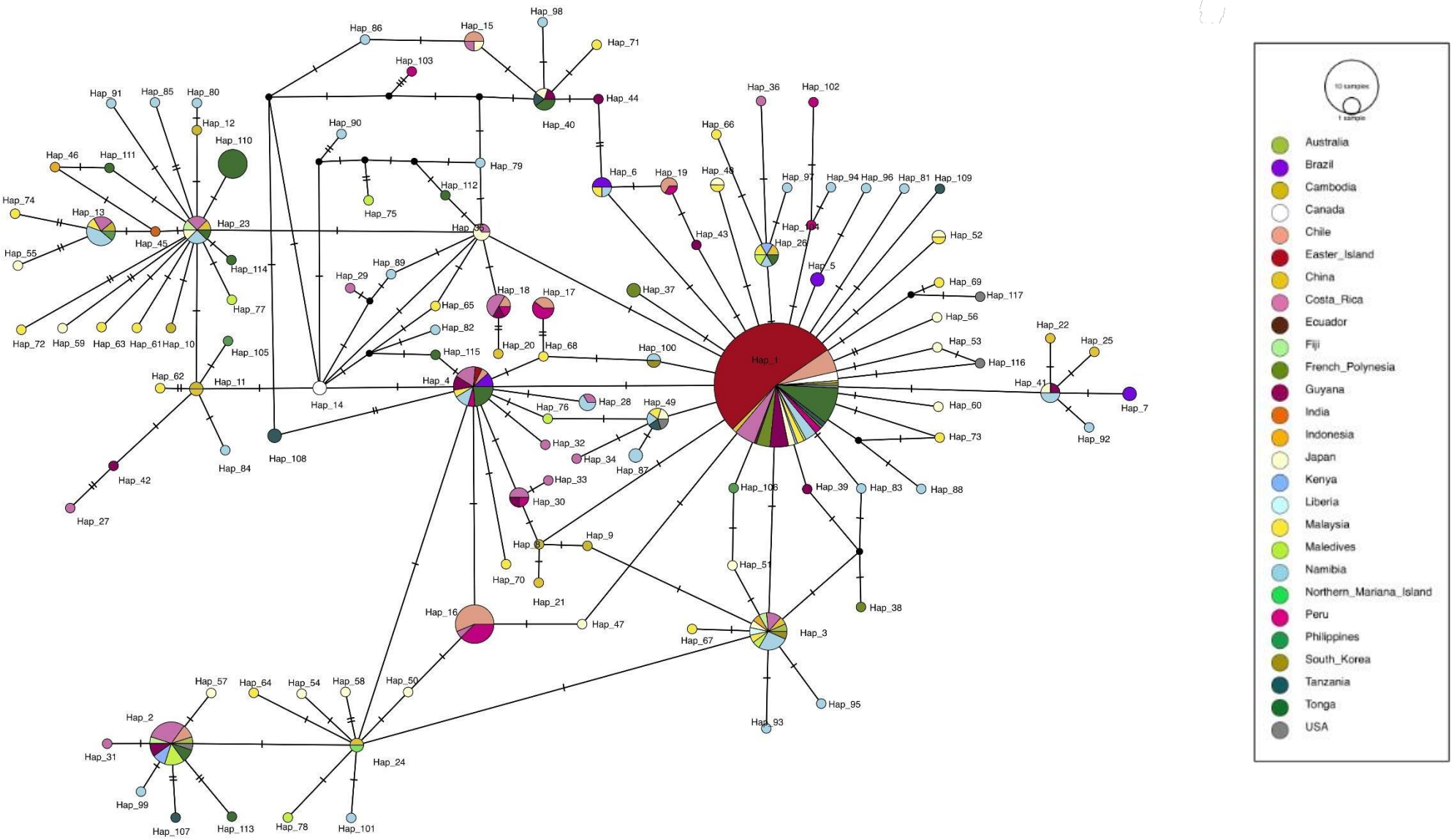
Hap_98
Hap_86
Hap_15
Hap_71
Hap_103
Hap_91
Hap_85
Hap_80
Hap_44
Hap_12
Hap_40
Hap_36
Hap_102
Hap_90
Hap_66
Hap_110
Hap_46
Hap_111
Hap_79
Hap_6
Hap_19
Hap_48
Hap_97
Hap_94
Hap_96
Hap_81
Hap_109
Hap_74
Hap_112
Hap_75
Hap_43
Hap_52
Hap_13
Hap_23
Hap_45
Hap_26
Hap_5
Hap_55
Hap_89
Hap_69
Hap_117
Hap_29
Hap_114
Hap_18
Hap_17
Hap_37
Hap_65
Hap_56
Hap_77
Hap_22
Hap_25
Hap_72
Hap_59
Hap_63
Hap_61
Hap_10
Hap_82
Hap_53
Hap_105
Hap_115
Hap_20
Hap_68
Hap_100
Hap_116
Hap_1
Hap_62
Hap_11
Hap_4
Hap_41
Hap_14
Hap_76
Hap_28
Hap_49
Hap_60
Hap_7
Hap_32
Hap_73
Hap_108
Hap_34
Hap_87
Hap_92
Hap_84
Hap_42
Hap_33
Hap_106
Hap_39
Hap_83
Hap_88
Hap_30
Hap_27
Hap_9
Hap_51
Hap_70
Hap_21
Hap_38
Hap_16
Hap_47
Hap_67
Hap_3
Hap_57
Hap_64
Hap_54
Hap_58
Hap_50
Hap_95
Hap_2
Hap_93
Hap_31
Hap_24
Hap_99
Hap_107
Hap_113
Hap_78
Hap_101
Australia
Brazil
Cambodia
Canada
Chile
Easter_Island
China
Costa_Rica
Ecuador
Fiji
French_Polynesia
Guyana
India
Indonesia
Japan
Kenya
Liberia
Malaysia
Maledives
Namibia
Northern_Mariana_Island
Peru
Philippines
South_Korea
Tanzania
Tonga
USA

Figure 3.B.2: Mutational haplotype network based on statistical parsimony of the CO1 marker gene showing genealogical relationships between 420 *Pantala flavescens* individuals from 27 countries. Each country is represented by a given color (see legend), haplotypes are shown as circles, while the size of the circles reflects the number of individuals per haplotype. Each perpendicular line between haplotypes represents a base substitution.

The ND3 (Figure 3.B.3) and ND1 (Figure 3.B.4) haplotype networks show 52 and 33 haplotypes, of which 48 and 30 are private, respectively. Especially the ND1 network shows a common haplotype in the center of the network (Hap_5) that is shared by individuals from 5/7 populations: Easter Island, Australia, Ecuador, Kenya and Namibia. China shows three private haplotypes and a shared haplotype with Namibian individuals (Hap_4). Tanzanian individuals display three private haplotypes as well, and a shared haplotype with Namibia.

The nuclear ITS haplotype network (Fig. 3.B.5) showed one common haplotype (Hap_7, n = 23), two additional shared haplotypes (Hap_1, n = 10 and Hap_2, n = 2) and 11 private haplotypes, with a maximum of nine substitutions between the most common haplotype and the more divergent private ones. However, haplotypes are shared between Easter Island, Namibia and Tanzania (Hap_7); Namibia, Tanzania and China (Hap_1) and between each a single individual from China and Easter Island (Hap_2). Australia, Ecuador and Kenya show only private haplotypes. Interestingly, in contrast to the mitochondrial networks for this nuclear marker, the Easter Island population (n = 6) displays four haplotypes, 2 private and 2 shared ones (Fig. 3.B.5).

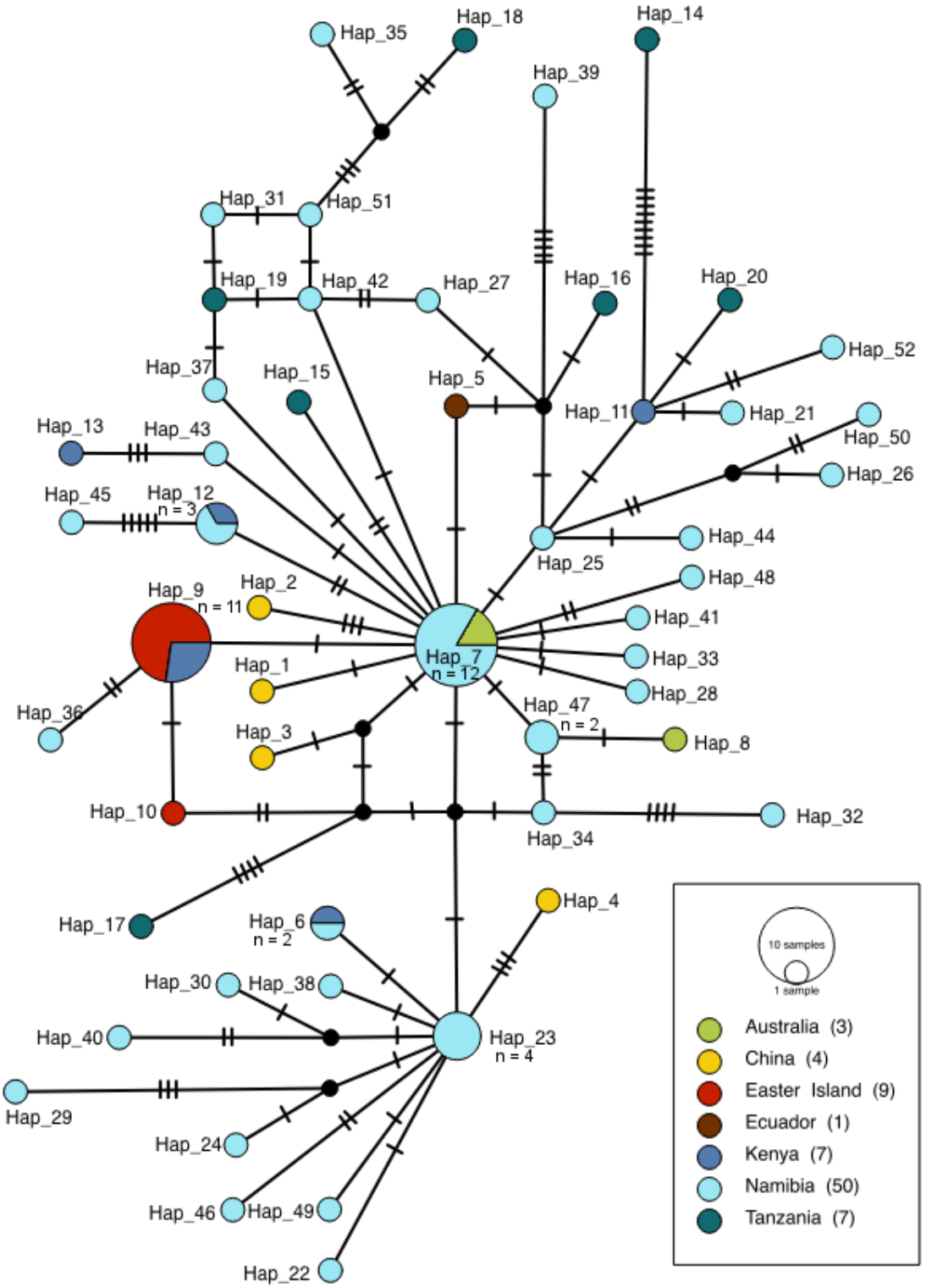

Figure 3.B.3: Mutational haplotype network based on statistical parsimony showing genealogical relationships between 73 *Pantala flavescens* individuals from seven countries for the ND3 marker gene. Each country is represented by a given color (see legend), the circles display the haplotypes, correlating in their size with the number of individuals. Each perpendicular line between haplotypes represents a base substitution.

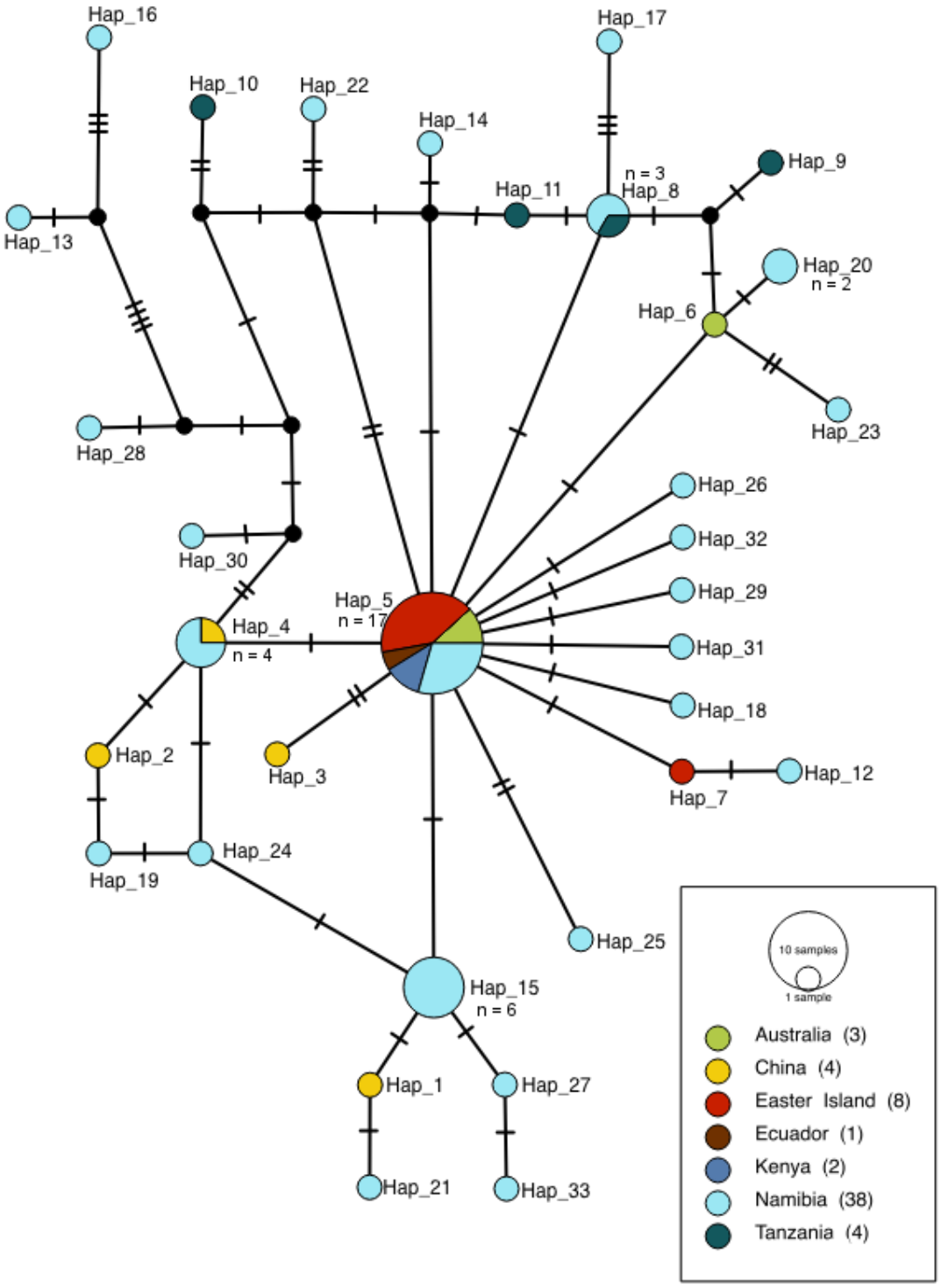

Figure 3.B.4: Mutational haplotype network based on statistical parsimony showing genealogical relationships between 60 *Pantala flavescens* individuals from seven countries for the ND1 marker gene. Each country is represented by a given color (see legend), the circles display the haplotypes, correlating in their size with the number of individuals. Each perpendicular line between haplotypes represents a base substitution.

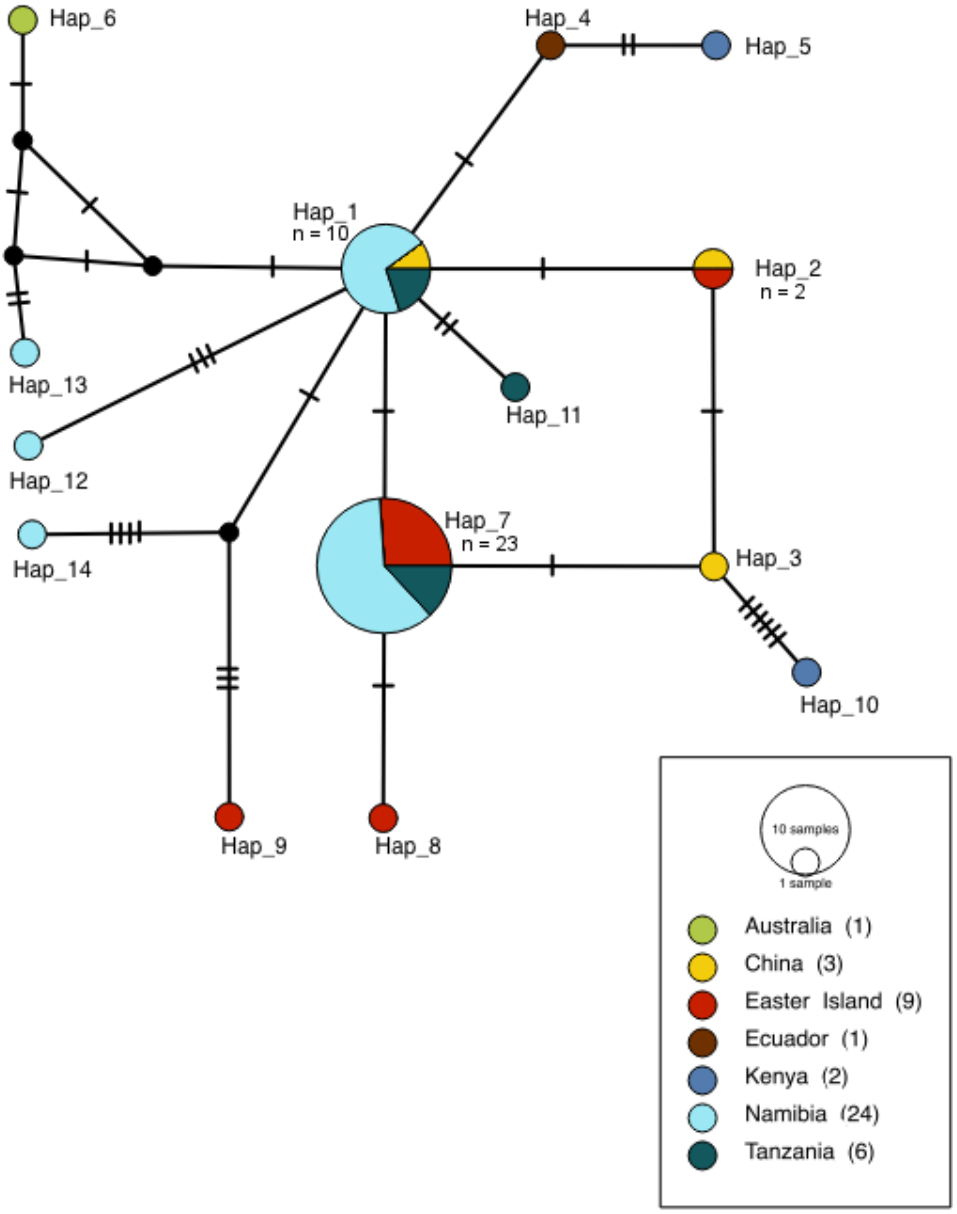

Figure 3.B.5: Mutational haplotype network based on statistical parsimony showing genealogical relationships between 47 *Pantala flavescens* individuals from seven countries for the nuclear ITS marker gene. Each country is represented by a given color (see legend), the circles display the haplotypes, correlating in their size with the number of individuals. Each perpendicular line between haplotypes represents a base substitution.

Amino acid substitutions

In total, ten out of 420 individuals (2.4 %) showed an altered amino acid sequence for CO1. The translated alignment for the ND1 gene fragment contains 162 amino acids, the protein coding region starts at position 40. The ND3 gene fragment consists of 211 aa, while the protein is located from position 71 to 189. Both nad proteins contain six and four sites with amino acid substitutions, respectively. For ND3, 11/73 studied individuals (15.1 %) display an amino acid sequence different from the consensus sequence; for ND1, even 30 % of individuals show at least one amino acid substitution within the protein coding sequence. For both NADH subunits, all animals showing an alternative aa sequence originate from African populations (Fig. 3.B.6).

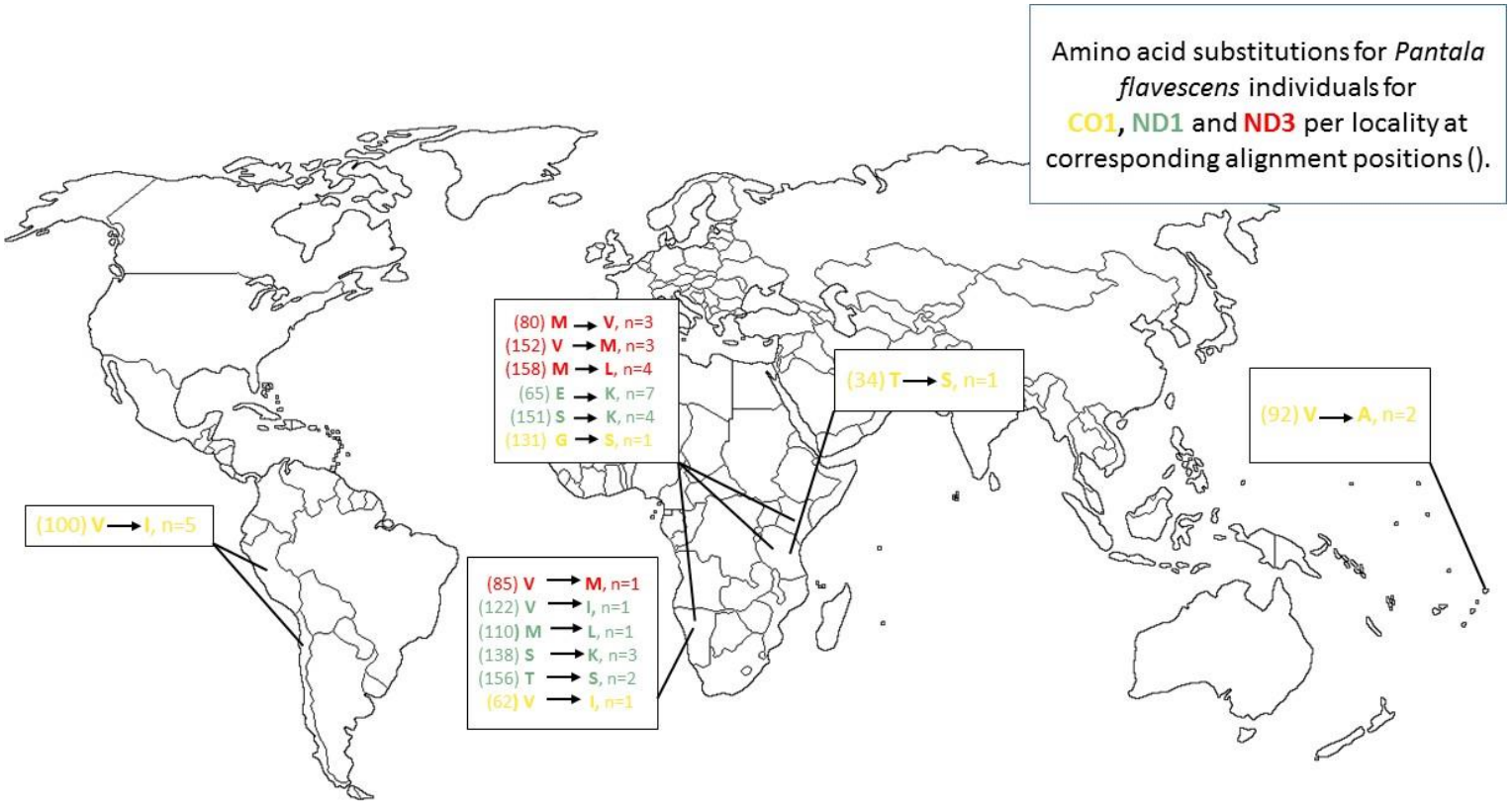

Figure 3.B.6: Worldwide distribution of amino acid substitutions for the analyzed gene fragments CO1 (yellow), ND1 (green) and ND3 (orange) between *Pantala flavescens* individuals. Alignment positions are given in brackets using single letter amino acid codes. For CO1, at position 34 the conserved threonine is replaced by a serine for a single individual from Tanzania; a single individual from Namibia exchanged a valine with an isoleucine at position 62; at position 92 valine is changed to alanine for two individuals from French Polynesia; five individuals from either Peru and Chile show an isoleucine instead of valine at position 100; at position 131 for one Namibian individual a serine is detected instead of glycine. For ND1, seven individuals from Tanzania and Namibia have a substitution from glutamic acid to lysine at position 65. At position 110, methionine is replaced by leucine and at position 122, valine is substituted by isoleucine in each a single individual from Namibia. At site 138, three Namibian individuals show a lysine instead of serine and five individuals from Tanzania and Namibia have a lysine instead of serine at position 155, as well. Two individuals from Namibia replace a threonine with serine at site 156. For ND3 four variable position were detected: 80 (methionine changed to valine by three Tanzanian and Namibian individuals), 85 (methionine replaces valine, n = 1 from Namibia), 152 (valine is substituted by methionine in three Tanzanian and Namibian individuals) and 158 (threonine is replaced by serine in two individuals from Namibia*).*

Population differentiation and gene flow

Genetic distances were calculated between populations as well as within all populations for the used markers. CO1 based data for the global population set is given in Table 3.B.5a and Table 3.B.5b.

The highest intrapopulational distances can be observed in Indonesia (1.269 %), followed by Malaysia (0.963 %), Namibia (0.936 %), Tanzania (0.933 %) and the Maldives (0.930 %). The lowest genetic diversity is found in the Easter Island population with 0.005 %. Comparably low genetic diversity is further detected in the population of Canada (0.270 %) and French Polynesia (0.268 %). There are several pairs of populations observable which display values larger than 1.0 %: Liberia vs. Fiji with 1.269 % as highest, followed by 1.256 % between Fiji and Malaysia, Fiji and Tonga with 1.244 %. Lowest values were detected between Cambodia and Namibia (0.003 %), Chile and Cambodia (0.141 %), Chile and Namibia (0.144 %), followed by Cambodia and Ecuador (0.169 %) and Namibia and Ecuador (0.170 %).

Genetic distances for the subset with additional markers ND1, ND3 and ITS are given in Table 3.B.6. It shows that information is marker dependent. For the Easter Island population subset, genetic diversity is slightly higher for the other observed mitochondrial markers (0.035 % for ND3 and 0.051 % for ND1). Values for the nuclear ITS are the highest among all analyzed markers (0.574 %). The latter is comparable to the Namibian population (0.503 %) that shows a greater diversity in all measures on the mitochondrial markers. Overall, ITS shows unusual high genetic distances in all populations, but highest in Kenya (3.724 %). Between populations, ITS genetic distances are highest when Kenya is compared to all other populations: Australia (2.527 %), Namibia (2.007 %), Easter Island (1.973 %), Tanzania (1.957 %), Ecuador (1.848 %) and China (1.842 %). However, genetic distances of the mitochondrial marker ND3, exhibits 0.0 % diversity within the Kenyan population. Whereas in the adjacent Tanzanian population values for the same marker reach 1.433 %, which is the highest value observed for ND3. In general, genetic distances for ND1 range from 0.0 (Kenya vs. Ecuador) up to 0.931 % (Tanzania vs China). ND1, known to be a suitable genetic marker for population genetic analyzes in Odonates, shows the lowest values (< 0.1) when populations of this diverse species are compared.

Table 3.B.5a: F_{ST} - values based on CO1 for *P. flavescens*. Asterisks indicate: p≤0,05 (lower left diadonal). Genetic distances (%) based on CO1 (upper right diagonal).

Country	1	2	**3**	4	5	**6**	7	8	9	10	**11**	**12**	13
1 Australia (n = 3)	*0.507*	0.613	0.846	0.451	0.639	0.339	0.669	0.647	0.338	0.508	0.442	0.563	1.100
2 China (n = 11)	0.0	*0.812*	0.879	0.444	0.650	0.319	0.698	0.742	0.317	0.783	0.458	0.578	1.079
3 Easter Island (n = 89)	0.907*	0.420*	*0.005*	0.628	0.934	0.582	0.847	0.890	0.580	0.822	0.713	0.800	0.689
4 Ecuador (n = 1)	0.0	0.0	0.0	*n/c*	0.487	0.170	0.531	0.537	0.169	0.564	0.310	0.411	0.761
5 Kenya (n = 4)	0.098	0.0	0.749*	0.0	*0.761*	0.429	0.786	0.752	0.429	0.791	0.563	0.627	1.138
6 Namibia (n = 48)	0.0	0.0	**0.162***	0.0	0.0	*0.936*	0.440	0.495	0.003	0.509	0.144	0.331	0.764
7 Tanzania (n = 8)	0.0	0.020	0.625*	0.0	0.032	0.025	*0.933*	0.797	0.438	0.761	0.567	0.670	0.969
8 Brazil (n = 8)	0.135	0.021	0.623*	0.0	0.090	0.038	0.069	*0.543*	0.495	0.749	0.620	0.678	1.028
9 Cambodia (n = 7)	0.149	0.022	0.781*	0.0	0.000	0.015	0.118*	0.215*	*0.846*	0.508	0.141	0.330	0.761
10 Canada (n = 6)	0.200	0.0	0.726*	0.0	0.031	0.0	0.0	0.067	0.097	*0.270*	0.611	0.690	0.931
11 Chile (n = 29)	0.066	0.087*	**0.416***	0.0	0.143*	**0.082***	0.055	0.079*	0.230*	0.011	*0.640*	0.456	0.903
12 Costa Rica (n = 40)	0.0	0.0	**0.292***	0.0	0.039	**0.020***	0.020	0.076*	0.085*	0.0	**0.046***	*0.791*	0.966
13 Fiji (n = 3)	0.0	0.0	0.900*	0.0	0.005	0.0	0.0	0.167	0.0	0.122	0.108	0.0	*0.846*
14 F. Polynesia (n = 9)	0.209	0.135	0.424*	0.0	0.099	0.029	0.113*	0.121*	0.243*	0.132	0.145*	0.088*	0.245
15 Guyana (n = 20)	0.011	0.0	**0.292***	0.0	0.034	0.010	0.0	0.026	0.145*	0.0	**0.039**	**0.004**	0.036
16 India (n = 1)	0.538	0.162	0.993	1.0	0.143	0.037	0.205	0.496	0.0	0.644	0.437	0.230	0.091
17 Indonesia (n = 2)	0.0	0.0	0.940*	0.0	0.0	0.0	0.047	0.238	0.0	0.247	0.246*	0.052	0.0
18 Japan (n = 24)	0.0	0.0	**0.294***	0.0	0.006	**0.004**	0.0	0.046	0.083*	0.0	**0.049***	**0.0**	0.0
19 Tonga (n = 40)	0.097	0.010	**0.295***	0.0	0.009	**0.010**	0.075	0.125*	0.049	0.0	0.135*	0.045*	0.011
20 South Korea (n = 2)	0.0	0.0	0.916*	0.0	0.0	0.0	0.0	0.065	0.061	0.205	0.062	0.0	0.0
21 Liberia (n = 1)	0.0	0.0	0.978	1.0	0.0	0.0	0.0	0.048	0.0	0.360	0.012	0.0	0.0
22 Malaysia (n = 25)	0.0	0.0	**0.247***	0.0	0.0	0.0	0.015	0.052	0.010	0.0	**0.088***	**0.026***	0.0
23 Maldives (n = 10)	0.0	0.049	0.694*	0.0	0.064	**0.064***	0.040	0.149*	0.149*	0.060	0.099*	0.009	0.0
24 N. Mariana I. (n = 1)	0.0	0.0	0.989	1.0	0.0	0.0	0.0	0.221	0.103	0.467	0.0	0.0	0.0
25 Philippines (n = 4)	0.169	0.032	0.870*	0.0	0.0	0.0	0.085	0.202*	0.0	0.111	0.201*	0.066	0.0
26 Peru (n = 19)	0.067	0.080*	**0.478***	0.0	0.087	**0.087***	0.065	0.061	0.190*	0.008	**0.0**	**0.065***	0.096
27 USA (n = 5)	0.0	0.0	0.645*	0.0	0.0	0.0	0.0	0.042	0.135	0.017	0.065	0.0	0.0

Color Scales

F_{ST}-values	Genetic distances
1.0	1.3
0.955	1.242
0.91	1.184
0.865	1.126
0.82	1.068
0.775	1.01
0.73	0.952
0.685	0.894
0.64	0.836
0.595	0.778
0.55	0.72
0.505	0.662
0.46	0.604
0.415	0.546
0.37	0.488
0.325	0.43
0.28	0.372
0.235	0.314
0.19	0.256
0.145	0.198
0.1	0.14
0.055	0.082
0.01	0.024
0.0	0.0

Table 3.B.5b: F_{ST} - values based on CO1 for *P. flavescens*. Asterisks indicate: p ≤ 0.05 (lower left diagonal). Genetic distances (%) based on CO1 (upper right diagonal)

Country	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 Australia (n = 3)	0.804	0.638	0.719	0.254	0.802	0.651	0.772	0.338	0.788	0.867	0.423	0.751	0.662	0.558
2 China (n = 11)	0.952	0.746	0.698	0.571	0.820	0.876	0.796	0.698	0.758	0.857	0.571	0.793	0.685	0.647
3 Easter Island (n = 89)	0.852	0.931	0.780	0.761	0.921	1.048	0.911	0.943	1.048	0.789	0.798	1.011	0.764	0.906
4 Ecuador (n = 1)	0.719	0.578	0.508	0.423	0.619	0.668	0.606	0.508	0.601	0.613	0.423	0.603	0.472	0.491
5 Kenya (n = 4)	1.020	0.774	0.796	0.648	0.877	0.853	0.865	0.621	0.729	0.904	0.648	0.814	0.739	0.714
6 Namibia (n = 48)	0.637	0.498	0.384	0.257	0.540	0.634	0.530	0.505	0.547	0.572	0.257	0.540	0.401	0.357
7 Tanzania (n = 8)	0.911	0.820	0.738	0.600	0.873	0.916	0.856	0.761	0.905	0.871	0.646	0.888	0.726	0.743
8 Brazil (n = 8)	0.958	0.812	0.812	0.635	0.899	0.863	0.882	0.647	0.877	0.885	0.692	0.872	0.749	0.758
9 Cambodia (n = 7)	0.635	0.497	0.381	0.254	0.538	0.635	0.529	0.508	0.548	0.571	0.254	0.539	0.400	0.355
10 Canada (n = 6)	0.804	0.758	0.804	0.423	0.876	0.770	0.850	0.508	0.940	0.867	0.592	0.878	0.717	0.728
11 Chile (n = 29)	0.748	0.614	0.522	0.339	0.669	0.748	0.658	0.593	0.686	0.712	0.367	0.670	0.535	0.485
12 Costa Rica (n = 40)	0.870	0.698	0.660	0.533	0.770	0.797	0.761	0.623	0.751	0.797	0.559	0.727	0.625	0.630
13 Fiji (n = 3)	0.635	1.069	0.888	1.015	0.975	1.244	0.973	1.269	1.256	0.571	1.015	1.174	0.819	1.117
14 F. Polynesia (n = 9)	*0.268*	0.947	0.888	0.635	0.970	1.066	0.962	0.888	1.149	0.825	0.761	1.079	0.838	0.888
15 Guyana (n = 20)	0.038	*0.560*	0.825	0.571	0.912	0.888	0.896	0.656	0.906	0.921	0.661	0.880	0.783	0.760
16 India (n = 1)	0.703	0.405	*n/c*	0.635	0.838	0.964	0.833	0.888	0.895	0.825	0.635	0.888	0.692	0.736
17 Indonesia (n = 2)	0.340	0.161	0.0	*1.269*	0.731	0.635	0.698	0.254	0.801	0.825	0.254	0.730	0.615	0.508
18 Japan (n = 24)	0.040	**0.0**	0.182	0.0	*0.836*	1.027	0.937	0.883	0.999	0.911	0.751	0.964	0.802	0.853
19 Tonga (n = 40)	0.102*	**0.047***	0.220	0.054	0.052*	*0.638*	0.998	0.584	0.987	1.053	0.761	0.971	0.882	0.827
20 South Korea (n = 2)	0.094	0.0	0.500	0.0	0.0	0.044	*0.507*	0.846	0.982	0.902	0.724	0.960	0.796	0.838
21 Liberia (n = 1)	0.208	0.0	1.0	0.0	0.0	0.0	0.0	*n/c*	0.761	0.952	0.508	0.793	0.768	0.660
22 Malaysia (n = 25)	0.034	**0.011**	0.011	0.0	**0.007**	**0.005**	0.0	0.0	*0.963*	1.019	0.761	0.950	0.863	0.839
23 Maldives (n = 10)	0.189*	0.080*	0.252	0.040	0.012	0.135*	0.0	0.0	0.077*	*0.930*	0.825	0.999	0.774	0.901
24 N. Mariana I. (n = 1)	0.548	0.072	1.0	0.0	0.0	0.168	0.0	1.0	0.0	0.0	*n/c*	0.761	0.635	0.558
25 Philippines (n = 4)	0.293*	0.143	0.0	0.0	0.055	0.057	0.078	0.0	0.0	0.132	0.067	*0.888*	0.826	0.819
26 Peru (n = 19)	0.140*	0.061*	0.323	0.177	**0.070***	**0.147***	0.002	0.0	**0.090***	0.101*	0.000	0.152*	*0.850*	0.714
27 USA (n = 5)	0.040	0.0	0.364	0.0	0.0	0.067	0.0	0.0	0.0	0.0	0.000	0.119	0.055	*0.710*

Color Scales

F_{ST}-values	Genetic distances
1.0	1.3
0.955	1.242
0.91	1.184
0.865	1.126
0.82	1.068
0.775	1.01
0.73	0.952
0.685	0.894
0.64	0.836
0.595	0.778
0.55	0.72
0.505	0.662
0.46	0.604
0.415	0.546
0.3	0.488
0.325	0.43
0.28	0.372
0.235	0.314
0.19	0.256
0.145	0.198
0.1	0.14
0.055	0.082
0.01	0.024
0.0	0.0

Table3.B.1: F_{ST} - values based on ND1, ND3 and ITS *for P. flavescens*. Asterisks indicate: p ≤ 0.05 (lower left diagonal) and genetic distances values based on ND1, ND3 and ITS (upper right diagonal).

Country	Genetic marker	Australia	China	Easter Island	Ecuador	Kenya	Namibia	Tanzania
Australia	ND1	*0.136*	0.428	0.094	0.068	0.068	0.503	0.602
	ND3	*0.211*	0.516	0.281	0.264	0.542	0.469	0.850
	ITS	*n/c*	1.332	1.482	1.334	2.527	1.319	1.277
China	ND1	0.0	*0.654*	0.385	0.359	0.359	0.744	0.931
	ND3	0.0	*0.875*	0.613	0.595	0.815	0.749	1.140
	ITS	0,333	*0.442*	0.590	0.663	1.842	0.563	0.497
Easter Island	ND1	0.0	0.018	*0.051*	0.025	0.025	0.476	0.593
	ND3	0.0	0.0	*0.035*	0.334	0.613	0.536	0.946
	ITS	1.0	0.666	*0.574*	0.811	1.973	0.535	0.498
Ecuador	ND1	0.0	0.0	0.0	*n/c*	0.0	0.452	0.567
	ND3	0.200	0.0	0.466	*n/c*	0.595	0.530	0.888
	ITS	0.0	0.082	0.0	*n/c*	1.848	0.692	0.608
Kenya	ND1	0.0	0.246*	0.123	0.0	*0.0*	0.452	0.567
	ND3	0.718*	0.457*	0.457*	0.894	*0.875*	0.725	1.129
	ITS	0.295	0.109	0.612	0.357	*3.724*	2.007	1.957
Namibia	ND1	0.0	0.0	0.0	0.0	0.002	*0.823*	0.922
	ND3	0.0	0.006	0.0	0.250	0.214*	*0.665*	1.049
	ITS	0.276	0.142	0.619	0.511*	0.0	*0.503*	0.443
Tanzania	ND1	0.0	0.185*	0.105	0.004	0.396	0.092	*0.864*
	ND3	0.0	0.0	0.0	0.606	0.274*	0.061*	*1.433*
	ITS	0.309	0.138	0.669	0.355*	0.0	0.0	*0.420*

Color Scales

F_{ST} -values	Genetic distances
1.0	3.7
0.950	3.515
0.9	3.33
0.850	3.145
0.8	2.96
0.750	2.775
0.7	2.59
0.650	2.405
0.6	2.22
0.550	2.035
0.5	1.85
0.450	1.665
0.4	1.48
0.350	1.295
0.3	1.11
0.250	0.925
0.2	0.74
0.150	0.555
0.1	0.37
0.050	0.185
0.0	0.0

As a measure of gene flow, F_{ST} values were calculated for each marker set. For the global CO1 data set, F_{ST} values are given in table 3.B.5a and 3.B.5b on the lower diagonal. Again, values range from 0.0 to 1.0. Please note that study sites with insufficient sample sizes are given in the table for completeness but are not discussed, as they are likely to show extreme values due to sampling bias. Highest significant values associated with population separation can be detected for Easter Island, when compared to Peru and Chile, respectively; the lowest value for the Easter Island population is 0.162 compared to Namibia. All other population comparisons show F_{ST}-values smaller than 0.1.

For ND1 and ND3 similar results were obtained within the data subset (Tab. 3.B.6): Easter Island vs. Kenya (0.123 ND1; 0.457 ND3) and vs. Tanzania (0.105 ND1) showed higher values as when Easter Island was compared to Namibia (0.0 ND1/ND3).

ITS values however do show genetic structuring: Overall, F_{ST} s range from 0.0 for ITS when the population of Namibia and Kenya are compared, up to 1.0 when Australia is compared to Easter Island. Between Easter Island and all three African populations, values range from 0.612 to 0.669, indicating restricted gene flow.

Migration estimates

The number of migrating individuals per generation (N_m) was estimated via F_{ST}-values based on mitochondrial CO1 and nuclear ITS, which is visualized in Fig 3.B.7 and 3.B.8. For CO1, migrants in varying numbers are detected between almost all populations. Only the Easter Island population is isolated, except for a very small amount of migrants exchanged with Namibia. In contrast, for the nuclear ITS marker, Easter Island exchanges migrants with all African populations, however only a small amount (1.5 to 2 individuals / generation). Interestingly, there seems to be no migration among closely located African populations. The high amount of gene flow that was observed for CO1 was not detected by the ITS region.

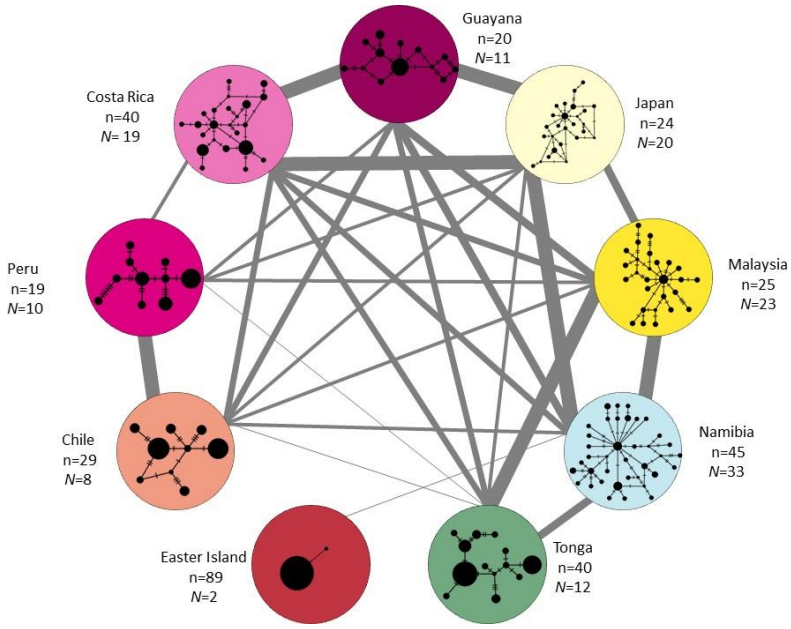

Figure 3.B.7: Number of migrants between populations based on CO1. Within each circle the haplotype network of the respective population is displayed. Connections between the circles display N_m for the given population pair.Connections vary in thickness representing the amount of migrants. Thin lines = low migration, thick lines = high migration.

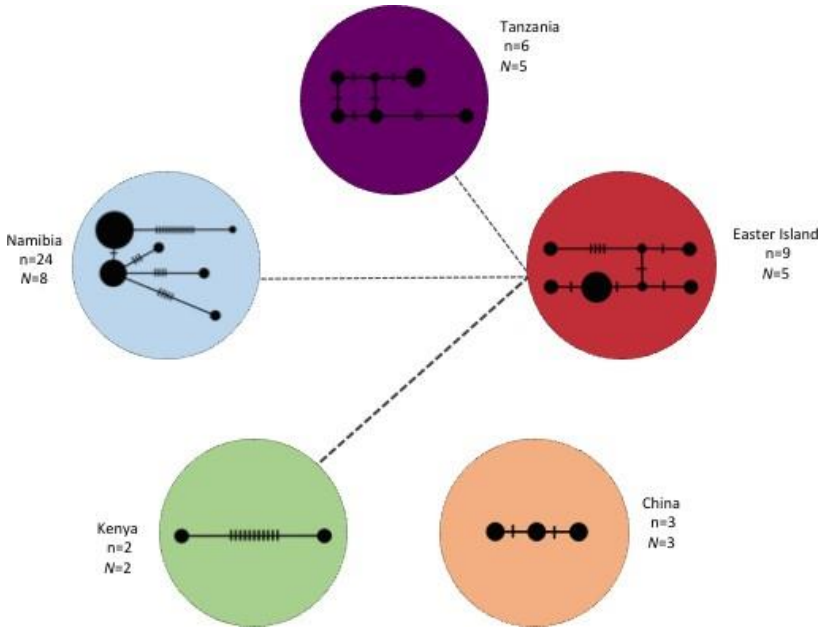

Figure 3.B.8: Number of migrants between populations based on ITS. Within each circle the haplotype network of the respective population is displayed. Connections between the circles display N_m for the given population pair.Connections vary in thickness representing the amount of migrants. Thin lines = low migration, thick lines = high migration.

Neighbor Joining phylogeny

An NJ tree (Figure 3.B.9) was calculated on the concatenated mitochondrial data set (CO1, ND1, ND3; 1557 bp). To construct an outgroup for tree rooting, the according genes were mined from the *Orthetrum triangulare melania* complete mitochondrial genome (AB126005, Yamauchi et al. 2004). Earliest branching events are represented by African individuals. The genetic information in this dataset seems to be insufficient to resolve the tree to full extend, since many taxa from clades of all studied populations appear polytomous. Still, a distinct Easter Island clade can be observed with African individuals as their closest neighbors. This is the only clade were individuals cluster by the same geographical origin. The single individual from Ecuador shares common ancestors with Namibia and China.

Figure 3.B.9: Neighbour Joining tree based on concatenated CO1, ND1 and ND3 sequences *for Pantala flavescens*.

Discussion

Population structure

A panmictic population is supposed to show no genetic structuring due to random mating at all (King & Stanfield 1997). However, comparing 420 individuals from every continent presents a former unknown genetic differentiation between (i) mainland and island populations, (ii) center and edge populations of this species' distribution range. This is even reflected on the protein level. A study with special focus on island populations corroborates the genetic differentiation between continental and insular populations (Alvial et al. 2018). Also Pfeiler and Markow (2017) reported a spatial structure when they compared populations from Malaysia, Guyana and Japan (the sequences were also included in the here presented data set). The population on Easter Island indicates significant measures for genetic isolation as also described in Alvial et al. (2018). Genetic distances and F_{ST}-values between mainland and islands and among island populations (e.g. Easter Island, Fiji) were the highest we observed in this study. This gives evidence, that though *Pantala flavescens* is capable of crossing open waters, it may not be its main migration mode.

The population connectivity of the cosmopolitan odonate *Pantala flavascens* gained scientific attention, when its exceptional migratory capability was revealed (Anderson 2009). Troast et al. (2016) proposed a global panmictic population structure, based on 49 individuals and the species´ distributional range area was not nearly met. In this study, only six haplotypes of which two are shared among the localities were found. Two of the used sequences were not correctly identified as *Pantala flavescens.* Further, the sequences from this study published on NCBI contain many wobbled sites leading to the assumption, that the large amount of private haplotypes we observed was hereby masked.

The results reveal a remarkable amount of private haplotypes for CO1, especially in Africa, which is usually a population genetic signal of local adaption and range expansion. In contrast, we find African individuals in almost all shared haplotypes and genetic distances and gene flow measures are lowest when the Namibian population is compared to all others. However, on the protein level we found the highest diversity in Africa with no shared amino acid substitution elsewhere. Consequently, the high similarity between the African and all other populations cannot be explained by recent

gene flow due to random mating, as more populations would show the same altered proteins otherwise. Furthermore, the nuclear results are not congruent with the mitochondrial data.

In this study, mitochondrial diversity is low on Easter Island and F_{ST} values as well as genetic distances show unexpectedly the highest similarity to Namibian individuals. However, our dataset contains populations from South and Central America that were more likely to be the origin of the colonization of Easter Island. Interestingly, for the usually more conserved ITS marker gene, this population exhibits more haplotypes than for the mitochondrial markers analyzed. Nuclear diversity measures are comparable or even higher than in Australia, China or African populations. Bias between nuclear and mitochondrial marker in population genetics is a well-known phenomenon (e.g. Leyrholm et al. 1999, Nyakaana et al. 1999), also in odonates (Damm & Hadrys 2012). In fact, such incongruences can enhance the understanding of the evolutionary history of the study species (Rubinoff & Holland 2005). These conflicts can be a sign for introgression, complex population structure, and sex-biased gene flow (see Bowen et al. 2005). For *P. flavescens* the last hypothesis can be rejected, as both males and females are known to migrate (Srygley 2002).

Genetic distances and gene flow measures observed in this study, as well as by other authors indeed suggest a genetic structuring within this species, however it cannot be explained by spatial geographical distances (alone). It is more likely that a complex population structure due to demographic events and phylogenetic history lead to the observed patterns.

Population expansion

The star-like shape of all haplotype networks and the negative values for the Tajima's D test (Table 3.B.3 and 3.B.4) indicate one or more population expansions. Typical genetic signals of a recent, rapid population expansion are shallow gene genealogies with one common haplotype and numerous, less common haplotypes that differ by a few mutational steps from the main one (Slatkin & Hudson 1991). Usually, these populations also exhibit low levels of nucleotide diversity, which we did not observe for *P. flavescens.* Low et al. (2017) describe a demographic expansion for *P. flavescens* in Peninsular Malaysia dated in the Pleistocene (190,000 – 260,000 years ago), associated with Pleistocene climate change events. The age of Easter Island is

comparably young from a geological point of view with estimated 110,000 – 240,000 years (Segers & Dumont 1993). The low genetic diversity here could be explained due to a founder effect by colonization combined with subsequent and ongoing isolation. However, due to the observed saturation of the CO1 gene, the value of these interpretations is questionable. The fact that there is still stronger similarity between African populations and Easter Island, than between other populations, is more likely a demographic and phylogenetic effect, than evidence for a global panmictic population.

Phylogenetic assumptions

The phylogenetic tree based on three mitochondrial markers, shows a distinct clade for the Easter Island population. African individuals are located at the base of the phylogeny. Indeed, phylogenies of libellulid dragonflies describe *Pantala* as a sister-genus to *Trithemis* (Ware et al. 2007). The most common ancestor between *Pantala* and *Trithemis* occurred most likely in Africa and was adapted to temporary pools (Damm et al. 2010). A time calibrated tree displaying the *Trithemis* radiation in Africa used *P. flavescens* as an outgroup. It depicts the last common ancestor between *Pantala* and *Trithemis* in the Miocene, 12.5 Mya (Damm et al. 2010). While *Trithemis* radiated into 40 African, two Madagascan and five Asian species (Pinhey 1970, Dijkstra 2007), the genus *Pantala* only consists of two species, the study target and *Pantala hymenaea.* The latter species is also migratory but restricted to the Americans (Paulson 2018). As migratory behavior is not known from *Trithemis* species, this trait must have evolved after the split of *Pantala* and *Trithemis*.

During this evolutionary period, *Trithemis* radiated strongly to all kinds of African habitats, which is still seen in present-day odonate communities (Damm et al. 2012). It is possible that due to concurrence between the related genera, *Pantala*'s range expansion out of Africa based on its migratory abilities was promoted. The fact that African individuals can be found through almost all clades of the phylogeny is likely due to the poor phylogenetic resolution of the saturated CO1 fragment.

Conclusion

First and foremost, CO1 is a prominent and valuable genetic marker but not without limitations. Global genetic assessments benefit largely from CO1 databases, as mining sequences is way more time and cost effective compared to extensive field studies. However, it might not be a suitable marker to study the genealogical relationships of *P. flavescens*. The unusual and partly conflicting genetic pattern revealed in this multi-marker study on 420 individuals probably result from a complex combination of population demographics and evolutionary processes that are not yet fully understood. Though gene flow could be possible over larger spatial scales than in any other odonate species, the assumption of a global panmictic population has to be rejected due to differentiation between (i) mainland and island populations, (ii) conflicting mtDNA and nDNA patterns and (iii) differences even on protein level. Regardless, its striking success in terms of abundance, distribution and adaptive traits, even *P. flavescens* is not secure against isolation processes. Further analyzes based on adequate genetic marker systems (e.g. RAD sequencing, transcriptomics) are highly recommended to shed more light onto the isolation and adaptation processes of the Easter Island population.

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4 Chapter 3: Conservation genetics of *Lestes macrostigma*

Factors influencing the spatio-temporal dynamic of the threatened dragonfly *Lestes macrostigma* (Odonata: Zygoptera): lessons from a 10 years monitoring with an emphasis of the conditions for species resilience

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Abstract

As part of a regional habitat restoration plan for the endangered damselfly *Lestes macrostigma,* 107 individuals of this stenotypic species were analysed based on the mitochondrial cytochrome c oxidase subunit I (CO1) over a seven year time period starting in 2010. Study area was the Camargue region in Southern France.

L. macrostigma is known to show great inter-annual abundance fluctuations. To establish a suitable management plan for this species, the reasons and consequences of these population dynamics has to be investigated.

By assessing the genetic diversity compared to other French populations, *L. macrostigma* exhibits a genetically impoverished population in the Camargue area. Further, a genetic bottleneck event in 2016 was detected. The data show a compensation of diversity parameters in the following year, though diversity parameters did not reach the values of the best year analysed (2015). Most individuals show a major shared haplotype, but we detected four year-specific private haplotypes, from which two were observed in 2017. Abundance data positively correlated with the amount of haplotypes detected per year. Though it could not be demonstrated that these fluctuations are based on varying precipitation (alone), water management of *L. macrostigma*'s exclusive brackish habitats should be taken into consideration. An alarming amount of climate change scenarios predict a pronounced decrease in precipitation and especially the warm seasons in the Mediterranean climate will be affected by drought periods. This displays an increasing risk for temporary wetland species to experience more and more population bottlenecks, which were already discovered by this study.

Introduction

The ongoing loss of biodiversity demands an increasing accuracy and an acceleration of conservation decision-making. To create modern management plans on endangered species, the often called 'crisis-disciple' conservation biology moved on to genetic approaches to conquer the numerous challenges it faces. These challenges are mainly of direct and indirect anthropogenic origin, like habitat destruction or fragmentation and climate change consequences (Avise 2008, DeSalle & Amato 2004, Sala et al. 2000). These adverse events result for instance in population decline, isolation processes or inbreeding in small populations, with consequently (local) extinction of species. Thereby, the loss of species as well as genetic diversity is irretrievable (Hartl 2000). Genetic diversity is the pivotal fundament to adapt to environmental changes - on the level of individuals, populations and species (Frankham et al. 2002, Hartl 2000). Conservation genetics aims to detect and interpret genetic diversity based on these levels to improve conservation actions (Avise 2008). Genetic monitoring of species of conservational interest has become state of the art among conservation managers (Schwartz et al. 2007). Especially long-term monitoring data sets are of great value to detect population dynamics and processes, however time-series studies are still rare or limited on the time-scale. Only long-term data sets can reliably distinguish between sampling error and reasonable changes in abundance, population genetic metrics or influence of environmental variability and are inevitable to study population dynamics (Forney 2000, Gerodette 1987). Particularly studies on a regional scale are lacking as conservationists call for large-scale global assessment. However, genetic results alone do not countervail against all problems natural ecosystems and their endangered inhabitants are faced with. Furthermore combinations of molecular methodology accompanied by classic ecological approaches provide powerful assistance in conservation decision-making.

Although not inscribed in the Habitats Directive, the odonate species *Lestes macrostigma* (Eversmann 1936) is nonetheless a species with strong heritage value. This value is recognized (i) on local scale as in Poitou-Charente (Cotrel et al. 2007), (ii) national as in France (Dommanget et al. 2008), Bulgaria (Marinov 2005), Spain (Rosas et al. 1992, Ocharan et al. 2006) and (iii) European scale (Sahlen et al. 2004). The issued conservation status for *Lestes macrostigma* is based on its fragmented habitat

but also on threats to its local development environments. This damselfly species is distributed from Portugal to Mongolia but restricted to exclusive temporary brackish habitats (Berquier & Andrei-Ruiz 2019, Boudot & Raab 2015) which are rarely and patchy distributed across this species distribution range. Further, these habitats face high anthropogenic pressure mainly due to increasing urbanization in coastal areas and regions with steppe lakes, and industrial and agricultural development (Kalkman 2014). Especially Mediterranean temporary ponds are disappearing (Zacharias & Zamparas 2010), as do other salted temporary waters. Climate change can display another major threat to this stenotypic species as numerous climate models predict a pronounced decrease in precipitation, especially during the warm season in the Mediterranean climate (Giorgia & Lionello 2008), putting particularly temporary waters at risk. Because *L. macrostigma* is known to show great inter-annual variation in population abundance and site occupancy (e.g. Florencio & Díaz-Paniagua 2012, Lambret et al. 2009, Borisov 2005), it is essential to study population dynamics in abundance and genetic diversity over-time on a regional scale. Therefore, the Camargue was chosen as study area as a part of a restoration plan for this species included in a wetland protection management directive. Monitoring efforts started in 2010 with special focus on both habitat and population developments. Habitat constitution was assessed regarding plant composition (presence / absence of *Bolboschoenus maritimus*, this species key-plant for oviposition), preferential macro-habitats Scirpion compacti (Atlantic littoral) and Scirpion compacto-littoralis (Mediterranean littoral), the intake of fresh water (phreatic, flood and / or rainwater supply), the presence of brackish standing water, the presence of protection against the wind (see Grand & Boudot 2010) and the water temperature during larval development. On the species level, abundance, oviposition and reproduction success and genetic parameters were observed on a ten years' time-scale.

The presented study is part of this restoration plan and deals with the genetic *status quo* and dynamics of 17 investigated *L. macrostigma* populations. In total 107 individuals were analysed by means of the cytochrome c oxidase subunit I (CO1) as genetic marker to examine if the variation in abundance between years is reflected in this species genetic diversity patterns.

Material and methods

Study area, main sites and climate

The main occupancy of *Lestes macrostigma* is the studied area in Southern France (Fig. 4.A.1). This area comprises the Camargue, i.e. the River Rhône delta, known since the middle of last century as one of few places where the species occurs in Western Europe (Askew 2004, Aguesse 1968). We also considered: (1) to the east, the Provence-Alpes-Côte d'Azur (PACA) region where the species was known to reproduce in the Crau area (i.e. the ancient delta of the River Durance) and around the *étang de Berre*, and even to disperse till the *bassin de Réaltor* (Bence & Bence, 1989); (2) to the west, the Occitanie region, where *L. macrostigma* has been cited from the vicinity of Montpellier (Cassagne-Méjean 1965).

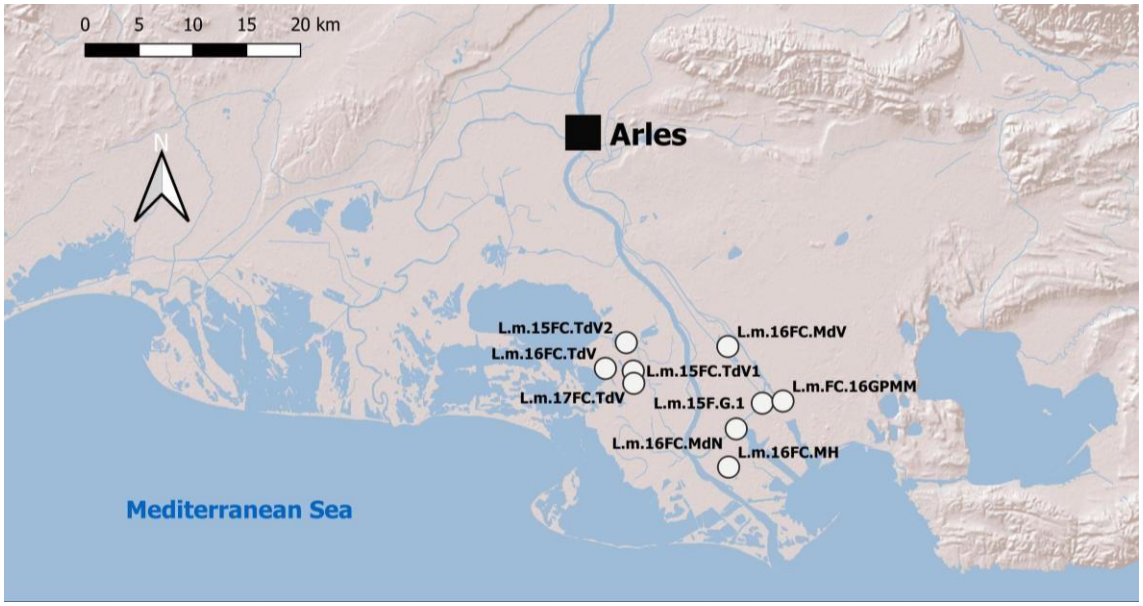

Figure 4.A.1: Study area of the long-term monitoring of *L. macrostigma* within the Camargue, France. Figure was kindly provided by PL.

Within the study area, Lambret et al. (2009) highlighted that *L. macrostigma* had not been recorded for 10 years at several sites; on another hand, most of the last records were made at two protected areas, namely the Regional Nature Reserve of the Tour du Valat (1845 ha; here after TdV) and the National Nature Reserve of the Marais du Vigueirat (919 ha; here after MdV), and as well as within the "green ring" of Marseille-Fos Euromediterranean port (2,648 ha; here after GPMM).

Mediterranean climate is characterized by rainy winters and dry summers. However, the rainfall events are also typically unpredictable, either in the time of occurrence and their amount of precipitation. Hence, there is a strong inter-annual variability in total precipitations, ranging from 217 to 1016 mm and mean ± SD = 559 ± 175 mm (n = 30; 1989-2018; meteorological station Météo France of 'Arles-Valat' n°13004003, 43.5100°N, 4.6938°E).

Water regimes and associated habitats

Since the containment of the Rhône River and the sea by huge dikes, the hydrological functioning of the Camargue is almost totally artificial (Mathevet 2004). The hydro-period of water bodies (discarding irrigation ditches and canals) depends on the type of land-use and, within natural areas, on the objectives of the wildlife managers. Hence, water bodies can be: (1) temporary with a natural water cycle, i.e. flooded from autumn to spring and dry from spring to autumn (*e.g.* temporary ponds in natural reserves); (2) temporary with a shifted water cycle, i.e. flooded from summer to winter-spring (*e.g.* temporary marshes managed for water fowl hunting); (3) temporary with a reversed water cycle, i.e. flooded from spring to autumn and dry from autumn to spring (rice fields); (4) semi-permanent or permanent, i.e. with very short drought period or flooded all year round (*e.g.* reed beds, other hunting marshes). The duration of hydro-period and the water levels of these water bodies depend on three flooding types: rainfalls, phreatic water up comes, and / or artificial from floodgates and irrigation network, either gravitational or by pumping. During years with regular or heavy rainfalls in spring, water bodies of type 2 can turn into type 4 (semi-permanent).

Natural habitats are conditioned by altitude, hydro-period and their distance to the sea (i.e. salinity). Depending on the interactions of those three gradients, the main wetland types consist of coastal brackish lagoons, temporary brackish ponds with *Juncus maritimus* and *Bolboschoenus maritimus*, and reed beds (*Phragmites australis*).

Surveys and monitoring

Depending on the observers' willingness and availability to invest themselves in the monitoring of the species, we followed a stepwise complexity in the monitoring

methodology (Lambret 2010). Three types of data were used: (1) Opportunistic data recorded in the frame of the following public / participative science programs and databases: the enquête on *Lestes macrostigma* of the Observatoire naturaliste des ecosystems méditerranéen (www.onem.com); the atlas of the dragonflies of PACA region (Papazian et al. 2017, www.faune-caca.bit); the atlas of the dragonflies of Occitanie / Languedoc-Roussillion region coordinated by the Opie Languedoc-Roussillion and the Écologiste de l'Euzière (www.atlas-papillules.zob); the Système d'information sur la nature et les paysages (SINP) in PACA region coordinated by the Conservatoire d'espaces naturels (CEN) PACA (www.silene-faune.znbioboaizebnob); and the www.obsnature-camargue.org by TdV. (2) Data recorded during the surveys realised in different protected areas (TdV, National Nature Reserve of Camargue, sites belonging to the Conservatoire du littoral and managed by the Natural regional parc of Camargue PNRC, A Rocha). (3) Data recorded along count transects in the MdV and the GPMM (monitoring *per se*) in the frame of the national survey of priority species (SONEP) launched by the Société française d'Odonatologie (SfO).

Information recorded during the surveys consisted of the species development stage (exuviae, emergents and tenerals, immature and mature adults), behavior (tandem i.e. male grasping female, copula, and oviposition), abundance, or a rough description of the habitat. Such information was always recorded during the monitoring. Additionally, the water levels of the monitored water bodies of the MdV were recorded every week by the conservation managers of the protected area.

Genetic tissue sampling

During the surveys, individuals were captured using an entomological net and a single mid-leg was taken as tissue sample. After the procedure all animals were released. This method is described as minimal invasive by Fincke & Hadrys (2005). Tissue samples were stored in 98 % EtOH and at -20 °C upon analyses. In total, 107 samples were taken at 17 population sites (see Table 4.A.2). In addition, individuals from the French Island of Corsica and the French Atlantic coast were analysed as "outgroups".

Molecular methods and analyses

To extract the genomic DNA of *L. macrostigma*, a standard phenol-chloroform protocol (Hadrys et al. 1992) was used. The primer LCO 1490 (fwd.) 5' GGT CAA CAA ATC ATA AAG ATA TTG G-3' and *L. macrostigma* specific Lestes-HCO (rev) 5' GTG ACC AAA AAA TCA AAA YAA A- 3' were used to amplify the CO1 barcode region and OdoCO1 (fwd.) 5' TAG ACG AGC ATA TTT TAC TTC AGC 3' and OdoCO1 (rev) 5' CCT AAA TCC ATT GCA CTT TTC3' were used for the OdoCO1 fragment (Tab. 4.A.1).

Table 4.A.1: Oligonucleotide primers used in this study for PCR and sequencing.

Primer	Locus	Sequence (5'-3')	Reference
HCO2198	*cox1*	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
LCO1490	*cox1*	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
Lestes_HCO	*cox1*	GTGACCAAAAAATCAAAAYAAA	Herzog et al., *submitted*
OdoCO1 fw	*cox1*	TACACGAGCATATTTTACTTCAGC	Herzog & Hadrys (2016)
OdoCO1 rev	*cox1*	CTTAAATCCATTGCACTTTTC	Herzog & Hadrys (2016)

Table 4.A.2: Sampling locations by year and region, used abbreviation and number of analysed individuals for *Lestes macrostigma.*

Region	Year	Location	Abbreviation	(n)
Total				**107**
Corsica	2011	Etang de Canettu	L.m.11CEdC	1
	2011	Lagune d'Arbitru	L.m.11CLdA	2
	2011	Lagune de Caniscione	L.m.11CLdC	2
				total: 5
Carmargue	2010	Trou du Pélobate	L.m.10FC.TdP	2
	2011	Salins du Caban (Baisse de Lhallu)	L.m.11FC.SdC	8
	2011	Marais du Vigueirat	L.m.11FC.MdV	2
	2016	Marais du Vigueirat	L.m.16FC.MdV	15
	2015	Grand Port Maritime de Marseille, Mare de la pompe	L.m.15F.G.1	7
	2016	Grand Port Maritime de Marseille	L.m.FC.16GPMM	5
	2016	Grand Port Maritime de Marseille, Mare du nid	L.m.16FC.MdN	10
	2016	Grand Port Maritime de Marseille, Baisse de la pègue	L.m.16FC.MH	12
	2015	Tour de Valat, Cerisières moyennes	L.m.15FC.TdV1	9
	2015	Tour du Valat, Sarcelles	L.m.15FC.TdV2	4
	2016	Tour du Valat	L.m.16FC.TdV	4
	2017	Tour du Valat	L.m.17FC.TdV	19
				total: 78
French Atlantic coast	2011	Ile de Noirmoutier	L.m.11FIN	3
	2011	Vendée, Réserve de Chanteloup	L.m.11FV.RdC	1
				total: 4

PCR products were sequenced at the Yale Sequencing Facility, USA. Sequences were checked and edited with Geneious version 8.1.8, (Kearse et al. n.s.). After aligning the sequences with the Geneious implemented software MUSCLE version 3.8.425 (Edgar 2004), they were checked with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for correct species identification. A statistical parsimony based haplotype network was constructed using the 95 % parsimony criterion by implement in TCS (Clement et al. 2000). The visualization of the network was concucted with PopArt (http://popart.otago.ac.nz). Genetic distances within and between populations were calculated using MEGA 7 (Tamura 2015). F_{ST}-values were generated using Arlequin version 3.5.22 (Excoffier & Lischer 2015). DnaSP version 5.10.01 (Librado & Rozas 2009) was used to assess the haplotypic diversity and the nucleotide diversity.

Results

Genetic analyses and genetic diversity

In this study 107 individuals of *L. macrostigma* from 17 different population sites (Table 4.A.2) were analyzed. The primer specific alignment lengths are 522bp (barcodeCO1) and 536 bp (OdoCO1), respectively. After concatenation the final alignment was 1058 bp long.

The haplotype network (Figure 4.A.2) shows nine different haplotypes. Most of the individuals (n = 84) show haplotype 1 which is present in all sample sites except for Corsica (EdC). The second most frequent haplotype is haplotype 2, with five individuals coming from four sample sites. Four haplotypes are private and shown by one to four individuals. Two private haplotypes originate from TdV 2017, meaning there were not detected the years before. The other two private haplotypes are found in GPMM in 2015 and 2016. Interestingly, if other haplotypes than the main haplotype Hap_1 occur, they are not shared among the Camargue region. Individuals from TdV 2015 and GPMM 2015 share haplotypes with individuals from Corsica and the French Atlantic coast, respectively, but not among each other. Further, no shared haplotypes were found within the Camargue region for the same year nor between years, except the main haplotype. The highest number of haplotypes (6) can be detected in 2015, followed by 2017 (3). Though 2016 was the most extensively sampled year (n=46), only

two haplotypes were detected (Fig. 4.A.2). The fluctuation of haplotypes compared to the sample size over the years is visualized in Fig. 4.A.3 and shows the slump for 2016 compared to the other years.

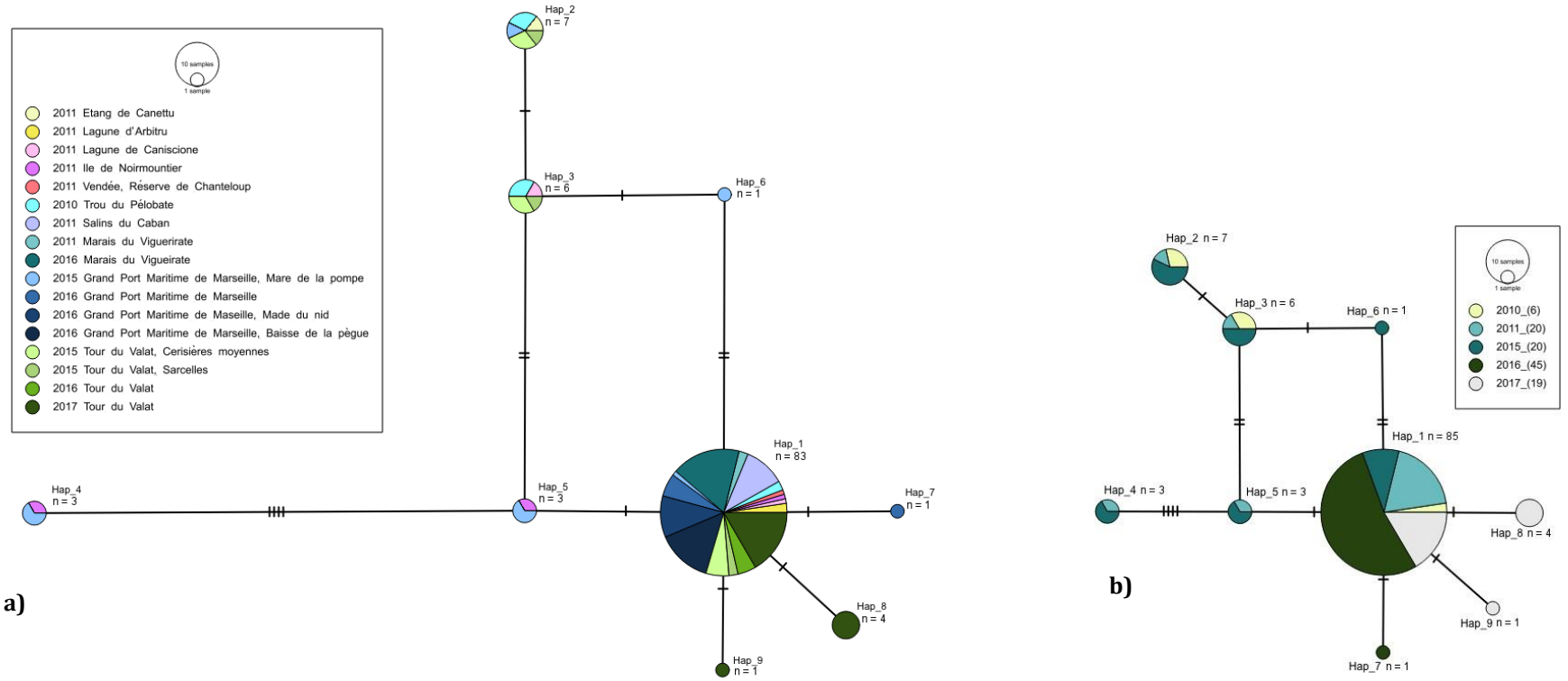

Figure 4.A.2: Mutational haplotype network based on statistical parsimony for *Lestes macrostigma* populations by means CO1 sequence information. Circles display haplotypes colored by their region and year of origin, resulting in pie charts if haplotypes are shared among populations. Circle sizes reflect the frequency of the given haplotype. Each black mark symbolizes one nucleotide substitution between inferred haplotypes. On the left, all analysed population sites and years are given (a), while on the right all populations were summarized by the respective years (b).

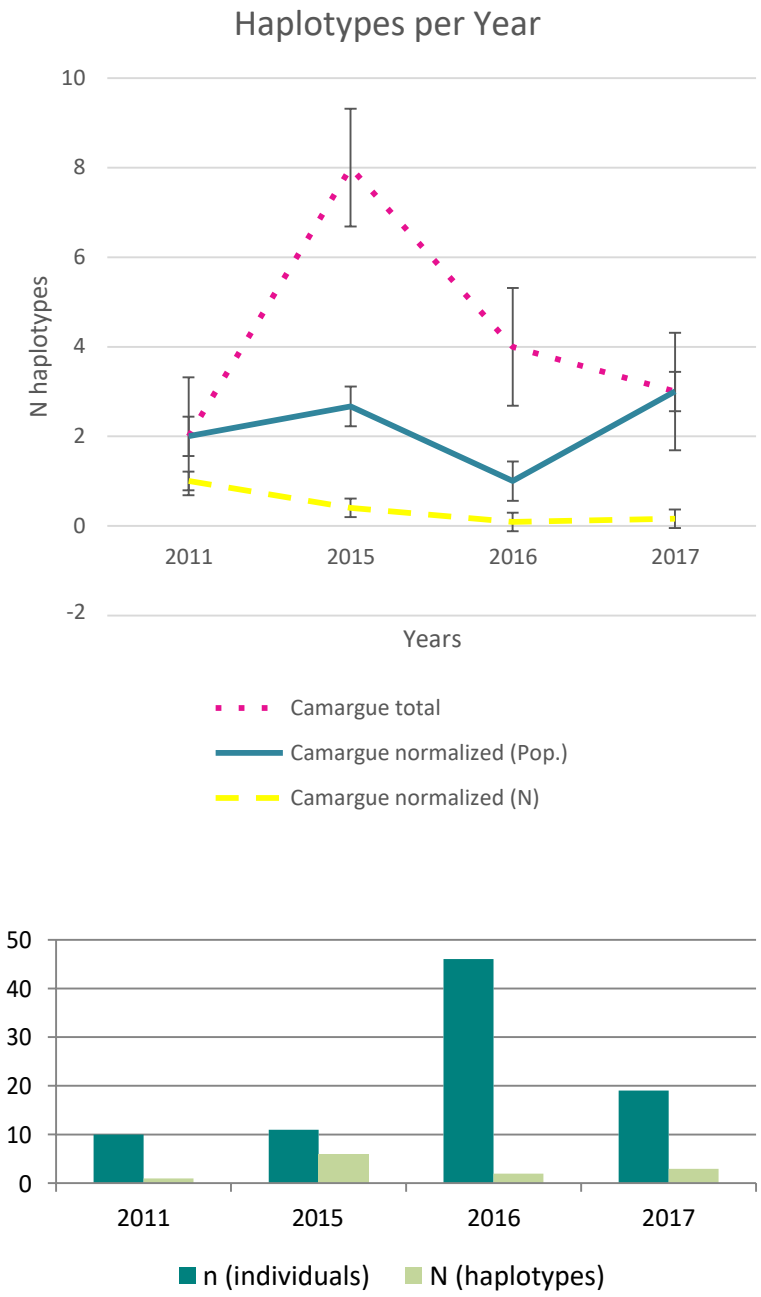

Figure 4.A.1: Haplotypes per year within the Camargue area in total, normalized by the number of populations analysed and normalized by the number of individuals analysed per year (above). Absolute numbers of individuals (n) and haplotypes (N) per year are given below.

Compatible with this results, haplotype diversity in 2016 ranges from 0.0 to 0.2, while in the year before varied from 0.667 to 0.905 (see Tab. 4.A.3). In 2017 higher values (0.433) were observed, too. However, the highest haplotype diversity was not detcted in the Camargue region but on Corsica and at the French Atlantic coast (both 1.0). Nucleotide diversity was extremly low in almost all populations ($\pi = 0$). The highest nucleotide diversities range from $\pi \approx 0.002$ % to $\pi \approx 0.003$ % and were all detected in 2015 within the Camague area, as well as on Corsica and at the French Atlantic coast (2011).

Sequence divergence within populations (Tab. 4.A.4) is also low and varied from 0.0 % (e.g. L.m.11FC.MdV, L.m.11FC.SdC, L.m.FC.16GPMM) to 0.34 % in GPMM in 2015. The second highest value was shown by L.m.11F.IN (0.32 %). Regarding the genetic distances between populations most of them are lower than 0.1 % indicating high relatedness. The highest sequence divergence between populations is 0.28 % when the French Atlantic coast (L.m.11F.IN) is compared to GPMM 2015. F_{ST}-values were calculated to detect the presence or absence of gene flow between sample sites of *L. macrostigma*. The F_{ST}-value range from 0 to 1, while a value of zero means complete mixis and one total isolation of populations. Table 4.A.4 shows on the upper right diagonal that many values are 0.00. The highest value is 0.659 ($p<0.01$) between Marais du Vigueirat 2016 and Ile de Noirmoutier (L.m.11F. IN.). Within the Camargue region high and significant values can be observed between the population of Marais du Vigueirat and GPMM (0.455*) and TdV (0.435*), respectively. Between years for the same population a F_{ST}-value of 0.499* can be observed between TdV 2015 and 2017.

Table 4.A.3: Number of individuals (n), number of haplotypes (H, total and private), haplotype diversity (HD) and nucleotide diversity in % (π) for the different *L. macrostigma* populations and years based on CO1.

country	year	location	(n)	H total/privat	HD (±SD)	π % (±SD)
Corsica	2011	Etang de Canettu	1	1/0	n/c	n/c
	2011	Lagune d'Arbitru	2	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2011	Lagune de Caniscione	2	2/2	1.000 (±0.500)	0.002 (±0.0014)
Carmargue	2010	Trou du Pélobate	2	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2011	Salins du Caban (Baisse de Lhallu)	8	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2011	Marais du Vigueirat	2	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2016	Marais du Vigueirat	15	1/0	0.200 (±0.154)	0.000 (±0.0000)
	2015	Grand Port Maritime de Marseille. Mare de la pompe	7	5/1	0.905 (±0.103)	0.003 (±0.0006)
	2016	Grand Port Maritime de Marseille	5	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2016	Grand Port Maritime de Marseille. Mare du nid	10	2/1	0.200 (±0.154)	0.000 (±0.0001)
	2016	Grand Port Maritime de Marseille. Baisse de la pègue	12	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2015	Tour de Valat. Cerisières moyennes	9	3/0	0.667 (±0.132)	0.001 (±0.0003)
	2015	Tour du Valat. Sarcelles	4	3/0	0.833 (±0.222)	0.002 (±0.0006)
	2016	Tour du Valat	4	1/0	0.000 (±0.000)	0.000 (±0.0000)
	2017	Tour du Valat	19	3/2	0.433 (±0.117)	0.000 (±0.0001)
French Atlantic coast	2011	Ile de Noirmoutier	3	3/0	1.000 (±0.272)	0.003 (±0.0012)
	2011	Vendée. Réserve de Chanteloup	1	1/0	0.000 (±0.000)	0.000 (±0.0000)

Table 4.A.4: Genetic distances (%) **within** and between populations of *Lestes macrostigma* (right diagonal) and F_{ST}-values (left diagonal) between populations. Significant F_{ST}-values based on 10,000 permutations are indicated by an asterics*.

nr.	year	location	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	2011	Corsica	**0.200**	0.000	0.327	0.000	0.481	0.000	0.214	0.333	0.426*	0,000	0,000	0.1587	0.377*	0.068	0.000
2	2010	Trou du Pélobate	0.133	**0.000**	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.043	0.055	0.000	0.000	0.000	0.000
3	2011	Salins du Caban (Baisse de Lhallu)	0.133	0.000	**0.000**	0.000	0.000	0.322*	0.000	0.000	0.000	0.318	0.472	0.000	0.051	0.492*	0.000
4	2011	Marais du Vigueirat	0.133	0.000	0.000	**0.000**	0.000	0.007	0.000	0.000	0.000	0.043	0.055	0.000	0.000	0.000	0.000
5	2016	Marais du Vigueirat	0.133	0.000	0.000	0.000	**0.000**	0.455*	0.000	0.042	0.000	0.435*	0.627	0.000	0.110	0.659*	0.000
6	2015	Grand Port Maritime de Marseille. Mare de la pompe	0.274	0.244	0.244	0.244	0.244	**0.343**	0.229*	0.348*	0.406*	0.038	0,000	0.184	0.431*	0.000	0.000
7	2016	Grand Port Maritime de Marseille	0.133	0.000	0.000	0.000	0.000	0.244	**0.000**	0.000	0.000	0.238	0.347	0.000	0.000	0.347	0.000
8	2016	Grand Port Maritime de Marseille. Mare du nid	0.142	0.009	0.009	0.009	0.009	0.253	0.009	**0.019**	0.019	0.334*	0.469	0.000	0.072	0.487*	0.000
9	2016	Grand Port Maritime de Marseille. Baisse de la pégue	0.133	0.000	0.000	0.000	0.000	0.244	0.000	0.009	**0.000**	0.391*	0.577*	0.000	0.089	0.603*	0.000
10	2015	Tour du Valat. Cerisières moyennes	0.172	0.153	0.153	0.153	0.153	0.278	0.153	0.163	0.153	**0.190**	0,000	0.199	0.392*	0.155	0.000
11	2015	Tour du Valat. Sarcelles	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	**n/c**	0.285	0.499*	0.068	0.000
12	2016	Tour du Valat	0.133	0.000	0.000	0.000	0.000	0.244	0.000	0.009	0.000	0.153	n/c	**0.000**	0,000	0.272	0.000
13	2017	Tour du Valat	0.158	0.025	0.025	0.025	0.025	0.269	0.025	0.034	0.025	0.178	n/c	0.025	**0.043**	0.515*	0.000
14	2011	Ile de Noirmoutier	0.272	0.190	0.190	0.190	0.190	0.271	0.190	0.199	0.190	0.285	n/c	0.190	0.215	**0.316**	0.000
15	2011	Vendée. Réserve de Chanteloup	0.133	0.000	0.000	0.000	0.000	0.244	0.000	0.009	0.000	0.153	n/c	0.000	0.025	0.190	**n/c**

Environmental conditions and abundance data

Rainfall over the analysed years varied from 249,2 mm in 2012-2013 to 670.8 mm in 2011-2012 (Fig. 4.A.4, Tab. 4.A.5). In 2015 524.7 mm were detected together with the greatest haplotype diversity. In the following year, 401.6 mm of rain occurred with the lowest diversity parameters. In 2017, less rainfall (273 mm) and an explosion in abundance can be detected. The number of species observed ranged from 99 in 2013-2014 and 5,000 in the years 2014 and 2017.

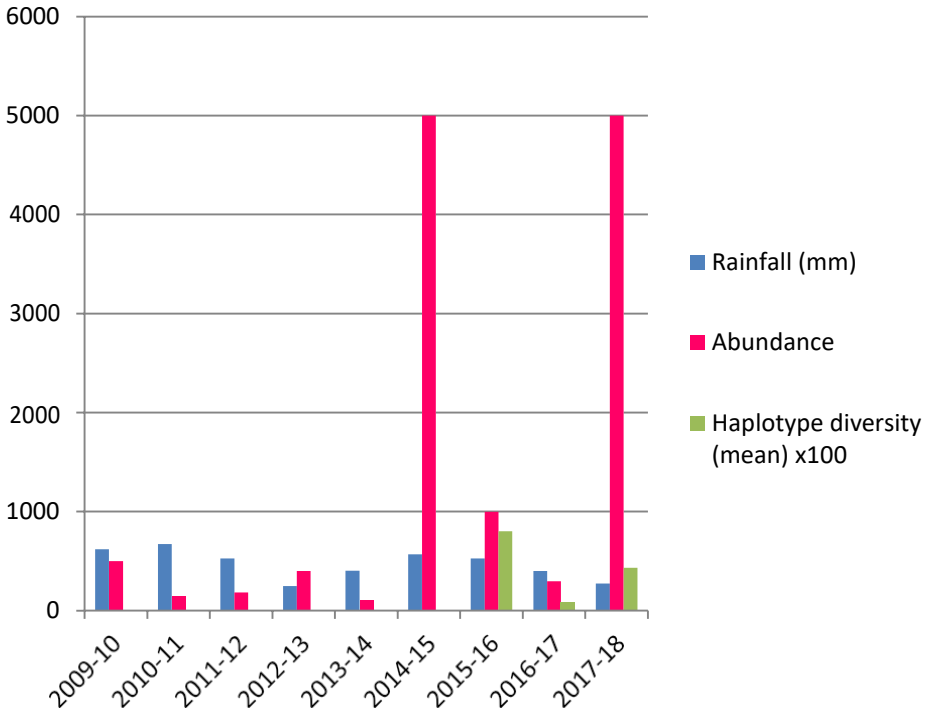

Figure 4.A.2: The cumulated amount of precipitation (mm) per year, the observed abundance of *L. macrostigma* and the detected mean haplotype diversity for the Camargue area (x100 for illustrational reason) over the analysed years.

Table 4.A.5: Precipitation data (mm) for the monitored years within the Camargue area, cumulated and separate for summer, fall, winter and spring, as well as the observed number of breeding sites and observed abundance for *L. macrostigma.*

Year	mm (rain)	Number of breeding sites	Abundance (individuals)
2009-10	**709.7**	**42**	**500**
summer	89.8		
fall	143.8		
winter	263.3		
spring	212.8		
2010-11	**726.5**	**31**	**148**
summer	55.7		
fall	155.7		
winter	277.4		
spring	237.7		
2011-12	**612.8**	**28**	**183**
summer	87.9		
fall	306.1		
winter	129.2		
spring	89.6		
2012-13	**291.9**	**24**	**400**
summer	42.7		
fall	119.5		
winter	18.4		
spring	111.3		
2013-14	**440.6**	**37**	**99**
summer	35.8		
fall	155.1		
winter	100.3		
spring	149.4		
2014-15	**705.6**	**55**	**5000**
summer	138.5		
fall	312.6		
winter	189		
spring	65.5		
2015-16	**723.5**	**15**	**999**
summer	198.8		
fall	142.2		
winter	163.4		
spring	219.1		
2016-17	**438.4**	**24**	**295**
summer	36.8		
fall	181		
winter	143.8		
spring	76.8		
2017-18	**312.9**	**70**	**5000**
summer	39.9		
fall	84.6		
winter	61.4		
spring	127		

Discussion

This genetic study on *Lestes macrostigma* aims to describe the population genetic dynamics within the Camargue area based on mitochondrial gene fragments (the barcode CO1 and OdoCO1 fragment). Understanding this species dynamics regarding abundance and genetic metrics displays a pivotal background for future management efforts and for classifications within risk assessments. The high potential of habitat destruction (due to tourism and land use) and adverse climate change effects threatening this region (e.g. Cramer et al. 2018, Sala et al. 2010, Giorgia & Lionello 2008), makes it mandatory to investigate this species ability to deal with population bottlenecks and their genetic consequences.

Overall, the Camargue population of *L. macrostigma* showed lower genetic diversity compared to the populations of Corsica and the Atlantic coast. Within the Camargue, population fluctuations in this species abundance and genetic diversity were detected between the different years observed. The highest number of haplotypes (6) was detected in 2015. The highest amount of year-wise private haplotypes (2) was observed in 2017. The most extensively sampled year 2016 showed lower numbers of haplotypes, though chances should have been higher to sample haplotypes of the years before. Therefore, we consider 2016 as a year where a genetic bottleneck occurred. These genetic bottlenecks could lead to the overall lower genetic diversity within the Camargue region. This was equally demonstrated in a European population comparison of this species (Herzog et al., *submitted*). Concordantly, this species abundance was higher in 2015 and 2017 than compared to 2016. The most private haplotypes were scored in 2017 where a population explosion occurred and 5,000 individuals were surveyed. In contrast, the year before, only 295 individuals were detected within the same area. Another popualtion explosion occured in 2014 were unfortunately genetic data is missing. The high inter-annual variation in population abundance and site occupancy of *L. macrostigma* is noticed in many studies (e.g. Florencio & Díaz-Paniagua 2012, Lambret et al. 2009, Borisov 2005). Dispersal events associated with these population explosions have been described (e.g. Schweighofer et al. 2010, Papazian 1995). However, the reasons for these fluctuations remain poorly understood. For a stenotypic species adapted to temporary waters, annual changes in precipitation are presumed to impact survival rates (Lambret et al. 2018). The timing

of precipitation is of great importance. Lambret et al. (2018) described that flooding in early spring of *L. macrostigma* temporary ponds reduces desiccation risk and egg mortality. Regarding the rainfall patterns observed in this study, no correlation between the cummulated amount of rainfall or the amount of rainfall during spring was detected. The amount of rainfall in 2017, where a drastic increase in abundance was observed, was lower than in years with lower abundance. Other factors influencing egg development and viability are e.g. temperature and photoperiod (Arrese & Soulages 2010, Rueda et al. 1990, Adkisson 1964) which were not surveyed for the respective years. To get a full understanding of the environmental factors shaping this species population dynamics, more abiotic variables should be taken into consideration.

The genetic analyses could not detect the described dispersal events (e.g. Schweighofer et al. 2010, Papazian 1995) within years with high population densities, as even in the small area of the Camargue F_{ST}-values for 2015 show less gene flow than for 2016. If the dispersal of this species is male-biased, maternal inherited mtDNA as used in this study would not be able to detect gene flow correctly (e.g. Prugnolle & de Meeus 2002). Sex-biased dispersal strategies are described for a few odonate species (Damm & Hadrys 2012, Beirinckx et al. 2006). One of these is *Trithemis arteriosa,* were sex-biased dispersal behavior was linked to extreme climate conditions (Damm & Hadrys 2012). Therefore we suggest further genetic monitoring approaches on a regional level to be conducted with nuclear markers, like microsatellites.

Conclusion

The endangered damselfly *L. macrostigma* is represented by a genetically impoverished population in the Camargue area. On top, we detected a genetic bottleneck event in 2016. The results show that this could be compensated in the following year, though diversity parameters did not reach the values of the best year analysed (2015). Though we could not demonstrate that these fluctuations are based on varying precipitation (alone), water management of the temporary waters this species is restricted to should be taken into consideration. Climate change scenarios predict that a pronounced decrease in precipitation is expected, especially during the warm season in the Mediterranean climate (Giorgia & Lionello 2008). Hence, there is

an increasing risk for temporary wetlands to experience a longer drought period which could be the cause for additional population declines, which may be not recoverable anymore.

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Genetic isolation within the stenotypic and patchy distributed damselfly *Lestes macrostigma* (Odonata): revealing stronger endangerment than expected

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Abstract

A major aim in conservation genetic studies is the identification of populations of a vulnerable species requiring special attention and protection. Genetic assessments can reliable detect isolated populations facing particular threats, e.g. bottleneck events, genetic drift and an increased risk of inbreeding. In this study, 250 individuals of the stenotypic damselfly *Lestes macrostigma* from Spain to Siberia were analyzed by means of mitochondrial and nuclear markers. This species has a strong niche conservatism and is restricted to brackish ecosystems with a special plant composition needed for reproduction. However, a unique population was recently detected in Siberia, Russia that is adapted to freshwater ecosystems instead. The special habitats of *L. macrostigma* are patchyly scattered across the distributional range (Europe to Mongolia) and affected by anthropogenic habitat destruction and fragmentation.

The chosen sequence markers (two CO1 fragments and ITS) shed light into possible historical demography. The results proposed three genetically distinct groups: the Spanish population (1), France (including the island of Corsica) and Lesbos (2), and an "eastern" group consisting of populations from Austria, Hungary, Ukraine, Serbia, Georgia, Russia and Samos (3). Haplotype frequencies and tests for neutral evolution suggested that both the Spanish and French groups underwent at least one bottleneck event and genetic drift. Moreover, in the Spanish population only private haplotypes are discovered. Therefore we recommend strict genetic monitoring attempts and a special consideration of these groups as management units in future risk assessment programs. Overall gene flow estimates express very low connectivity and high degrees of genetic sub-structuring, reflecting the restricted habitat of this brackish pond specialist.

Introduction

The protection of all levels of biodiversity is no longer an exclusive scientific interest, but has arrived to take its place at the center of society. Today, the loss of biodiversity proceeds at the local, regional, national and global levels with unprecedented pace (Meadows 2016). Among major drivers are habitat destruction and fragmentation due to land use and climate change (Sala et al. 2010, Henle et al. 2004, Watts et al. 2006). Europe's single biodiversity hotspot, the Mediterranean basin, is coincidently the region experiencing the most anthropogenic impact (e.g. Cuttelod et al. 2009, Cramer et al. 2018). Future modeling scenarios predict that Mediterranean biomes will experience the greatest change in biodiversity, due to the substantial influence of land use and climate change (Sala et al. 2010).

Biological diversity is composed of ecological diversity, species diversity and genetic diversity (Heywood 1995). Frankham et al. (2002) highlighted that human-related influences can reduce species to population sizes where they are especially prone to stochastic effects (e.g. environmental, demographic and genetic). Based on the correlation between heterozygosity and population fitness, it is essential to study and maintain genetic diversity for species to assure a potential response to environmental changes of their habitats (Watts et al. 2006, Reed & Frankham 2003). Habitat fragmentation hampers the dispersal and therefore the genetic exchange among populations of a species, which can lead to genetically poor, isolated populations. Furthermore, Reed and Frankham (2003) explicitly linked the loss of genetic diversity to an increased risk of inbreeding, which is associated with a higher risk of extinction (Watts et al. 2006).

These concerns are especially valid for highly stenotopic species with strong nich conservatism (e.g. Matern et al. 2009). The insect order Odonata (dragonflies and damselflies) comprises numerous stenotypic species (Corbet 2004). Odonates are well known as models for ecological and evolutionary research (Córdoba-Aguilar 2008) and genomic tools are increasingly applied in various studies (Bybee et al. 2016). They are considered prime indicators for the evaluation of freshwater ecosystems (Bried & Samways 2015, Kalkmann 2008). Genetics can bring a relevant insight in the fate of endangered species (e.g. Monroe & Britten 2014, Watts et al. 2006), studies on rare-

species ecology and conservation genetics are underrepresented and necessary in the context of changing environments (Bried & Samways 2015).

The damselfly *Lestes macrostigma* (Eversmann, 1836) (Odonata: Lestidae) is distributed from Portugal to Mongolia but only occurs in restricted areas, making its distribution area highly fragmented (Fig. 5.B.1). This fragmentation is due to the combination of the species ecology and the availability of the corresponding habitat: across its whole range *L. macrostigma* perennial populations mainly reproduce in temporary brackish waters (Boudot & Raab 2015, but see Berquier & Andrei-Ruiz 2019). As a rare example, in Siberia the species is usually found around fresh waters (Kosterin 2015). Mediterranean temporary ponds are disappearing at a high rate (Zacharias & Zamparas 2010), as do other salted temporary waters because of increasing urbanization in coastal areas and regions with steppe lakes, and industrial and agricultural development (Kalkman 2014). An additional threat is climate change: various scenarios expect a pronounced decrease in precipitation, especially during the warm season in the Mediterranean climate (Giorgia & Lionello 2008). Finally, *L. macrostigma* shows great inter-annual variation in population abundance and site occupancy (e.g. Florencio & Díaz-Paniagua 2012, Lambret et al. 2009, Borisov 2005). Hence, *L. macrostigma* populations are globally decreasing, suggesting the need of more monitoring of this species. Although the species is still considered as “Least Concerned” in the Red List of Threatened Species by the International Union for Conservation of Nature (IUCN) (Kalkman 2014), at lower scales, its IUCN threat category varies from “Near Threatened” to “Endangered” (e.g. UICN France et al. 2016, Kalkman et al. 2010, Riservato et al. 2009). The species is even legally protected in Hungary (Környezetvédelmi Minisztérium, 2001).

In this study, we focus on the conservation genetics of this threatened damselfly. We aimed to assess (1) the overall genetic diversity of this stenoecious species, especially in order to contribute to future assessments of the species´ extinction risk; (2) the impact of habitat fragmentation across the distribution range and potential migration routes, especially to fine-tune the implementation of future habitat restoration programs and to consider possible reintroduction actions; and (3) whether the habitat difference between Siberia and the rest of the species distribution range is associated with the status as sub-species, thereby possibly redefining the species

conservation strategy. Consequently, we assessed the population structure of *L. macrostigma* based on genetic analyses of populations coming from ten different countries and sampled at 34 different sites from Spain to Siberia, Russia. The selected genes for the genetic analyses are fragments of the Cytochrome c oxidase subunit I (CO1) and of the Internal transcribed spacer (ITS).

Material and Methods

Sample collection

From the first European congress on odonatology (2-5 July 2010, Vairão-Vila do Conde, Portugal), a large odonatologist network has been repeatedly invited to sample *L. macrostigma* wherever encountered. Hence, tissue samples were obtained from Austria, Cyprus, France (including Corsica), Georgia, Greece (Lesbos and Samos), Hungary, Russia (Siberia), Serbia, Spain and Ukraine (Fig. 4.B.1, Tab. 4.B.1) by using generally the minimal-invasive sampling method as described in Fincke & Hadrys (2005) where a single mid leg is used for tissue sampling. After shipment, tissue samples were stored in 98 % EtOH at -20 °C upon analyses.

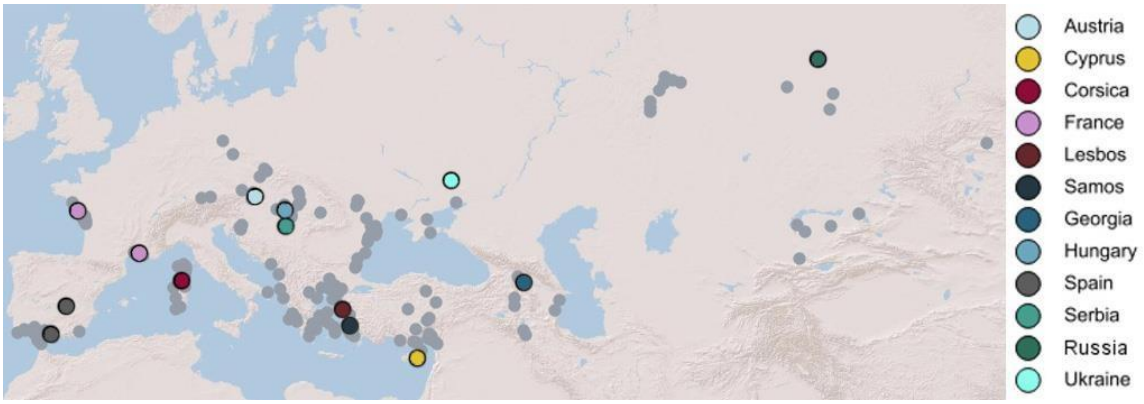

Figure 4.B.1: Current known occurrence (sensu from 1990 onward; after Boudot & Kalkman 2015, updated 2019) of *Lestes macrostigma* (grey dots) and distribution of sampling sites.

Table 4.B.1: Sample sizes per genetic marker and sampling sites (country and region) including sampling year, coordinates, habitat (type of water and dominant plant species used for oviposition) and estimated abundance of *Lestes macrostigma* during sampling. Abbreviations: Temp. = temporary; brack. = brackish*; B. mar*. = *Bolboschoenus maritimus*; *J. mar*. = *Juncus maritimus.* Abundance classes: 9 = few individuals, 99 = dozens, 999 = hundreds, 9999 = thousands; "corrected" abundance is given between square brackets when sampling has been done at the end of the flying period or when individuals were likely to disperse from another site where the species was more abundant.

Region	Year	Coordinates	Habitat	Abundance	Odo CO1	HCO LCO (barcode CO1)	Odo ITS
Austria					**3**	**3**	**4**
Burgenland	2010	47.8811°N 16.8811°E	Temp. brack. (*B. mar.*)	9*	3	3	4
Cyprus					**6**	**6**	**5**
Famagusta	2012	35.0397°N 33.9607°E	Temp. brack. (*J. mar.*)	999	6	6	5
France					**109**	**107**	**24**
Noirmoutier	2011	46.9903°N 02.2629°O	xxxxxxxx	[9999]	3	3	2
Vendée	2011	46.5547°N 01.7902°O	Temp. brack. (*B. mar.*)	999	1	1	/
Camargue	2010	43.4564°N 04.8361°E	Temp. brack. (*J. mar.*)	999	2	2	3
	2011	43.5141°N 04.7836°E	Temp. brack. (*J. mar.*)	999	2	2	2
		43.4556°N 04.8331°E	Temp. brack. (*J. mar.*)	99	8	8	2
	2015	43.4936°N 04.6753°E	Temp. brack. (*B. mar.*)	999	9	9	/
		43.5173°N 04.6673°E	Temp. brack. (*B. mar.*)	999	4	4	2
		43.4670°N 04.8230°E	Temp. brack. (*J. mar.*)	999	7	7	/
	2016	43.4959°N 04.6434°E	Temp. brack. (*B. mar.*)	99	7	4	/
		43.4456°N 04.7931°E	Temp. brack. (*B. mar.*)		18	10	/
		43.4139°N 04.7844°E	Temp. brack. (*J. mar.*)	9 [999]	16	11	7
		Grand Clos	Temp. brack. (*B. mar.*)	99	12	5	3
		43.5141°N 04.7836°E	Temp. brack. (*J. mar.*)	999	15	15	3
	2017	43.4835°N 04.6759 °E	Temp. brack. (*B. mar.*)	99	/	21	/
Corsica	2011	41.4237°N 09.2221°E	Lagoon** (B. mar.)	99	1	1	/
		41.4810°N 09.0198°E	Lagoon** (B. mar.)	9	2	2	/
		41.4879°N 08.9945°E	Lagoon** (B. mar.)	99	2	2	/
Georgia					**2**	**2**	**/**
Kverno-Kartli	2015	41.4453°N 45.2019°E	Perm. fresh (B. mar.)***		2	2	/
Greece**					**5**	**5**	**2**
Lesbos	2011	39.2075°N 26.2580°E	Temp. brack. (*B. mar.*)	XX	4	4	1
Samos		37.7092°N 27.0141°E	Temp. brack. (*J. mar.*)	99	1	1	1
Hungary					**37**	**37**	**19**
Bács-Kiskun	2010	46.8683°N 19.1703°E	Temp. brack. (*B. mar.*)	9 [999]	5	5	6

Region	Year	Coordinates	Habitat	Abundance	Odo CO1	HCO LCO (barcode CO1)	Odo ITS
		46.8311°N 19.1808°E	Temp. brack. (*B. mar.*)	9 [999]	4	4	/
		46.8094°N 19.1903°E	Temp. brack. (*B. mar.*)	99 [999]	7	7	/
		46.7931°N 19.1738°E	Temp. brack. (*B. mar.*)	9 [999]	5	5	/
		46.7661°N 19.1544°E	Temp. brack. (*B. mar.*)	99 [999]	9	9	9
		46.7223°N 19.1748°E	Temp. brack. (*B. mar.*)	999	7	7	4
Russia					**6**	**5**	**/**
Nowosibirsk	2015	56.5775°N 76.6031°E	Temp. fresh (Carex sp.)		6	5	/
Serbia					**2**	**2**	**/**
Vojvodina	2014	45.5131°N 20.2983°E	Temp. brack. (*B. mar.*)		2	2	/
Spain					**36**	**11**	**32**
Andalucía	2018	37.0364°N 04.8439°O	Temp. brack. (*B. mar.*)	999	5	1	5
		36.6591°N 04.4692°O	Temp. brack. (*B. mar.*)	99	6	1	6
Castilla	2016	39.4034°N 03.2511°O	Temp. brack. (*B. mar.*)	99	6	4	3
	2018	38.8405°N 01.5568°O	Temp. brack. (*B. mar.*)	99	19	5	18
Ukraine					**16**	**13**	**/**
Donesk	2016	48.8721°N 37.6133°E	Brack. (Carex sp.?)	XX	16	13	/
total:					**222**	**191**	**88**

The genomic DNA of tissue samples for 250 *L. macrostigma* individuals was extracted by using a standard phenol-chloroform protocol (Hadrys et al. 1992). For the amplification of the CO1 barcode region the standard barcoding primers were used (HCO/LCO; Folmer et al. 1994, see Table 4.B.2). As amplification success varied across populations due to a substitution in the reverse primer binding site, the reverse primer was adapted (rev) 5'-GTG ACC AAA AAA TCA AAA YAA A-3'. From then it was combined with the standard barcoding forward primer (LCO 1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'). The OdoCO1 fragment was amplified using the primers and temperature regime as described in Herzog & Hadrys (2017). In addition, the nuclear Internal Transcribed Spacer (ITS) was amplified for a data subset following the conditions of Damm & Hadrys (2010). Sanger sequencing of forward and reverse sequences was performed at DNA Analysis Facility on Science Hill at Yale University, New Haven, CT. For visual inspection, the obtained sequences were checked, edited and forward and reverse chromatograms were assembled with GENEIOUS software version 8.1.8 (Kearse et al., n.s.). After aligning the sequences with MUSCLE version

3.8.425 (Edgar 2004), all sequences were checked for correct identification by BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 4.B.2: Oligonucleotide primers used in this study for PCR and sequencing.

Primer	Locus	Sequence (5'-3')	Reference
HCO2198	*cox1*	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
LCO1490	*cox1*	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
Lestes_HCO	*cox1*	GTGACCAAAAAATCAAAAYAAA	This study
OdoCO1 fw	*cox1*	TACACGAGCATATTTTACTTCAGC	Herzog & Hadrys (2016)
OdoCO1 rev	*cox1*	CTTAAATCCATTGCACTTTTC	Herzog & Hadrys (2016)
ITS-Odo fw	*ITS I+II*	CGTAGGTGAACCTGCAGAAG	Damm & Hadrys (2010)
ITS-Odo rev	*ITS I+II*	CTCACCTGCTCTGAGGTCG	Damm & Hadrys (2010)

Statistical and phylogenetic analyses

DnaSP software version 5.10.01 (Librado & Rozas 2009) was used to calculate haplotype and nucleotide diversity. Genetic distances within and between the populations were assessed with the program Mega 7 (Kumar et al. 2015). A statistical parsimony haplotype network was constructed using the 95 % parsimony criterion implemented in TCS (Clement et al. 2000) for each marker. The visualization of the networks was realized in PopArt (Leigh & Briard 2015). Population sub-structuring was determined by means of F_{ST}-values and using analysis of molecular variance (AMOVA) as implemented in Arlequin software version 3.5.22 (Excoffier & Lischer 2015. AMOVA estimates the amount of genetic variation among predefined groups (Φ_{CT}), among populations within groups (Φ_{SC}), and among populations compared to the overall dataset (Φ_{ST}). To analyze the distribution of genetic variation, different models of grouped populations were compared as described in Table 4.B.5 according to geographical and ecological (e.g. habitat type) assumptions. DnaSP software version 5.10.01 (Librado & Rozas 2009) was used to calculate Tajima`s D (Tajima 1989) and Fu`s Fs (Fu 1997) to test for selective neutrality. Significant negative Tajima`s D and Fu`s Fs values would suggest the presence of selection or the occurrence of population expansion after a recent bottleneck. To test for a correlation between geographic and genetic distance (isolation by distance) a Mantel test was performed using the IBDWS software version 2.6 (Jensen et al. 2005, Rousset 1997) by keeping default settings and 1000 randomizations.

A character-based DNA barcode for the concatenated CO1 fragment was calculated with the Caos software (Sarkar et al. 2008) to generate population specific barcodes. Nucleotide substitutions occurring only in single individuals per population were ignored and pure diagnostic characters listed (Rach et al. 2008). Available CO1 sequences for other European Lestids were mined from NCBI GenBank (https://www.ncbi.nlm.nih.gov/) and BOLD Systems databanks (http://www.boldsystems.org/) and added to the barcode CO1 alignment to construct a phylogenetic tree. The dataset included *Lestes virens* (Accession numbers: KF369424, FBAQU1427), *Lestes sponsa* (AB708308 - AB708312, LC366662), *Lestes dryas* (KM53704, KM532506, KM531276, KM 528476, KM535968, KM532810, KU875364) and *Sympecma fusca* (KF369553) as outgroup. Phylogenies of species were inferred by Neighbor Joining (NJ) and Maximum Likelihood (ML) algorithms. The NJ tree was calculated via GENEIOUS treebuilder using 1000 bootstrap replicates. RaxML 8.2.4 (Stamatakis 2014) was used to calculate ML trees, using the gamma GTR model (Tavare 1986) and 750 bootstraps under the MRE-based Bootstopping criterion.

Results

The final CO1 alignments included 218 individuals for OdoCO1 (596 bp) and 192 individuals for the barcode CO1 fragment (525 bp). The nuclear marker gene ITS was sequenced on a subset of 88 individuals, resulting in a 669 bp long alignments. (Sequences will be uploaded to GenBank and accession numbers will be provided upon manuscript acceptance). The most parsimony informative sites are detected for the OdoCO1 fragment (38), followed by the barcode CO1 fragment (17). The more conserved ITS alignment contained only seven variable sites. The OdoCO1 alignment showed a deletion at position 536, 42 nucleotide positions upstream the expected stop codon in 83 specimen examined (38 %), leading to an altered amino acid (aa) composition for the last eleven amino acids in the translated protein (Fig. 4.B.2).

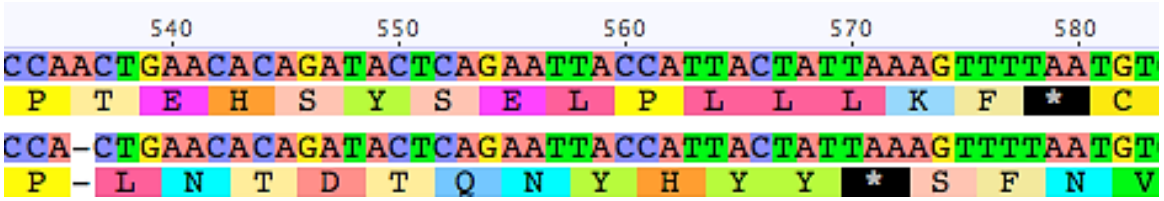

Figure 4.B.2: Nucleotide and translated protein sequences (single letter code) for the 3'-end of cox1 for *Lestes macrostigma*. The conventional sequence (above) and the sequence with deletion (position 536 for the OdoCO1 alignment) below, which is shown by 83 individuals across almost all populations.

Genetic diversity parameters and haplotype distribution

Overall genetic diversity was low to moderate, depending on the genetic marker. Indeed, though France was the most extensively sampled region (109 samples), it shows the highest number of haplotypes for HCO LCO Co1 only (9). For OdoCO1, seven haplotypes were detected in France, which is the same amount as in Hungary and less compared to Spain with nine haplotypes at far lower sample sizes (37 and 32, respectively).

Overall haplotype diversity ranged from 0.772 for OdoCO1, 0.650 for HCO LCO CO1 and 0.153 for ITS (Tab. 4.B.3). Population-wise haplotype diversity ranges for OdoCO1 from 0.0 (Georgia) to 1.0 (Austria and Serbia) followed by 0.933 in Siberia and 0.833 on Lesbos; for HCO LCO CO1 values range as well from 0.0 (Georgia, Serbia) to 0.833 in the Lesbos population. Again, France showed lower values (0.275 for OdoCO1; 0.293 for HCO LCO CO1) compared to Hungary (0.713, 0.718) and to Spain (0.528 for OdoCo1, but 0.164 for HCO LCO CO1). Nucleotide diversity ranged from 0.058 for ITS, 0.333 for HCO LCO CO1 to 0.938 for OdoCO1. It showed the highest values in the Lesbos population (0.980 odoCO1; 0.667 HCO LCO CO1) for both CO1 markers, while for ITS the population on Corsica showed the maximum value of 0.240.

Significant negative Tajima´s D values were displayed in France (-2.288**, -1.966*) and Spain (-2.057*, -2.239**) indicating an excess of rare haplotypes and that these populations underwent a recent demographic expansion after a bottleneck. The proportions of the different haplotypes among populations are shown in Fig. 4.B.3 visualizing the differences in haplotype frequency among France, Corsica and Lesbos compared to all other "Eastern" populations and the special status of the Spanish population. The latter comprises only private haplotypes (given in grey-scales).

Table 4.B.3: Number of individuals (n), number of haplotypes (H, total and private), haplotype diversity (HD), nucleotide diversity in % (π), Tajima´s D with p-value (*p<0.01, **p<0.001) and FU´s F for the analyzed genetic markers OdoCO1, HCO LCO CO1 and ITS.

Country	Genetic marker	(n)	H total/private	HD (±SD)	π % (±SD)	Tajima's D (P)	FU's Fs
total	**odoCO1**	**218**	**37/28**	**0.772 (±0.025)**	**0.938 (±0.0002)**	**-0.605**	**-3.950**
	HCO LCO	**192**	**17/13**	**0.650 (±0.031)**	**0.333 (±0.0031)**	**-1.235**	**-5.835**
	odoITS	**88**	**8/5**	**0.153 (±0.052)**	**0.058 (±0.0002)**	**-2.190****	**-3.619**
Austria	odoCO1	3	3/2	1.000 (±0.272)	0.224 (±0.0007)	n/c	-1.216
	HCO LCO	3	2/0	0.667 (±0.314)	0.127 (±0.0006)	n/c	0.201
	odoITS	4	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
Corsica	odoCO1	5	3/1	0.700 (±0.218)	0.202 (±0.0008)	-1.048	-0.186
	HCO LCO	5	3/0	0.700 (±0.218)	0.305 (±0.0009)	0.699	0.276
	odoITS	/	/	/	/	/	/
Cyprus	odoCO1	6	2/1	0.181 (±0.090)	0.127 (±0.0034)	n/c	n/c
	HCO LCO	6	3/0	0.733 (±0.155)	0.292 (±0.0006)	0.862	0.540
	odoITS	5	2/2	0.400 (±0.078)	0.240 (±0.0014)	-1.093	2.202
France	odoCO1	104	8/5	0.275 (±0.056)	0.121 (±0.0004)	-2,288**	-2.310
	HCO LCO	103	9/5	0.293 (±0.060)	0.132 (±0.0003)	-1.863	-4.815
	odoITS	24	3/1	0.163 (±0.099)	0.062 (±0.0004)	-1.996*	-0.430
Georgia	odoCO1	2	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
	HCO LCO	2	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
	odoITS	/	/	/	/	/	/
Lesbos	odoCO1	4	4/1	0.833 (±0.222)	0.980 (±0.0028)	1,873	1.747
	HCO LCO	4	3/0	0.833 (±0.222)	0.667 (±0.0022)	-0.817	0.961
	odoITS	1	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
Samos	odoCO1	1	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
	HCO LCO	1	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
	odoITS	1	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
Hungary	odoCO1	37	10/5	0.713 (±0.051)	0.217 (±0.006)	-1.574	-1.537
	HCO LCO	37	5/2	0.718 (±0.029)	0.291 (±0.0003)	0.709	0.772
	odoITS	20	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
Russia	odoCO1	6	5/3	0.933 (±0.122)	0.503 (±0.0020)	-1.422	-1.283
	HCO LCO	5	2/0	0.400 (±0.237)	0.076 (±0.0004)	-0.816	0.090
	odoITS	/	/	/	/	/	/
Serbia	odoCO1	2	2/0	1.000 (±0.500)	0.168 (±0.0008)	n/c	0.000
	HCO LCO	2	1/0	0.000 (±0.000)	0.000 (±0.0000)	n/c	n/c
	odoITS	/	/	/	/	/	/
Spain	odoCO1	32	9/9	0.528 (±0.105)	0.142 (±0.0003)	-2.057*	-6.079
	HCO LCO	11	5/5	0.164 (±0.164)	0.291 (±0.0010)	-1.007	-1.027
	odoITS	32	4/2	0.181 (±0.090)	0.075 (±0.0004)	-2.239**	-1.159
Ukraine	odoCO1	16	4/1	0.517 (±0.132)	0.097 (±0.0002)	-1.055	-1.478
	HCO LCO	13	3/1	0.590 (±0.122)	0.127 (±0.00039	0.096	-0.021
	odoITS	/	/	/	/	/	/

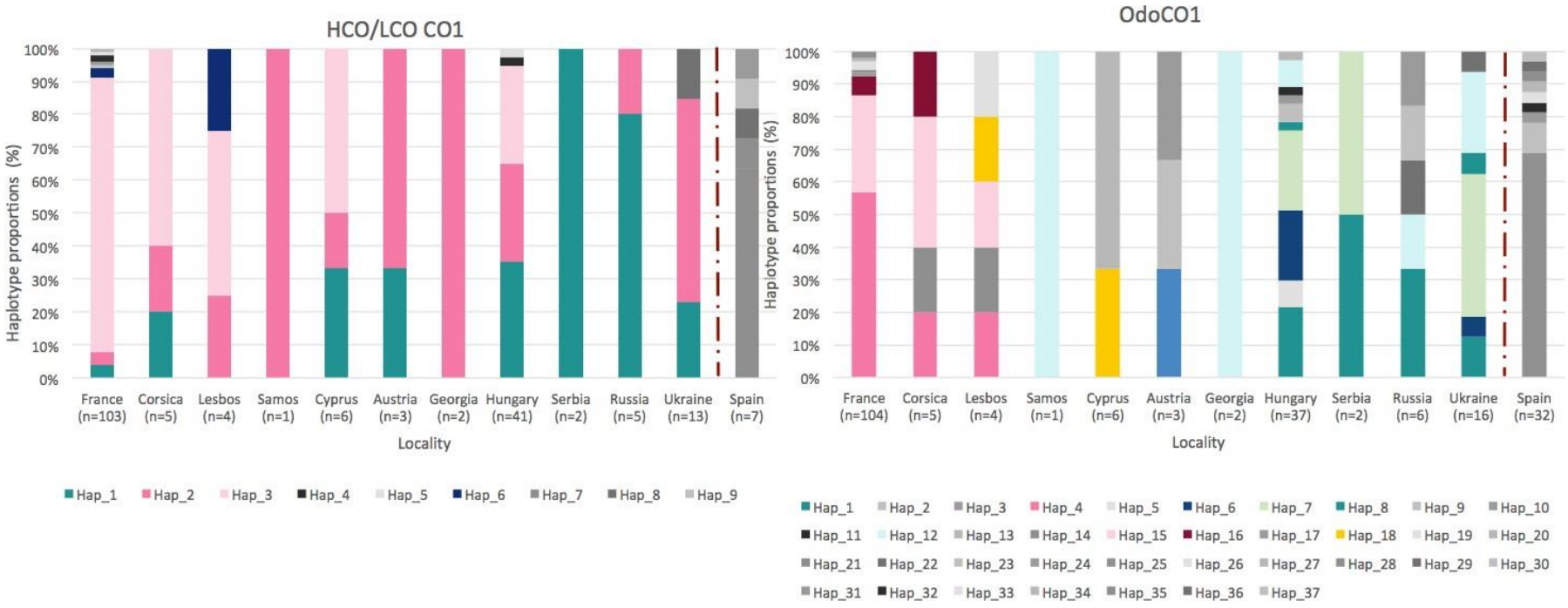

Figure 4.B.3: Proportions of mitochondrial haplotype frequencies across the distribution area of Lestes macrostrigma, for HCO LCO CO1 on the left and OdoCO1 on the right. All shared haplotypes are colored according to their legend, private haplotypes are given in grey-scales and black. Sample sizes per region can be found beyond the locality label in brackets. Please note that the Spanish population exhibits only private haplotypes (therefore seperated by a dotted line).

Genealogical relationships

Statistical parsimony haplotype networks are shown in Fig. 4.B.4 and 4.B.5. All markers showed a majority of private haplotypes: 76.5 % for HCO LCO CO1, 75.7 % for OdoCo1 and 62.5 % for ITS. For OdoCO1 out of a total of 218 individuals, 37 unique haplotypes were detected (a). The two most widespread haplotypes were each shared between five countries: Haplotype Hap_1 was found in Austria, Hungary, Serbia, Russia and Ukraine, while Hap_12 was present in Hungary, Ukraine, Greece (Samos), Georgia and Siberia. The French populations shared three haplotypes with individuals from Corsica and/or Lesbos and showed five private haplotypes. Out of 104 examined French samples, 59 individuals had haplotype Hap_4, followed by 33 individuals with Hap_15. Private haplotypes were only shown by up to three specimen. In total, eight haplotypes were detected in the French populations. The Spanish population showed only private haplotypes, but nine haplotypes were detected at a much smaller sample size than compared to France. The same is true for the Hungarian samples, where ten haplotypes were detected in the set of 37 individuals. In total, two unsampled haplotypes were present for OdoCO1. Overall, the network visualized three groups: Spain; France, Corsica and Lesbos; and an “Eastern” group with Austria, Hungary, Ukraine, Serbia, Siberia, Georgia and Samos.

The special situation of the Spanish population was also detected with the HCO LCO barcode CO1 marker, where genetic resolution is lower as for OdoCO1, resulting in only 17 haplotypes in total (Fig. 4.B.4b). Here, five haplotypes were detected in the set of seven Spanish individuals and all of them were private. However, for this marker the French populations shared haplotypes with all members of the “Eastern” group. For the nuclear ITS marker gene eight haplotypes were detected in the set of 88 analyzed individuals, with the majority of them showing Hap_1 (79 individuals). Spain showed two private haplotypes and shared one haplotype each with France and Hungary. Also Cyprus exhibited two private haplotypes.

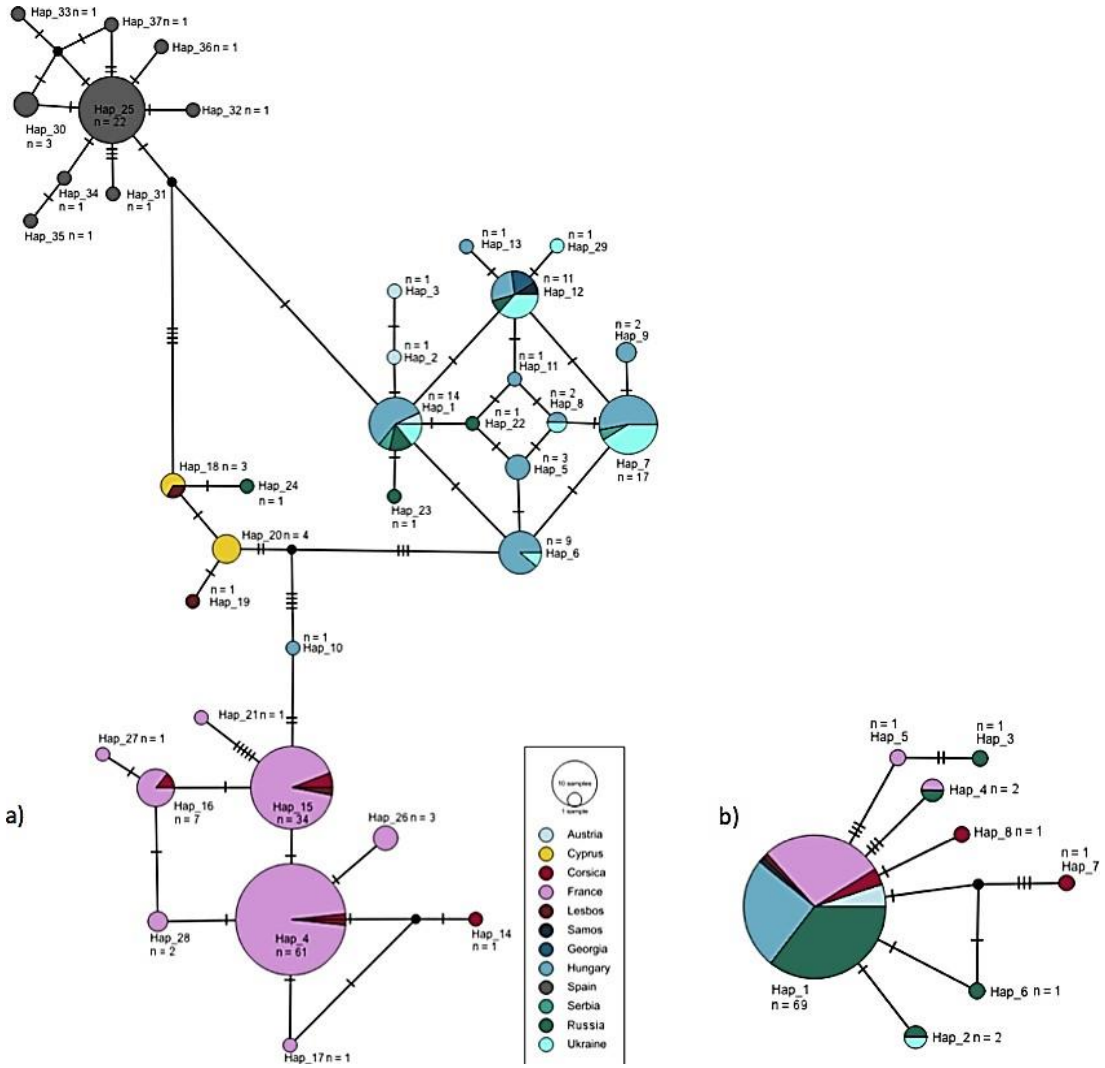

Figure 4.B.4: Mutational haplotype network based on statistical parsimony for *Lestes macrostigma* populations based on OdoCO1 (left) and ITS (right) sequence information. Circles display haplotypes colored by their region of origin, resulting in pie charts if haplotypes are shared among populations. Circle sizes reflect the frequency of the given haplotype. Little black circles depict unsampled haplotypes and each black mark symbolizes one nucleotide substitution between inferred haplotypes.

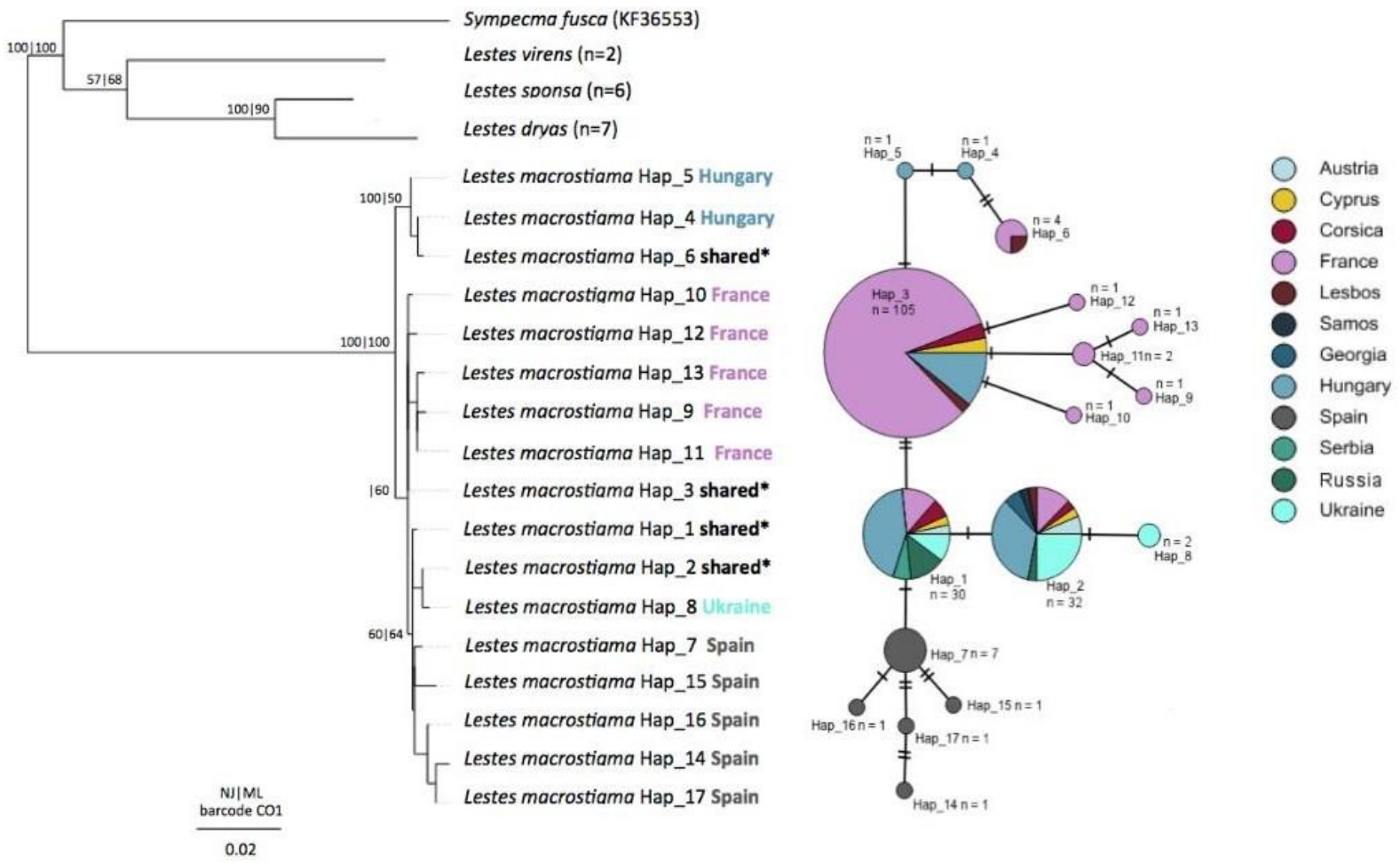

Figure 4.B.5: HCO LCO CO1 based phylogenetic tree including bootstrap values >50 of available European *Lestes* species (including sample sizes) additional to the 17 inferred haplotypes for *Lestes macrostigma* with *Sympecma fusca* as outgroup. Species branches where collapsed except for *L..macrostigma*. The color code of the haplotype network based on statistical parsimony for Lestes macrostigma matches the tree. Circles display haplotypes colored by their region of origin, resulting in pie charts if haplotypes are shared among populations. Circle sizes reflect the frequency of the given haplotype**.**

Population differentiation, structure and gene flow

Genetic distances within and between populations, as well as F_{ST}-values are displayed in table 4.B.4 in heat-map style for all used marker genes. Highest genetic distances within a population were detected in Lesbos (0.980 % for OdoCo1, and 0.666 % for HCO LCO CO1). Lowest values can be were found in Georgia (0.0 % for all markers). Between populations, low values for genetic distances (green shaded) were observed mainly within two groups: France, Corsica and Spain showed values from 0.125 % to 0.308 % for OdoCO1 between each other; Samos, Austria, Georgia, Hungary, Serbia, Siberia and Ukraine showed values from 0.0 % to 0.336 % for OdoCO1. Genetic distances between populations of these two groups were high (yellow shaded) and ranged between 1.621 % and 1.781 % for OdoCO1, and between 0.266 % and 0.571 % for HCO LCO CO1. Lesbos and Cyprus could not be included in these groups, though Lesbos showed lower distances to France/Corsica and higher values to the Eastern group. For the Cypriote population it was the opposite, with higher distances (1.375 % to 1.455 % for OdoCO1) compared to France and Corsica, and lower distances (ranging from 0.812 % to 1.008 %) to the Eastern group.

Strong genetic sub-structuring was observed by means of F_{ST}-values: high values (pink shaded) for both CO1 markers, reaching values up to 0.934 (Georgia vs. Cyprus) for OdoCO1 and 1.0 -indicating complete isolation- (Serbia vs. Samos and Georgia) for HCO LCO CO1. Significant low F_{ST}-values were observed between France and Corsica (0.019 for OdoCO1), while between France and all other populations values ranged between 0.574 (vs. Lesbos) and 0.889, with most of them being significant. The Spanish population showed also evidence for isolation by F_{ST}-values, scoring high and mostly significant values between 0.603 (compared to Russia) and 0.902 (vs. Corsica) to all other observed populations. Moderate non-significant values were detected for OdoCO1 between selected population combinations of the Mediterranean islands: Corsica and Lesbos (0.259), Lesbos and Samos (0.257) and Cyprus and Lesbos (0.353). Within the Eastern group consisting of Austria, Hungary, Serbia, Georgia, Russia and Ukraine, low values (between 0.0 and 0.207) were present indicating gene flow, despite a significant F_{ST}-value between Austria and Hungary of 0.342 for OdoCO1.

Analyzes of molecular variance (AMOVAs) were conducted both ungrouped (population-wise) and between different models of grouped populations to test genetic structuring based on OdoCO1 as the most informative marker (see Table 4.B.5). By means of AMOVA a significant overall Φ_{ST}-value was detected when comparing genetic variation among all populations for both CO1 markers. Inferred groups by habitat differences (temporary brackish vs. temporary freshwater) and by plant species as dominant available oviposition substrate (*B. maritimus* vs. *J. maritimus*) did not result in significant values and were left out in table 4.B.5. The percentage of variation explained among groups was rising with the number of groups inferred based on genetics. The most variation (82.97 %) is explained among populations based on OdoCO1 ($p<0.00001$) and corroborated an isolated population structure for *Lestes macrostigma*. Concordantly, Mantel tests for the three used markers showed no significant correlation between geographic and genetic distances for this species.

Table 4.B.4: Genetic distances (%) within and between populations of *Lestes macrostigma* (upper right diagonal) and F_{ST}-values (lower left diagonal) between populations given in heat-map style. Significant F_{ST}-values based on 10000 permutations are indicated by an asterics*.

Country	Genetic marker	France	Corsica	Lesbos	Samos	Cyprus	Austria	Georgia	Hungary	Serbia	Russia	Spain	Ukraine
France	odoCO1	**0.589**	0.125	0.744	1.711	1.375	1.707	1.711	1.621	1.627	1.622	0.308	1.699
	HCO LCO	**0.142**	0.237	0.377	0.571	0.264	0.512	0.571	0.349	0.395	0.430	0.739	0.560
	odoITS	**0.062**	n/c	0.031	0.031	0.150	0.031	n/c	0.031	n/c	n/c	0.066	0.180
Corsica	odo CO1	0.019*	**0.201**	0.815	1.781	1.445	1.770	1.781	1.686	1.697	1.685	1.686	1.766
	HCO LCO	0.168	**0.304**	0.428	0.380	0.247	0.342	0.380	0.289	0.266	0.289	0.612	0.383
	odoITS	n/c	**n/c**	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c
Lesbos	odo CO1	0.574*	0.259	**0.980**	1.386	0.714	1.386	1.386	1.318	1.302	1.246	1.292	1.376
	HCO LCO	0.190	0.0	**0.666**	0.619	0.444	0.587	0.619	0.492	0.523	0.542	0.870	0.626
	odoITS	n/c	n/c	**n/c**	0.0	0.119	0.0	n/c	0.0	n/c	n/c	0.037	0.149
Samos	odo CO1	0.885	0.839	0.257	**n/c**	1.008	0.336	0.0	0.158	0.084	0.364	0.577	0.052
	HCO LCO	0.751	0.200	0.0	**n/c**	0.349	0.063	0.0	0.283	0.190	0.152	0.536	0.073
	odoITS	n/c	n/c	n/c	**n/c**	0.119	0.0	n/c	0.0	n/c	n/c	0.037	0.149
Cyprus	odo CO1	0.870*	0.878*	0.353	0.920	**0.0**	1.008	1.008	0.958	0.924	0.812	0.913	0.997
	HCO LCO	0.283*	0.0	0.0	0.163	**0.292**	0.306	0.349	0.275	0.222	0.247	0.568	0.349
	odoITS	0.145	n/c	n/c	n/c	**0.239**	0.119	n/c	0.119	n/c	n/c	0.156	0.269
Austria	odo CO1	0.884*	0.852*	0.507*	0.333	0.884	**0.224**	0.336	0.313	0.252	0.420	0.577	0.325
	HCO LCO	0.725*	0.315	0.266	0.0	0.25	**0.126**	0.063	0.257	0.126	0.114	0.473	0.107
	odoITS	0.0	n/c	n/c	n/c	0.0	**0.0**	n/c	0.0	n/c	n/c	0.037	0.149
Georgia	odo CO1	0.889	0.873*	0.479	0.0	0.934*	0.571	**0.0**	0.158	0.084	0.364	0.577	0.052
	HCO LCO	0.767*	0.416	0.266	0.0	0.375	0.0	**0.0**	0.283	0.190	0.152	0.536	0.073
	odoITS	n/c	n/c	n/c	n/c	n/c	n/c	**n/c**	n/c	n/c	n/c	n/c	n/c
Hungary	odo CO1	0.865*	0.832*	0.691	0.0	0.753	0.342*	0.144	**0.216**	0.152	0.359	0.545	0.174
	HCO LCO	0.426*	0.0	0.152	0.0	0.0	0.075	0.192	**0.290**	0.205	0.221	0.552	0.294
	odoITS	0.0	n/c	n/c	n/c	0.310*	0.0	n/c	**0.0**	n/c	n/c	0.037	0.151
Serbia	odo CO1	0.878	0.826*	0.370*	0.0	0.865*	0.207	0.0	0.0	**0.168**	0.308	0.493	0.105
	HCO LCO	0.672*	0.195	0.147	1.0	0.073	0.368	1.0	0.0	**0.0**	0.038	0.346	0.175
	odoITS	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	**n/c**	n/c	n/c	n/c
Russia	odo CO1	0.861*	0.768*	0.435	0.0	0.678	0.064	0.032*	0.207	0.0	**0.504**	0.605	0.360
	HCO LCO	0.693*	0.342	0.363*	0.500	0.232	0.151	0.622	0.065	0.0	**0.0**	0.384	0.155
	odoITS	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c	**n/c**	n/c	n/c
Spain	odo CO1	0.887*	0.902*	0.784*	0.737*	0.861*	0.720*	0.760*	0.651*	0.704*	0.603*	**0.142**	0.567
	HCO LCO	0.772*	0.517*	0.528*	0.458	0.487*	0.479*	0.552*	0.473*	0.339	0.450*	**0.290**	0.522
	odoITS	0.0	n/c	n/c	n/c	0.126	0.0	n/c	0.0	n/c	n/c	**0.074**	0.177
Ukraine	odo CO1	0.887*	0.885*	0.730*	0.0	0.853*	0.538*	0.063	0.048	0.0	0.326*	0.751*	**0.096**
	HCO LCO	0.751*	0.511*	0.543*	0.0	0.456*	0.0	0.0	0.246*	0.413	0.306	0.609*	**0.126**
	odoITS	0.600	n/c	n/c	n/c	n/c	1.0	n/c	1.0	n/c	0.0	0.500	**n/c**

Colore Scales

F_{ST}-values	Genetic distances
1.0	1.78
0.975	1.735
0.95	1.69
0.9	1.6
0.875	1.555
0.85	1.51
0.8	1.42
0.775	1.375
0.75	1.33
0.7	1.24
0.675	1.195
0.65	1.15
0.6	1.06
0.575	1.015
0.55	0.97
0.5	0.88
0.475	0.835
0.45	0.79
0.4	0.7
0.375	0.655
0.35	0.61
0.3	0.52
0.275	0.475
0.25	0.43
0.2	0.34
0.175	0.295
0.15	0.25
0.1	0.16
0.075	0.115
0.05	0.07
0.0	0.0

Table 4.B.5: Distribution of genetic variation via hierarchical AMOVA for HCO LCO CO1 (grey-shaded) and OdoCO1 (rest). Inferred groups based on genetics or geography are yellow shaded.

Source of Variation	d. f.	Sum of Squares	Variance Components	% of Variation	*P*	Fixations Indices
total	**191**	**166.979**	**1.058**			
among populations	11	76.075	0.553 Va	52.28		
within populations	180	90.904	0.505 Vb	47.72	<0.0001	Φ_{ST} = 0.829
total	**219**	**663.859**	**3.975**			
among populations	11	523.019	3.298 Va	82.97		
within populations	208	140.814	0.677 Vb	17.03	<0.0001	Φ_{ST} = 0.522
Model I (France, Corsica, Lesbos) vs. (Samos, Cyprus, Austria, Hungary, Ukraine, Georgia, Serbia, Russia, Spain)						
total	**217**	**658.702**	**4.947**			
among groups	1	419.096	3.171 Va	64.10	<0.0001	Φ_{CT} = 0.640
among populations within groups	10	116.871	1.180 Vb	23.86	<0.0001	Φ_{SC} = 0.664
within populations	217	122.735	0.595 Vc	12.04	<0.0001	Φ_{ST} = 0.879
Model II (France, Corsica, Spain) vs. (Lesbos, Samos, Cyprus, Austria, Hungary, Ukraine, Georgia, Serbia, Russia)						
total	**219**	**663.859**	**4.470**			
among groups	1	246.497	1.376 Va	30.80	0.1358	Φ_{CT} = 0.307
among populations within groups	10	276.521	2.416 Vb	54.06	<0.0001	Φ_{SC} = 0.781
within populations	208	208	0.677 Vc	15.14	<0.0001	Φ_{ST} = 0.848
Model III (France, Corsica, Lesbos) vs. (Spain) vs.(Cyprus) vs.(Samos,Austria,Hungary,Ukraine,Georgia,Serbia,Russia)						
total	**219**	**663.859**	**4.468**			
among groups	3	504.085	3.562 Va	79.71	<0.0001	Φ_{CT} = 0.797
among populations within groups	8	18.933	0.229 Vb	5.14	0.0019	Φ_{SC} = 0.253
within populations	208	140.841	0.677 Vc	15.15	<0.0001	Φ_{ST} = 0.848
Model IV (France, Corsica, Lesbos) vs.(Spain)vs.(Cyprus)vs.(Samos)vs. (Austria,Hungary,Ukraine,Georgia,Serbia,Russia)						
total	**219**	**663.859**	**4.451**			
among groups	4	504.596	3.536 Va	79.45	<0.0001	Φ_{CT} = 0.794
among populations within groups	7	18.422	0.237 Vb	5.34	0.0098	Φ_{SC} = 0.259
within populations	208	140.841	0.677 Vc	15.21	<0.0001	Φ_{ST} = 0.847

Barcode analysis

Population-specific barcodes were generated by selecting 25 positions from the concatenated CO1 sequences (Fig. 4.B.6). In addition, position 1060 is visualized to demonstrate that the deletion at this site is evenly distributed among populations.

	11	251	299	344	422	428	441	455	482	501	504	526	529	565	613	616	712	784	802	865	889	905	943	1006	1012	1060
France	A/T	T/C	A/G	A/G	A/G	G	G	A/G	C/T	C/A	A	T	A	T	G	A	A	T	T	A	T	G	C	G	C	A/-
Corsica	A/T	T	A/G	A	A/G	G	G	A	C	C	A	T	A	T	G	A	A	T	T	A	T	G	C	A	C	A/-
Lesbos	A/T	T/C	A/G	A	A/G	G	G	A/G	C	C	A	T	G	C/T	A/G	A	A	T/C	C/T	A/G	C/T	A/G	C/T	A	A/C	A/-
Cyprus	A/T	T	A/G	A	A/G	G	G	A	C	C	A	T	A	C	A	A	A	C	C	G	C	G	T	A	A	A/-
Austria	A	T	A/G	A	G	G	G	A	C	C	A	T	G	C	A	G	G	T	C	G	C	G	T	A	C	A
Georgia	A	T	A	A	G	G	G	A	C	C	A	T	G	C	A	G	G	T	C	G	C	A	T	A	C	A
Hungary	A/T	T/C	A/G	A/G	A/G	G	G	A	C	C	A	T	G/A	C/T	A	G/A	A/G	T	C	A/G	C/T	A/G	T	A	C	A/-
Samos	A	T	A	A	A	G	G	G	C	C	A	T	A	C/T	A	G	G	T	C	G	C	A	T	A	C	A
Serbia	A	T	G	A	G	G	G	A	C	C	A	T	G	C	A	G	G	T	C	G	C	A	T	A	C	A/-
Russia	A	T	G	A	G	G	G	A	C	C	A	T	G/A	C	A	G/A	A/G	T/C	C	G	C	A/G	T	A	A/C	A
Ukraine	A	T	A/G	A	G	G	G	A	C	C	A	T	G	C	A	G	G	T	C	G	C	A/G	T	A	C	A/-
Spain	A	T	G	A	G	A	A/G	A	C	C/A	A/T	T/G/A	A	C	A	G	G	T	C	G	C	G	T	A	C	A/-

Figure 4.B.6: Character-based DNA barcodes for *Lestes macrostigma* populations based on concatenated CO1 sequences with character states (nucleotides) at 25 selected positions. The deletion at position 1060 is symbolized by „-“.

Discussion

Identifying genetic patterns of habitat specialists provides valuable informations not only about health and history of the species under investigation but also about the habitat itself. Species restricted to patchy habitats are especially prone to effects of environmental change. In this context, the damselfly *Lestes macrostigma* is an excellent indicator for patchy, brackish environments. In this study the application of three sequence markers combined with -and integrated into- an ecological framework allowed insights into population sub-structuring, habitat connectivity, isolation processes and anthropogenic effects on the environment itself.

Diversity and phylogenetic analysis

The overall genetic diversity of *L. macrostigma* is moderate and higher than compared to other stenotypic European odonate species, like *Ortethrum coerulescens* (Herzog et al., *in prep*.) or *Nehalennia speciosa* (Bernard et al. 2011), at least for the CO1 marker genes. The majority of genetic variation was detected with the OdoCO1 marker gene and among populations, resulting in 37 haplotypes. For *O. coerulescens*, an odonate species with a higher expected dispersal capacity, only 22 haplotypes where detected in a comparable study design (Herzog et al., in prep.). Nevertheless, the haplotype diversity was associated with a high proportion of private haplotypes (76.5 % for HCO LCO CO1, 75.7 % for OdoCO1), usually an indication for a lack of gene flow and/or local adaptation (e.g. Foudrilis 2019, Sjöstrand et al. 2014). With the nuclear ITS marker, little genetic variation among populations could be detected, though it was successfully used in population genetic studies before (e.g. Bower et al. 2009, Gomez-Zurita & Vogler 2003). However, there are other studies on odonates where this marker was discarded due to lack of information (Damm & Hadrys 2012).

Genetic diversity among regions varied and this did not just reflect variation in sample sizes. Regarding populations with larger sample sizes, Hungary was the most diverse (HD =0.7), followed by Spain and Ukraine. Strikingly, the French region, though it was the most extensively sampled, showed the lowest diversity parameters. This is likely due to a recent bottleneck event, as suggested by the significant negative Tajima´s D value. Based on the OdoCO1 haplotype distribution and frequencies, the following genetic groups can be distinguished: (1) Spain; (2) continental France, Corsica

and Lesbos (Greece, Anatolia); and (3) an "eastern" group including all other sites, from Samos (Greece, Anatolia) to Novosibirsk (Siberia, Russia), with the Cypriote population intermediate between group (2) and (3).

The phylogenetic analysis comparing *L. macrostigma* to other European Lestids was calculated because available phylogenies were based on different markers than used in this study (Dijkstra & Kalkman 2012, Dumont et al. 2010). Due to lack of publicly available CO1 sequences for *Lestes barbarus*, this species could not be included in the European phylogeny. The NJ and ML trees displayed the other *Lestidae* in a single clade, where *Lestes sponsa* and *Lestes dryas* derived from a more recent common ancestor that again shares ancestry with *Lestes virens*. The *Lestes macrostigma* haplotypes constitute a distinct clade derived from the common ancestor that all included Lestids share. This is in contradiction with Dijkstra & Kalkman (2012) and egg clutch patterning characteristics (Matushkina et al. 2016). Within *L. macrostigma,* a Spanish clade and a French clade could be distinguished, though the French populations also shared haplotypes for HCO LCO CO1. Given that for the more variable OdoCO1 France together with Corsica and Lesbos formed a distinct clade (data not shown but congruent with haplotype network), the hypothesis of different local subspecies should be elucidated in the future based on additional genetic markers. A morphological examination of specimens from these three groups would further help to test this assumption. Sequence divergence between the French group and the "Eastern" group of up to 1.8 % are close to the 1.97 % sequence divergence between the well-recognised subspecies *Orthetrum c. coerulescens* and *Orthetrum c. anceps* based on the same genetic marker (Herzog et al., in prep.).

Population structure, demography and biogeography

The overall genetic structure revealed that western populations (including Lesbos) are highly isolated from each other. Furthermore, they are also isolated from the eastern populations.. Given *L. macrostigma´s* dispersal behaviour during years with demographic explosion (e.g. Schweighofer et al. 2010, Papazian 1995) a limited amount of gene flow can be expected within the eastern group and between the French Mainland and the island of Corsica. This limited gene flow reflects the species

fragmented habitat structure (Fig 5.B.1). However, the eastern group (3) is the most diverse and seems to be connected at least by a stepping-stone model.

Lestes macrostigma is a lowland species, ca. 75 % of altitudinal records ranging from sea level to 100 m a.s.l., ca. 22 % ranging from 100 to 1000 m a.s.l. and mostly referring to brackish lakes on plateau elevations, and remaining ca. 3 % ranging from 1,000 to 1,780 m a.s.l. corresponding to single vagrants (J.-P. Boudot, *pers. com*.). There are no high mountainous reliefs between Siberia, Ukraine and Hungary. Hence, populations located to the West of the ancient Panonian sea (i.e. Parathethys, which ranged from the Alps to the Aral Sea during the Miocene [ca. 23-5 Myr] and even more during Pliocene [5-2.5 Myr]) are connected to those of Eastern Europe and Asia by Danube's valley, between Carpathians and Balkans (Dinaric Alps). Georgia's population also belongs to the Eastern group, being possibly connected to the other populations of this group despite the Greater Caucasus through the Caspian Sea coast and Koura's valley. Then, between the eastern group and the French group (2) the Alps most likely display a geographical barrier in between. Anatolia cannot be considered as a geographical barrier given that the species is found across these mountains (Kalkman & Van Pelt 2006a,b) and that the Cypriote population cannot clearly be assigned to any group. French populations share at least some haplotypes with the Eastern group for the HCO LCO CO1 marker, but in a different composition. Here, the excess of private haplotypes is most likely based on genetic drift due to a bottleneck event followed by ongoing geographical isolation. Regarding the Spanish group (1), this population had only private haplotypes for both CO1 fragments, which indicates drift and probably an even longer isolation from the French group (2) through the Pyrenees. This is also indicated by the significant negative Tajima´s D value for this population. Genetic distances are lower between the Spanish and French populations (0.7 % and 0.3 % for the CO1 markers, respectively) than compared to the eastern group (1.8 %) suggesting closer relatedness and less divergence time between them. However, the Pyrenees formed about the same time as the Alps about 50-100 Myr. The estimated mutation rate for the CO1 gene in other insects varies largely from 1.15 % to 19 % / Myr (e.g. Ney et al. 2018, Gratton et al. 2008, Clarke et al. 2001, Knowles 2000, Brower 1994), with higher values in the more recent studies. Based on these range of mutation rates the observed 0.7 % divergence between group 1 and 2 would translate into a

separation between ~37,000 and ~600,000 years ago. Even for the conservative divergence estimates the Pyrenees would still constitute a geographical barrier.

The habitat type of *L. macrostigma* in the Iberian Peninsula does not differ from that of other populations, except for Siberia (Tab. 4.B.1). Nevertheless, the pluviometric calendar of the Iberian Peninsula differs from the typically Mediterranean one, with highest rainfalls occurring in autumn and not spring (Martin-Vide & Lopez-Bustins 2006), the period of peculiar importance for aquatic larval growth (Lambret et al. 2018). Hence, the species is known to locally disappear during several years (Díaz-Martínez et al. 2018, Cano-Villegas & Conesa-García 2010, Ferreras-Romero et al. 2005), leading to very small population sizes and recurring bottleneck events during unfavourable periods. This temporal disappearing scheme, followed by recolonization or population explosion applies also to the French populations (Lambret et al., *in prep*.). In conclusion, dry periods in the western Mediterranean climate are likely to affect this species abundance thereby causing recurrent bottleneck events, leading to genetic drift and lower genetic diversity.

The chosen genetic markers did not reflect the Siberian population´s adaptation to fresh water instead of brackish water, which is a unique feature across this species´ distribution range (Tab. 4.B.1). To identify the gene(s) responsible for adaptation to either fresh/brackish water, more advanced genetic studies e.g. comparative transcriptomics are needed. Alternatively, we hypothesize that *L. macrostigma* originates from Asia, where its ecological niche might be wider than in Europe, and has colonised the Mediterranean and the Iberian Peninsula either by using Mediterranean Islands as stepping stones or before the formation of the Alps and Pyrenees. A postglacial westbound range expansion was shown for other zygopteran species as e.g. for *Coenagrion scitulum* (Swaegers et al. 2014). The latter requires much greater divergence time as reflected by genetic divergence of the populations; however it is possible that, given the narrow ecological niche of this species, diversification was prevented due to phylogenetic niche conservatism as described e.g. for *Megaloprepus caerulatus* (Feindt et al., *submitted*)

Implications for this species´ conservation

The isolation and possible sub-species status of the "Spanish" (1) and the "French" (2) groups, motivates to consider them as distinct management units during next extinction risk assessments (IUCN Red Lists). This is further motivated given that climate change scenarios predict a pronounced decrease in precipitation, especially during the warm season in the Mediterranean area (Giorgia & Lionello 2008). Already effects of dry summers could be shown for a riverine odonate species (Herzog & Hadrys 2016) in the region. Especially brackish species are prone to dry summers. Hence, there is an increasing risk for temporary water bodies to face drought episodes as soon as early spring, i.e. during the development of aquatic larvae. Climate change represents an increased risk of local extinction and therefore a reinforcement of the bottleneck effect for these two genetically distinct groups. More dramatically, recolonization potential of these populations is uncertain as the haplotype composition is uniquely found within these groups and may not be recovered if extinct.

For the same reasons, future connectivity and habitat restoration programs, i.e. temporary pond creation or appropriate water management, in areas where the species is not currently present, should consider the genetic findings of this study when re-introducing the species is planned. Eggs should be sampled from populations within the same genetic group. Another possibility would be to introduce some specimens from the Eastern group (3) in group (1) and (2) populations to increase the genetic diversity of these isolated populations. However, this is highly debatable if local haplotypes reflect local adaptation and if the risk of outbreeding depression is real (e.g. Huff et al. 2011, Montalvo & Ellstrand 2001). In Frankham et al. (2011) decision assistance about the probability of outbreeding depression is provided. Translocation experiments for genetic rescue should first, only be considered if future local monitoring projects (similar to Herzog & Hadrys 2017) detect an ongoing decrease in genetic diversity and second, conducted in a manageable, closed system under strict and frequent monitoring efforts.

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5 Summarized results and discussion

A. Odonate mitogenomes and new genetic markers

(Studies within Chapter 1 and references therein)

In the different studies of this thesis, four Mitogenomes could be announcend for the targeted species (*Anax imperator, Ischnura elegans, Megaloprepus caerulatus* and *Pantala flavescens*). The typical metazoan set of 37 genes (13 protein-coding genes, 22 tRNA genes, and 2 rRNA genes) was detected in the same gene order as in other odonate mitogenomes. However, the analysed mitogenomes varied largely in length and revealed with 16,087 bp the longest mitogenome of *Anax imperator*, as well the most compact mitogenome with 14,847 bp for *Pantala flavescens* among anisopterans. These length differences were mainly due to the variable length of the A+T-rich control region, ranging from 160 bp for *P. flavescens* to 1291 bp for *A. imperator.* Further, a premature stop codon was detected for P. flavescens in the *nad4* gene. Between *nad4* and *nad4l* a 207 bp long non-coding spacer region is located.

Comparing all generated mitogenomes to available mitogenome data, inconsistend number and size of intergenic spacer regions were detected, rejecting the theory that spacer are features to distinguish between anisoptera and zygoptera, or at the family level. Phylogenies were consistent to described gene tree phylogenies.

New sequence markers despite the barcode CO1 gene were successfully tested for population genetic studies. In this thesis the suitability of the NADH dehydrogenase subunit 1 and 3 gene for global population studies on the cosmopolitan dragonfly *P. flavescens* was demonstrated. For *L. macrostigma* the traditional CO1 barcoding primer (HCO 2198) was adapted due to a substitution in the reverse primer binding site and called Lestes HCO. Combined with the standard barcoding forward primer amplification of the CO1 barcode gene fragment was successful. However, the alternative OdoCO1 fragment showed greater divergence in population comparisons making it more valuable for comparisons on a low taxonomic level.

B. **The genetics of the cosmopolitan, migratory odonate *Pantala flavescens***

(Studies within Chapter 2 and references therein)

In the scope of this thesis, a first global multi-marker study with 27 populations from every continent of *P. flavescens´* distribution range revealed a higher population structure than expected, given this species migratory capacities. Based on the traditional barcode CO1 fragment, the genetic diversity (in total up to 0.834 HD), gene flow (the majority of F_{ST}-values range from 0.0 to 0.2) and the number of migrants between populations detected was high. Only the Easter Island population showed extremely low diversity measures (HD = 0.022 based on CO1, two haplotypes out of 89 individuals) and restricted gene flow with all other analysed populations, except for Namibia (F_{ST}-value = 0.162*, while to all other populations values from 0.292 up to 0.993). With the nuclear ITS marker however, higher numbers of haplotypes at a smaller sample size were observed on Easter Island (four haplotypes out of nine individuals). Furthermore, diversity measures were at a comparable level with other studied populations. ITS based F_{ST}-values indicated restricted gene flow, where by CO1 vast numbers of migrants were estimated. Interestingly, no migration among closely located African populations was detected with ITS. The African populations show the highest amount of non-synonymous mutations leading to 13 amino acid substitutions in total. For all other studied populations, only Peru and Chile, as well as French Polynesia show one altered protein sequences each. All these various findings reject the theory of a panmictic population structure for *P. flavescens* which was likely enunciated by the use of a single marker at an insuffiecent sample size. On top, the unproportional distribution of nucleotide substitutions and amino acid substitutions indicates a considerable degree of saturation for the CO1 gene. Therefore it might not be a suitable marker to study the genealogical relationships of *P. flavescens*.

Phylogenetic analyses were conducted based on the concatenated mitochondrial markers. Overall resolution was poor resulting in a mostly unresolved tree. Nonetheless, the NJ tree comprises a distinct Easter Island clade, which indicates intermitted gene flow for a respective amount of time.

C. Conservation genetic assessments of the endangered damselfly *Lestes macrostigma*

(Studies within Chapter 3 and references therein)

The main findings of the local-scale genetic monitoring of *L. macrostigma* using CO1 sequence data of 107 individuals, were the highly fluctuating population sizes across the analysed years ranging from dozens to thousands of individuals. This leads again to fluctuation in genetic diversity parameters (2015: 6 haplotypes, 2016: 3 haplotypes, 2017: 4 haplotypes) and genetic bottlenecks. More specific, a genetic bottleneck event in 2016 was detected. The data show a compensation of diversity parameters in the following year (2017), though diversity parameters did not reached the values of the best year analysed (2015). Most individuals show a major shared haplotype, but four year-specific private haplotypes, from which two were observed in 2017, were detected. Abundance data was positively correlated with the amount of haplotypes detected per year. These results were associated with fluctuating precipitation and drought periods and were compared to populations covering the entire distribution range of this species and are discussed in a geographic context.

Based on the chosen sequence markers (two CO1 fragments and ITS) three genetically distinct groups can be detected: the Spanish population (1), France (including the island of Corsica) and Lesbos (2), and an “eastern” group consisting of populations from Austria, Hungary, Ukraine, Serbia, Georgia, Russia and Samos (3). Haplotype frequencies and tests for neutral evolution suggested that both the Spanish and French groups underwent at least one bottleneck event and genetic drift. This is consistent whith the findings of the local ten years monitoring data. Moreover, in the Spanish population only private haplotypes were discovered. Therefore strict genetic monitoring attempts and a special consideration of these groups as management units in future risk assessment programs is recommended. Overall gene flow estimates express very low connectivity (F_{ST}-values up to 0.885) and high degrees of genetic sub-structuring (82.97 % of the variation is explained among populations, $p<0.0001$), reflecting the restricted habitat of this brackish pond specialist.

D. General conclusion

The alarming results of current research on insect biodiversity and especially the decline of flying insects, the world most diverse animal group, call for new integrative strategies to measure, evaluate and protect the adaptive potential on the species and population level. Therefore not only undirected monitoring approaches but studies explaining the causal mechanisms behind the scene are demanded which take different adaptive strategies of the given species into account in order to establish optimally suited and tailored management plans.

Accordingly, the presented dissertation focusses on two odonate species with very different ecological niches and distributional dynamics leading to individual adaptive strategies. The cosmopolitan, abundant and migratory dragonfly *Pantala flavescens* displays hereby an ecological generalist. This species has an unique set of features to protect itself against local environmental changes. Due to their migratory behavior, individuals of this species are highly mobile and have the ability to physically escape unfavourable conditions immediately. They show the shortest larval development of all known odonate species, thereby being optimally adapted to temporary waters generated by heavy rains. Their ecological tolerance even enables larval development in artificial anthropogenic habitats such as swimming pools, garden ponds and fountains. All these features make *Pantala flavescens* the most widespread and most abundant dragonfly species in the world – which one would expect to be optimally adapted to survive even in times of the Anthropocen. The genetic studies initially agree with this and show, at first glance, high intraspecific diversity (up to 3.7 % for ITS, 1.4 % for ND1, 1.3 % for CO1 and 1.1 % for ND3). Nonetheless, the results show that some of the 27 populations studied globally, despite their high mobility and potential for intercontinental gene flow, undergo local adaptation processes. In particular, the Easter Island population is genetically depleted at mitochondrial level with only one haplotype on 89 animals (however, a single individual shows one additional haplotype). Since phenotypic differences and adapted behavioral strategies have been described locally, it should be obvious that this population has different conservational needs than continental populations. As the only dragonfly species on Easter Island, *P. flavescens* presumably holds an environmental key position and the particularity of this special population should not be overshadowed by the generally good

performance of this species in a global risk assessment. The phylogenetic analyses comprise a distinct Easter Island clade, which even indicates intermitted gene flow over an evolutionary significant period of time. Further, the discrepancy in mitochondrial and nuclear data indicates that the (genetic) consequences of this species' extraordinary migration potential are not as fully understood and more complex than Odonatologists believe.

The general assumption of experts, that *P. flavescens* displays a potential "winner" within this period of mass extinction is questioned by the results of this dissertation and maybe too superficial – at least on local levels.

In contrast to *P. flavescens*, the second target species, *L. macrostigma*, is an endangered, stenotopic damselfly adapted also to temporary but brackish ecosystems. The availability and distribution of this exclusive ecosystem is highly limited. Though being generally highly mobile organisms, *L. macrostigma* does not have the same high dispersal capacities as *P. flavescens*. The fragmented habitat structure and low connectivity is reflected by this species population genetics, displaying low genetic diversity measures (between 0 % for ITS, 0.589 % for the barcode CO1 fragment and up to 1.3 % for OdoCO1) and isolated populations (F_{ST}-values up to 0.885). Three genetically distinct groups were discovered: the Spanish population (1), France (including Corsica) and Lesbos (2), and an "eastern" group comprised of populations from Austria, Hungary, Ukraine, Serbia, Georgia, Russia and Samos (3). Two of these groups, namely France and Spain show significant negative Tajima´s D values indicating a recent bottleneck event. Further, the high amount of private haplotypes found here (for Spain 100 %, nine out of nine) is a signal for local adaptation processes. The monitoring data shows that local climate impacts the populations' abundance and its haplotype diversity. If adverse climate change effects occur on a local level, *L. macrostigma* populations have not the same chance to leave these conditions as *P. flavescens* could, due to their narrow ecological niche and scarcely distributed habitats. Therefore, fluctuations in precipitation and drought periods (which will increase in the Mediterranean area) have a high impact even on small spatial scales by affecting the limited number of suitable habitats within a larger region. If this would lead to a local extinction of a population, this would an irretrievable loss of genetic diversity in consequence, due to the unique genetic composition of French and Spanish

populations. Though expected to display a potential "loser" within this period of massive anthropogenic environmental change, the potential risk these two groups are facing was highly underestimated before the presented studies. Therefore these two groups, should be considered as distinct management units during next extinction risk assessments (IUCN Red Lists).

Table 5.D.1 compares the two studied species and sums up their relevant ecological and genetic features .

Table 5.D.1: Comparison of the two studied species *P. flavescens* and *L. macrostigma* and relevant ecological and genetic features

	P. flavescens	***L. macrostigma***
Adaptive strategy	habitat generalist	habitat specialist
Habitat choice	temporary waters	temporary, brackish waters
Dispersal capacity	High	low
population connectivity	unclear, high based on mtDNA low based on nDNA	low
Isolated populations	ves, on Easter Island	yes
Genetic diversity	high (total: 0.711 to 0.962 HD)	low to moderate (total: 0.153 to 0.772 HD)
Conservation status	least concern , but should be revised for the Easter Island population	should be revised for the Spanish and French population

Under the current circumstances of rapid environmental change, one can summarize that the adaptive strategy of *P. flavescens* is (as expected) in total more favourable to this species persistence. However, even this potential "winner" faces local isolation processes regardless its outstanding migratory potential and global dominance. This demonstrates once again the necessity of smaller scale and regionally tailored risk assessments in conservation management. This was done for *L. macrostigma* and revealed a higher risk of (local) extinction than expected by the respective authorities for this potential "loser". If the genetic substructuring is not taken into account in future management plans, this loser will likely get lost when climate change effects become more sensible.

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Curriculum vitae

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CONFERENCES & TEACHING

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Since 2012:	Teaching assistant for undergraduates/graduates in practical classes focusing on Molecular Biology and Conservation Genetics, as well as field work methods at the ecological field trip „Crau-Camargue-Mittelmeer". (TiHo Hannover, ITZ)

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List of publications

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9 Appendix

Chapter 2: The genetics of the cosmopolitan, migratory odonate *Pantala flavescens*

Table A. 1: Oligonucleotide primers used for PCR and sequencing of the whole mitogenome of *P. flavescens.*

Name	Locus	Sequence (5'-3') Primer 1	Sequence (5'-3') Primer 2
ND2	nad2	CCTGTGCTTCGTACACCAAA	ATTGCCTAAGTCTCGACCATC
ND2/COX1a	nad2 - cox1	CCAGCTATCACTTGATTAAT	TGGAAGAAACACCAGCTAAGT
COX 1a	cox1	AGATTTACAGTCTATTGCCTAAGTCTC	TGGAGTTCTAGGGATAATTTATGCTAT
COX1b	cox1	TGCTCAAGAGAGAGGTAAAAAGG	AGTGCCATGGGTTTAAGCTC
COX1a/COX1b	cox1	CACTTAGCTGGTGTTTCTTCCA	TTGTTGCTGATGTAAAATAGGCA
COX1b/COX 2	cox1 - cox2	GGCATTAGTGTCTCAACGTCA	TTTAATTGGGCCCACGTTGC
COX 2	cox2	CAGAAAAGTGCCATGGGTTT	AACGATCTACTCTCAATGAAGAAATT
COX 2/ATP8	cox2 - atp8	GCATCATTGAGTGGCTGAAAG	TGGTGCTATTTGTGGAATCAAGA
ATP8	atp8	AACGATCTACTCTCAATGAAGAAATTA	TTTTCAGTATTTGATCCATCAACA
ATP6	atp6	CAAATCTATTTTCAGTATTTGATCCAT	AGTAGATCAAAGCCCTTGACC
ATP6/ATP8	atp8 - atp6	CGGCCAGGATTATTCTTCGG	TGGTGCTATTTGAATCAAGA
COX3	cox3 - trnaG	AACCCTAGAATCTGCTGTATCAA	TTTTGGTAATTGTAACAACTGTCATT
ND3	nad3	TTATGGCCACAGGGTTTCAT	TCAAAATGGGGCTGCTAACT
ND3/ND5a	nad3 - nad5	ATTGCAATCAGTTTCGACCTG	GGATGATGTGAGTATTTTGGAGC
ND5a	nad5	TTTTAGCGGTTTAATTCCGTTT	GCACACATAAAAAGGAGAGCC
ND5b	nad5	CACCTGCACACATAAAAAGGA	TTGCATTATTGACACCACAAATC
NAD5a/NAD5b	nad5 - trnH	ACAAGCAGTTAAACCCCGTG	ACCTTGGTTACTGCTGGTGT
ND4	nad4	GAGGAAGAACCTCCCACTCA	TCCTAAACCTAACGCTCCTTCA
ND4l	nad4l	CAGAGGTAAAACATAATGGGATCA	TCTCTGCATCAGTCAAATTTTATCA
ND4l/ND6	nad4l - nad6	AAATGACCAACCTCTCAAAA	TTGGTCGTAAAGGTCCTTGAT
ND6	nad6	TCTGCATCAGTCAAATTTTATCAGT	AACCATTACGAATTCAACACCC
ND6/CYTB	nad6 - cob	AATCAAGGACCTTTACGACCAA	TTGAGGGTGTTGGAAGATCAA
CYTB	cob	TCAAGGACCTTTACGACCAA	TGGTGAATAAAAATCCCCTCC
CYTB/ND1	cob - nad1	TCCATTCCACCCTTATTTCTCCT	TGCGGGGTACACTTCCTC
ND1	nad1 - trnL1	CGACCTGTTGAAGACCCTTA	AAAATTGGAGATAGAAACCGACC
16S rRNAa	l-rRNA	TCGTTAAGCTGCAAGTACTATCTGT	TGTATTTGCCGAGTTCCTTATTT
16S rRNAb	l-rRNA	ACAGGCGGGAACTGTATTTG	TGAATCGCACAGTTAAATTTTCA
16S rRNAb/12S rRNA	l-rRNA - s-rRNA	ATGCTACCTTTGCACGGTCA	ATCGCCCGTCGCTCTTATTT
12S rRNA	s-rRNA	TCAAATTAAACTGAATCGCACAG	ATTAAACCGATTTTTCCCCTC
CR	A + T-rich (control) region - trnM	GCACGATTTTAACCGGAGAT	TCCTTATGAAACAGAATCTGCAA

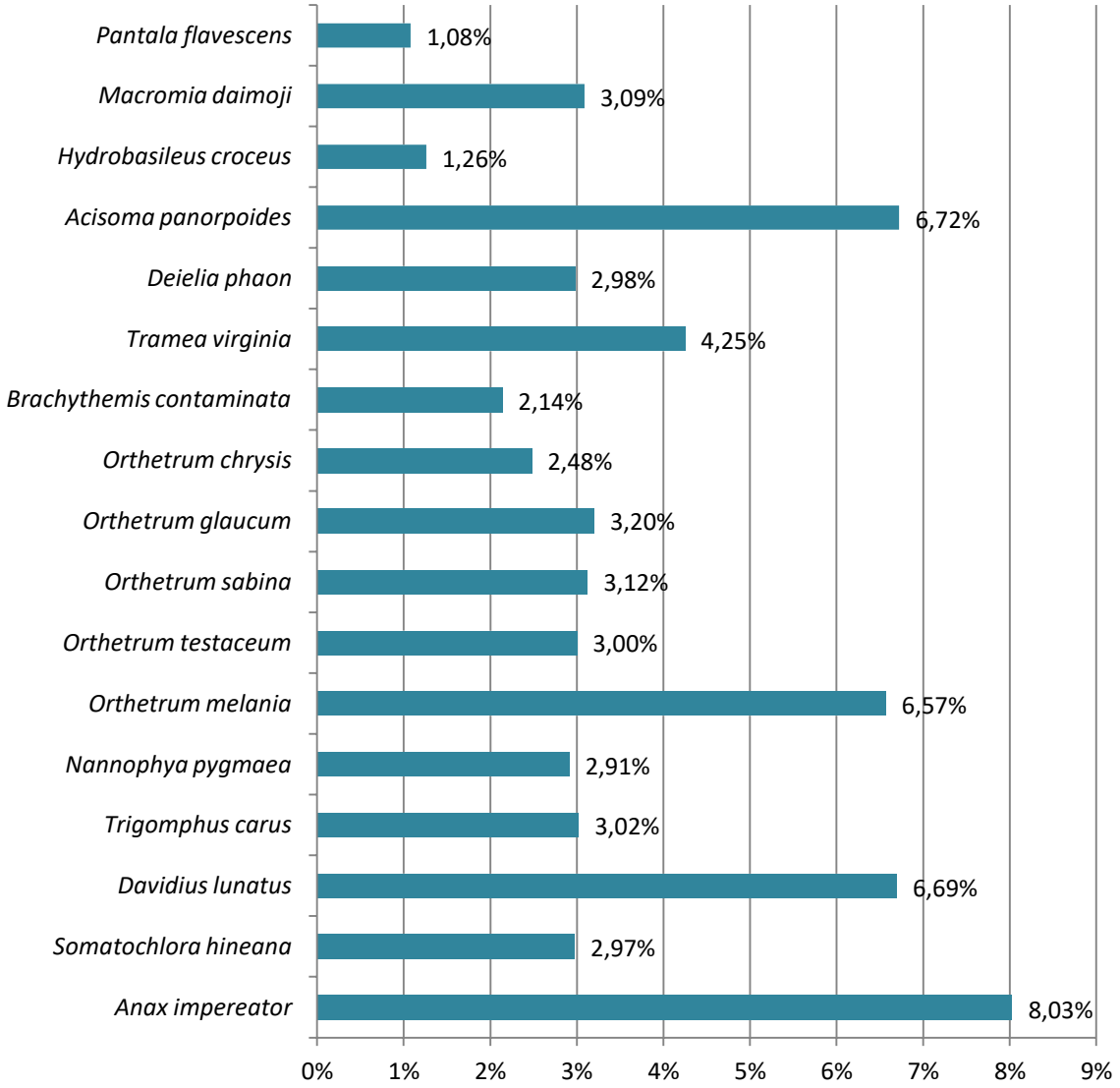

Figure A. 1: Control region length relative to the complete length of the mitochondrial genome for *P. flavescens* and 16 other anisopteran mitogenomes.

Table A. 2: Control region length for *P. flavescens* and 16 other anisopteran mitogenomes and the complete length of the mitochondrial genome of the respective species.

species	CR length (bp)	complete length (bp)	Ratio (%)
Anax impereator	1291	16087	8,03%
Somatochlora hineana	467	15705	2,97%
Davidius lunatus	1065	15913	6,69%
Trigomphus carus	457	15135	3,02%
Nannophya pygmaea	440	15112	2,91%
Orthetrum melania	1032	15715	6,57%
Orthetrum testaceum	455	15162	3,00%
Orthetrum sabina	474	15176	3,12%
Orthetrum glaucum	486	15184	3,20%
Orthetrum chrysis	374	15088	2,48%
Brachythemis contaminata	322	15056	2,14%
Tramea virginia	651	15321	4,25%
Deielia phaon	455	15281	2,98%
Acisoma panorpoides	1058	15742	6,72%
Hydrobasileus croceus	190	15088	1,26%
Macromia daimoji	469	15198	3,09%
Pantala flavescens	160	14842	1,08%

Table A. 3: Number of migrants (N_m) between 27 populations of *P. flavescens* calculated based on CO1 F_{ST}-values according to Wright´s formula.

Country	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 Australia																											
2 China																											
3 Easter Island																											
4 Ecuador																											
5 Kenya	2,294																										
6 Namibia			1,294																								
7 Tanzania		12,244			7,617	9,604																					
8 Brazil	1,606	11,615			2,526	6,388	3,396																				
9 Cambodia	1,432	10,881				16,562	1,861	0,912																			
10 Canada	1,000				7,817			3,503	2,333																		
11 Chile	3,542	2,640			1,493	2,785	4,293	2,910	0,836	22,131																	
12 Costa Rica					6,141	12,175	12,505	3,043	2,679		5,148																
13 Fiji					47,009			1,246		1,800	2,061																
14 French		1,598			2,264	8,404	1,957	1,822	0,777	1,641	1,477	2,598	0,769														
15 Guyana	22,770				7,062	25,576		9,299	1,470		6,150	65,195	6,737	6,307													
16 India		1,294			1,500	6,465	0,972	0,254		0,138	0,322	0,838	2,500	0,106	0,367												
17 Indonesia							5,073	0,800		0,762	0,764	4,570		0,486	1,299												
18 Japan					39,559	64,517		5,181	2,756		4,834			6,020		1,127											
19 Tonga	2,321	25,523			28,552	26,011	3,089	1,751	4,866		1,600	5,335	22,052	2,198	5,061	0,888	4,410	4,529									
20 South Korea								3,579	3,850	0,971	3,796			2,403		0,250			5,401								
21 Liberia								5,000		0,444	21,339			0,950		0,000											
22 Malaysia							16,472	4,560	23,765		2,586	9,358		7,140	22,091	21,913		35,567	53,283								
23 Maledives		4,806			3,633	3,633	6,017	1,429	1,423	3,938	2,287	28,752		1,073	2,857	0,743	5,938	21,246	1,598			3,011					
24 Northern								0,882	2,188	0,286				0,207	3,243	0,000			1,238		0,000						
25 Philippines	1,225	7,602					2,698	0,985		2,000	0,994	3,548		0,604	1,503			4,335	4,175	2,941			1,638	3,500			
26 Peru	3,484	2,880			2,636	2,636	3,571	3,872	1,066	30,053		3,593	2,345	1,542	3,838	0,525	1,163	3,345	1,454			2,517	2,224		1,398		
27 USA								5,646	1,598	14,310	3,624			6,079		0,437			3,493						1,845	4,337	

Table A. 4: Number of migrants (N_m) between nine populations of *P. flavescens* with more than 20 individuals calculated based on CO1 F_{ST}-values according to Wright´s formula. The different shades of blue correlate with the line width connecting populations pairs in the Figure 3.B.7.

Country	1	2	3	4	5	6	7	8	9
1 Easter Island									
2 Namibia	1,294								
3 Chile		2,785							
4 Costa Rica		12,175	5,148						
5 Guyana		25,576	6,150	65,195					
6 Japan		64,517	4,834	≥64,517	≥64,517				
7 Tonga		26,011	1,600	5,335	5,061	4,529			
8 Malaysia		≥64,517	2,586	9,358	22,091	35,567	53,283		
9 Peru		2,636	≥64,517	3,593	3,838	3,345	1,454	2,517	

< 2,0	2,0 - 5,0	5,0 - 15,0	15,0 - 40,0	>40,0

Table A. 5: Number of migrants (N_m) between nine populations of *P. flavescens* based on F_{ST}-values generated from nuclear ITS data according to Wright´s formula. These values are visualized in Figure 3.B.8.

	Australia	China	Easter Island	Ecuador	Kenya	Namibia	Tanzania
Australia							
China	0,333						
Easter Island	1.000	0,666					
Ecuador	0	0,082	0				
Kenya	0,295	0,109	0,612	0,357			
Namibia	0,276	0,142	0,619	0,511	0		
Tanzania	0,309	0,138	0,669	0,355	0	0	

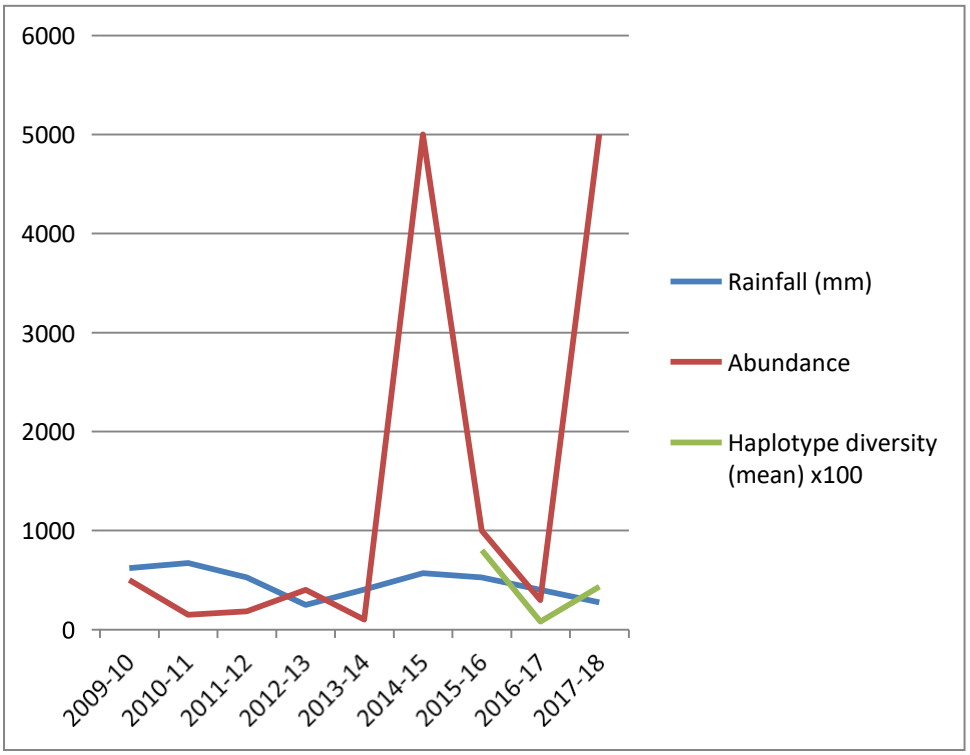

Figure A. 2: The cummulated amount of precipitation (mm) per year, the observed abundance of *L. macrostigma* and the detected mean haplotype diversity for the Camargue area (x100 for illustrational reason) over the analysed years.

Table A. 6: Precipitation data (mm) per month and per year for the Camargue study area

	Precipitation (mm) per month												
Year	January	February	March	April	May	June	July	August	September	October	November	December	Total
2009-10	85,2	106,2	32,4	140,3	32,5	25,3	2,4	4,4	83,4	87,5	23,3	86,8	709,7
3_winter	85,2	106,2	17,5									54,4	263,3
4_spring			14,9	140,3	32,5	25,1							212,8
1_summer						0,2	2,4	4,4	82,8				89,8
2_fall									0,6	87,5	23,3	32,4	143,8
2010-11	115	85,3	71,5	46,2	97,9	74,6	5,2	17,7	38,6	84,2	53,9	36,4	726,5
3_winter	115	85,3	52,1									25	277,4
4_spring			19,4	46,2	97,9	74,2							237,7
1_summer						0,4	5,2	17,7	32,4				55,7
2_fall									6,2	84,2	53,9	11,4	155,7
2011-12	32,4	22,5	110,6	12,4	2,2	39,7	55,8	4,4	31,8	51,2	247,4	2,4	612,8
3_winter	32,4	22,5	74,1									0,2	129,2
4_spring			36,5	12,4	2,2	38,5							89,6
1_summer						1,2	55,8	4,4	26,5				87,9
2_fall									5,3	51,2	247,4	2,2	306,1
2012-13	9,4	0,4	8	44,8	35,6	26,7	15,1	25,8	46,5	33,2	33,8	12,6	291,9
3_winter	9,4	0,4	3									5,6	18,4
4_spring			5	44,8	35,6	25,9							111,3
1_summer						0,8	15,1	25,8	1				42,7
2_fall									45,5	33,2	33,8	7	119,5
2013-14	29,1	8,6	59,7	73,5	54	14,1	16,6	8,5	11,9	37,8	69,7	57,1	440,6
3_winter	29,1	8,6	51,9									10,7	100,3
4_spring			7,8	73,5	54	14,1							149,4
1_summer						0	16,6	8,5	10,7				35,8
2_fall									1,2	37,8	69,7	46,4	155,1

2014-15	103,3	69,7	26,4	19,8	23,2	28,1	27,5	85,9	60,3	16	190,6	54,8	705,6
3_winter	103,3	69,7	16									0	189
4_spring			10,4	19,8	23,2	12,1							65,5
1_summer						16	27,5	85,9	9,1				138,5
2_fall									51,2	16	190,6	54,8	312,6
2015-16	52,1	60,8	67,2	113,8	1,6	82,4	7,6	148,8	42,6	104,1	22,1	20,4	723,5
3_winter	52,1	60,8	45,7									4,8	163,4
4_spring			21,5	113,8	1,6	82,2							219,1
1_summer						0,2	7,6	148,8	42,2				198,8
2_fall									0,4	104,1	22,1	15,6	142,2
2016-17	23	75,4	43,8	24	40,2	9,6	6	3,2	28	99,8	64	21,4	438,4
3_winter	23	75,4	40,8									4,6	143,8
4_spring			3	24	40,2	9,6							76,8
1_summer						0	6	3,2	27,6				36,8
2_fall									0,4	99,8	64	16,8	181
2017-18	31	13,6	44,2	63,5	28,3	26,6	2	12,5	11,8	2,2	61,4	15,8	312,9
3_winter	31	13,6	12,6									4,2	61,4
4_spring			31,6	63,5	28,3	3,6							127
1_summer						23	2	12,5	2,4				39,9
2_fall									9,4	2,2	61,4	11,6	84,6
2018-19	93,1	65,6	51,1	66,7	46,4	12,6	21,8	56,5	34	244,7	138,9	16,8	848,2
3_winter	93,1	65,6	51,1									0,4	210,2
4_spring			0	66,7	46,4	12							125,1
1_summer						0,6	21,8	56,5	33,4				112,3
2_fall									0,6	244,7	138,9	16,4	400,6
Total général	573,6	508,1	514,9	605	361,9	339,7	160	367,7	388,9	760,7	905,1	324,5	5810,1

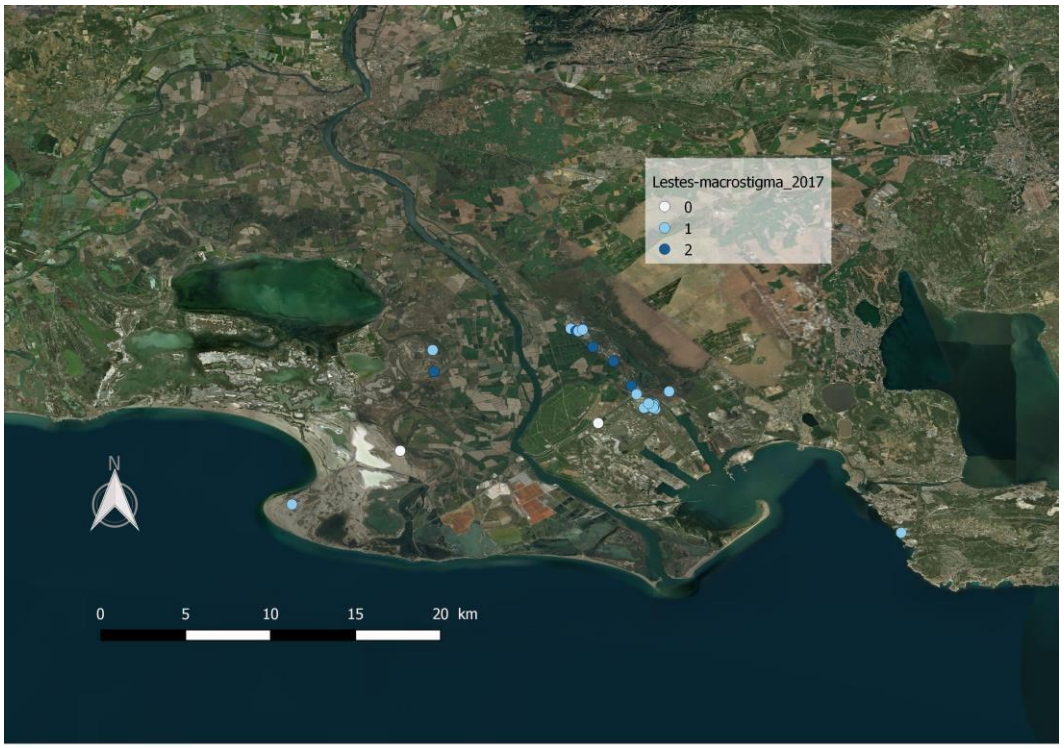

Figure A 3: Abundance and distribution of *L. macrostigma* populations within the Camargue study area for the year 2017.

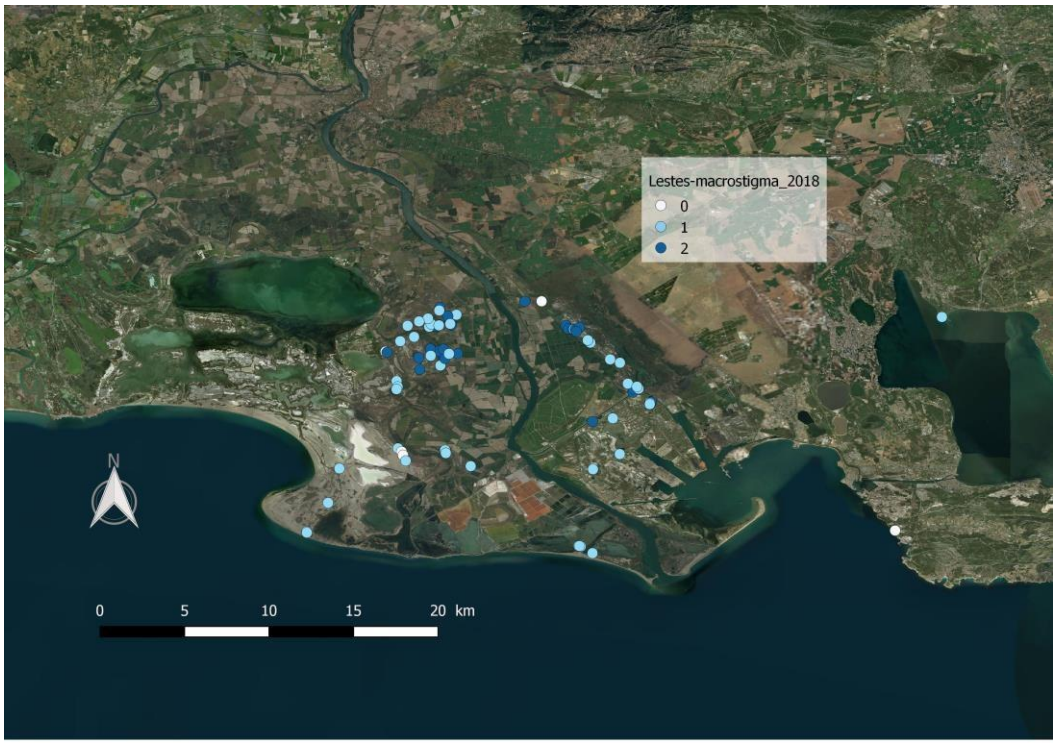

Figure A 4: Abundance and distribution of *L. macrostigma* populations within the Camargue study area for the year 2018.

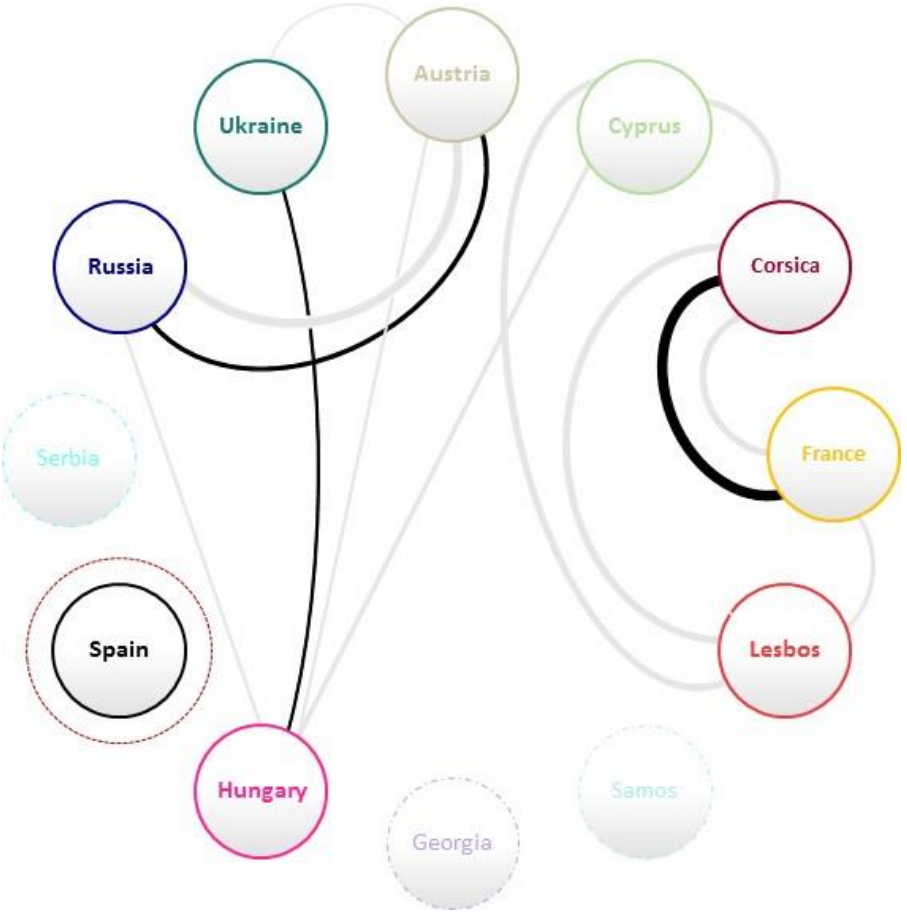

Figure A 5: Number of migrants (N_m) between 12 populations of *L. macrostigma* calculated based on CO1 F_{ST}-values (grey: HCO LCO C01; black: OdoCo1 data) according to Wright´s formula. The different line widths connecting populations pairs represent the number of migrants per generation.