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Molecular Phylogeny and Phylogeography of Reed Warblers and Allies (Aves: Acrocephalidae)

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Zusammenfassung

Rohrsänger und Artverwandte (Familie Acrocephalidae) blicken auf eine lange Geschichte der Klassifikation und kontinuierlichen Revision zurück. Aufgrund einer beachtlichen Ähnlichkeit in der Morphologie und schnelle Radiation konnten frühere Studien nicht alle phyloge netischen Beziehungen zwischen und innerhalb der Gattungen lösen . In dieser Studie wurden DNA-Sequenzen von acht mtDNA und Kerngenen (insgesamt, 6.280 nt) sowie die Analysen des genomischen ISSR-Fingerprintings verwendet, um die Verwandtschaftsverhältnisse unter den 35 Arten von Rohrsängern zu rekonstruieren . Die Ergebnisse sind kongruent mit früheren molekular genetischen Analysen (Leisler *et al*, 1997; Helbig und Seibold, 1999; Fregin *et al*, 2009), sie stellen jedoch einige der Schwesterbeziehungen deutlicher heraus als zuvor. Basierend auf den Analysen der Hauptklade von *Acrocephalus*, *Iduna* (außer *I. aedon*) wurden *Hippolais*, *Nesillas* und *Calamonastides* bestätigt. Die gegenwärtige Situation der Taxonomie von *Calamonastides gracilirostris* als eine monotypische Gattung wurde durch Phylogenierekosntruktion aufgezeigt. Obwohl die Integration von *Chloropeta natalensis* und *Chloropeta similis* in die Gattung *Iduna* nicht vollständig bewiesen werden konnte , kann eine enge Beziehung dennoch nicht ausgeschlossen werden. Es konnte im Gegensatz zur Vermutung von Fregin *et al*. (2009) kein Beleg für die Aufnahme von *aedon* in die Gattung *Iduna* gefunden werden; aus diesem Grund wird die Wiedereinführung der Gattung *Phragamaticola* für diese Spezies vorgeschlagen. Eine molekulare Uhr Analyse bestätigt die Hypothese von der schnellen Radiation der Familie im mittleren Miozän (12,5 Millionen Jahre). Diese kurze Evolutionszeit verursachte kurze Knoten an der Basis der Verzweigungen.

Darüber hin aus wurden phylogeographische Muster sowie die Evolutionsgeschichte des eurasischen Teichrohrsänger s (*Acrocephalus scirpaceus*) und Sumpfrohrsänger s (*Acrocephalus palustris*) untersucht. Zwei mtDNA Loci (*cyt b* und *COI*), eine Kombination von phylogeographischen Softwareprogrammen , molekulare Datierung und populationsgenetische Methoden wurden angewendet , um diverse Fragen über die genetische Vielfalt dieser beiden Langstreckenzieher aus der Familie der Rohrsänger in Bezug auf ihre geografische Verbreitung zu beantworten.

Bei der Untersuchung von Unterschiede in der mtDNA von mehr als 400 Individuen von *A. scirpaceus* aus 20 unterschiedlichen Orten konnten drei Abstammungslinien identifiziert werden. Diese entsprechen den drei Unterarten und teilten sich vor 0,4 Millionen Jahren wie folgt auf : eine über Asien (*A. s. fuscus*); eine über Europa und Nordafrika (*A. s. scirpaceus*); und eine über Ost - Afrika und Süd -West-Asien (*A. s. avicenniae*). Die Ergebnisse aus BEAST zeigen, dass die dritte Klade eine basale Position einnimmt und von ihren Schwesterarten, den afrikanischen Rohrsängern *A. baeticatus*, vor ca. 0,6 Millionen getrennt wurde. Diese ursprüngliche Unterart (avicenniae) könnte in einer der afrikanischen Refugien, wahrscheinlich weniger bewaldete Refugien des äthiopischen Hochlandes, während des letzten glazialen Maximums überlebt haben . Darüber hinaus konnte durch die DNA -Analyse eine hohe Anzahl von Identifikationsfehlern zwischen Rohrsängern bei der Feldidentifikation festgestellt werden. Insgesamt wurden 6,8% der eurasischen Rohrsänger von den Beringern falsch identifiziert und dadurch der falschen Art zugeordnet. *A. palustris* war die am meisten falsch identifiziert Spezies; auf der Ebene der Subspezies, 72,7 % der Proben wurden falsch identifiziert. Basierend auf der molekularen Analyse konnten wir erstmals die asiatische Unterart *A. s. fuscus* in Mitteleuropa (Treysa, Deutschland) nachweisen.

Im Gegensatz zu den Ergebnissen von *A. scirpaceus* wiesendie phylogeographische Struktur von ca. 230 Individuen aus 10 Brut-, Zug- und Überwinterungspopulationen von *A. palustris*, nur eine schwache Divergenz auf. Die Analysen auf der Grundlage von zwei mtDNA Loci konnten keine Subpopulationen feststellen und zeigten eine Besetzung eines einzigen Gletscherrefugiums während des Pleistozäns. Einr hoher Ausmaß von Genfluss zwischen Brut- Populationen (Nm = 13,69) ist ein weiterer Faktor , der zu einem hohen Grad an Vermischung führt . Geringe Unterschiede der Nukleotide, ein kaum entwickelter phylogenetischer Baum, ein sternförmiges Haplotypen- Netzwerk, eine unimodal Mismatch Verteilung und die Zeit bis zum letzten gemeinsamen Vorfahren (TMRCA = 0,45 MYA) sind alle jüngeren Ursprungs und gehen auf die letzten Eiszeiten zurück.

Summary

Reed warblers and allies (family Acrocephalidae) present a long history of classification and successive revisions. Due to a remarkable similarity in morphology and rapid radiation, the previous studies failed to solve all phylogenetic relationships between and within the genera. In this study DNA sequences from eight mitochondrial and nuclear loci (total, 6280 nt) as well as genomic ISSR fingerprinting were implemented to reconstruct the phylogenetic relationships among 35 species of reed warblers. The results are congruent with previous molecular analyses (Fregin *et al.* 2009; Helbig & Seibold 1999; Leisler *et al.* 1997) but support some of the sister-group relationships more strongly. Based on the analyses, the major clades of *Acrocephalus*, *Iduna* (except *I. aedon*), *Hippolais*, *Nesillas* and *Calamonastides* were recovered. The current taxonomic position of *Calamonastides gracilirostris* as a monotypic genus in the tree was supported. Although the inclusion of *Chloropeta natalensis* and *Chloropeta similis* in genus *Iduna* was not robustly supported, their close relationship could not be rejected. No support was found for inclusion of *aedon* in genus *Iduna* as suggested by Fregin *et al*. (2009), hence the resurrection of genus *Phragamaticola* for this species is proposed. A molecular clock analysis confirmed the hypothesis of the rapid radiation of the family from middle Miocene (12.5 MYA). This short evolutionary time causes low nodal support at the base of the some clades.

Furthermore, phylogeographic patterns and evolutionary history of the Eurasian reed warbler (*Acrocephalus scirpaceus*) and marsh warbler (*Acrocephalus palustris*) were investigated. Two mitochondrial loci (*cyt b* and *COI*) and a combination of phylogeographic tools, molecular dating, and population genetic methods were employed to address several questions regarding the genetic diversity of these two long-distance migratory reed warblers across their widespread geographic distribution range.

Investigation of mtDNA diversity among more than 400 individuals of *A. scirpaceus* from 20 sampling sites recovered three lineages. They correspond to three subspecies and split about 0.4 million years ago: one spanning Asia (*A. s. fuscus*); one encompassing Europe and Northern Africa (*A. s. scirpaceus*); and a third, including Eastern Africa and South-western Asia (*A. s. avicenniae*). Results from BEAST further suggested that the third clade has the basal position and diverged from its sister species African reed warbler *A. baeticatus* ca. 0.6 million years ago. This primitive subspecies (*avicenniae*) may have survived in one of the African refugia, likely low forest refugium of the Ethiopian Highlands during the Last Glacial Maximum. Moreover, DNA analysis could detect high number of identification errors between reed warblers in the field. In total, 6.8% of Eurasian reed warblers were misidentified as the wrong species. *A. palustris* was the most misidentified species; and at the subspecies level, it increased to 72.7% of the specimens. Based on the molecular analysis we could also report a first evidence of the Asian subspecies *A. s. fuscus* in Central Europe (Treysa, Germany).

In contrast to the results of *A. scirpaceus*, the phylogeographic structure in ca. 230 individuals from 10 breeding, migrating and wintering populations of *A. palustris* presented a shallow divergence. The analyses based on both mtDNA loci failed to detect any population subdivision and indicate occupation of a single glacial refugium during the Pleistocene glaciation. High levels of gene flow among breeding populations (Nm = 13.69) is another factor which leads to high degree of admixture. Low nucleotide diversity, shallow phylogenetic tree, star-like haplotype network, unimodal mismatch distribution and the time to the most recent common ancestor (TMRCA = 0.45 MYA) all point to their recent origin during the last glacial periods.

Abbreviations

AFLP	Amplified Fragment Length Polymorphism
BI	Bayesian inference
nt	nucleotides
COI	cytochrome oxidase 1
cpDNA	Chloroplast DNA
cyt b	cytochrome *b*
DNA	deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FIB5	beta fibrinogen intron 5
GTR	general time reversible
IOC	International Ornithological Congress
ISSR	Inter-Simple Sequence Repeats
LDH	Lactate Dehydrogenase
ML	Maximum Likelihood
MP	Maximum Parsimony
mtDNA	mitochondrial DNA
MYA	Million Years Ago
MYO	Myoglobin
ND2	NADH dehydrogenase subunit 2
ODC	ornithine decarboxylase
PCR	polymerase chain reaction
PP	posterior probability
RAD	Restriction site Associated DNA
RAG-1	Recombination activating gene 1
RAPD	Random Amplified Polymorphic DNA
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
ssp	subspecies
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats
Taq	*Thermus aquaticus*
TEMED	Tetramethylendiamine
VNTR	Variable Number Tandem Repeat

Publications

This thesis is based on the following manuscripts:

Arbabi, T., Gonzalez, J., Wink, M. A re-evaluation of phylogenetic relationships within Reed Warblers (Aves: Acrocephalidae) based on eight molecular loci and ISSR profiles. Molecular Phylogenetic and Evolution, DOI: http://dx.doi.org/10.1016/j.ympev.2014.05.026.

Arbabi, T., Gonzalez, J., Witt, H-H., Klein, R., Wink, M. Mitochondrial phylogeography of the Eurasian Reed Warbler (*Acrocephalus scirpaceus*) and a first genetic report of *A. s. fuscus* in Central Europe. Ibis, DOI: 10.1111/ibi.12174.

Arbabi, T., Gonzalez, J., Wink, M. Mitochondrial evidence for genetic diversity and low phylogeographic differentiation in the Marsh Warbler *Acrocephalus palustris* (Aves: Acrocephalidae). Organisms Diversity & Evolution, DOI: 10.1007/s13127-014-0177-3.

1 Introduction

1.1 General introduction

1.1.1 Avian taxonomy

The earliest known comprehensive study and classification of birds dates from the writings of Aristotle (384–322 BC). Aristotle spent several years travelling and wrote his observations in the book 'On the history of animals'. In this book he categorized 140 kinds of birds in five groups of: 1. birds of prey; 2. swimming birds; 3. pigeons and doves; 4. swifts, swallows and martins; 5. songbirds. He described in detail the external appearance, anatomy, behaviour, distribution and biology of the birds. His works greatly influenced his successors and followers and was the most important source of information for the next 1500 years (Wink 2014). Carl Linnaeus, the father of modern taxonomy, developed the Latin binomial system in 1758. Linnaeus like Aristotle classified organisms according to their structural similarities. The classification systems of both Aristotle and Linnaeus started with the same two groups of plants and animals. Linnaeus called these groups 'kingdoms' and divided them into five levels of class, order, genus, species, and variety. Later the classification modified according to describing the evolutionary relationships, originated from the influence of the evolutionary ideas of Charles Darwin (1809-1882). For the first time, skull was used for determining common inherited features of birds by Thomas Henry Huxley (1825–1895) in his paper 'On the classification of birds' (Huxley 1867).

The new method of molecular biology changed the study of avian systematic from being based on phenotype to genotype. The DNA-DNA hybridization was the first DNA based taxonomy started with the work of Charles G. Sibley and Jon E. Ahlquist in 1975, while they were developing techniques for comparing avian proteins by electrophoresis as a way to understand the genetics of hybrid populations. With further developments and improvements in techniques, they provided new evidence in classification of birds called 'Sibley-Ahlquist taxonomy' (Sibley *et al.* 1988; Sibley & Monroe 1990). Nowadays DNA hybridization has been replaced by DNA sequences. Developing the new molecular techniques of polymerase chain reaction (PCR) by Kary Mullis (Mullis & Faloona 1987), development of techniques for comparing the sequence data of mitochondrial and nuclear DNA and improving the knowledge of analytical methods (Fig. 1.1) offer more consistent results and prepare the possibility of constructing phylogenetic trees (e.g. in Fig. 1.2) for showing more precisely the evolutionary relationships among taxa.

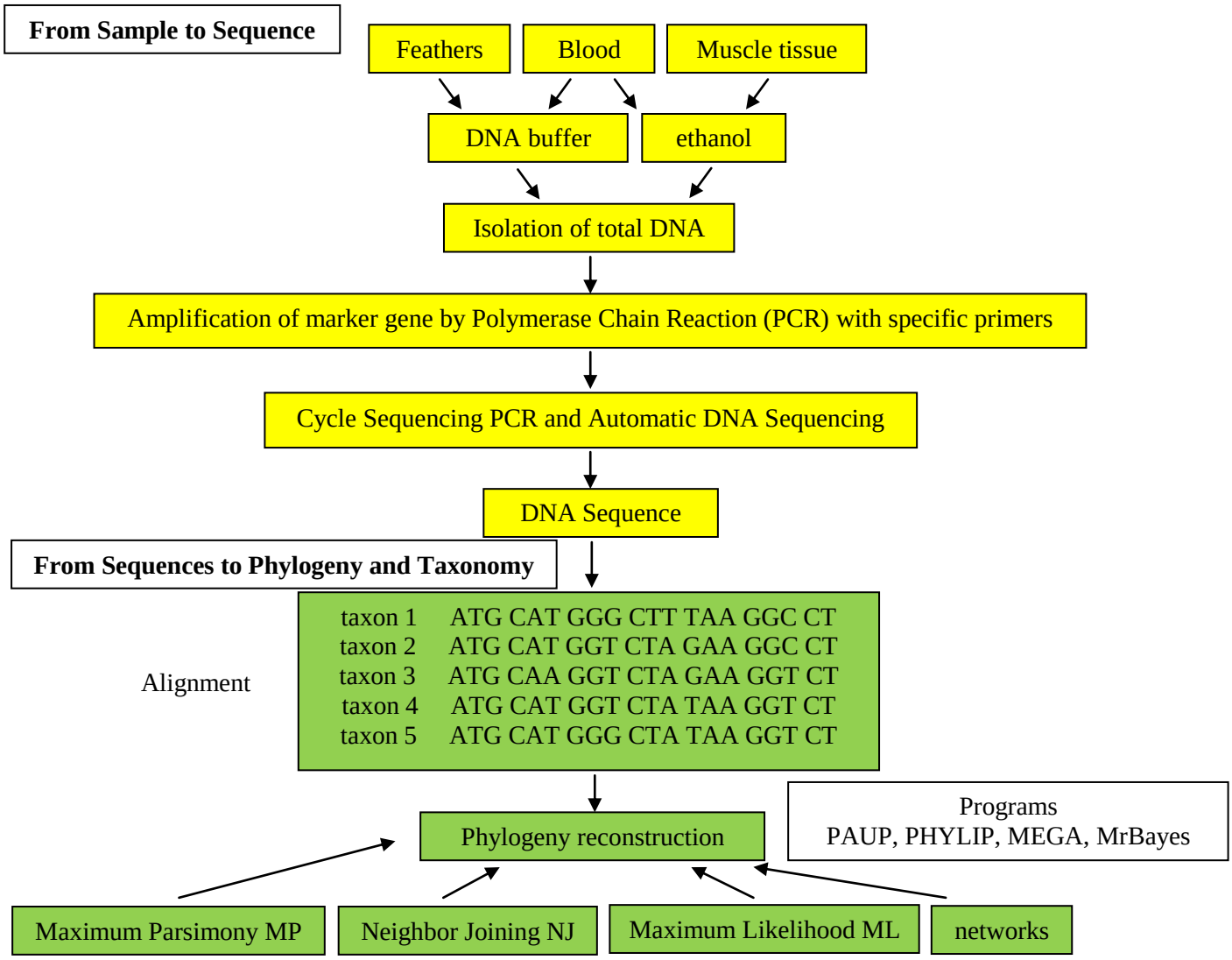

Fig. 1.1 From sample to DNA sequence and phylogeny (Storch *et al.* 2013; Wink 2006).

1.1.2 Phylogeny and phylogeography

Molecular phylogenetic study the evolutionary relationships between taxa by analyzing the changes occurring in DNA sequences (Wink 2007). However, when the different lineages diverged in a short time or when the studied markers contain insufficient information, phylogenetic relationships will remain unresolved. Obtaining more independent data such as different mitochondrial and nuclear loci can be a solution (Wink 1998). Independence is essential to resolve the phylogenetic tree. Linked loci, e.g. using only mitochondrial genes which have the same history, cannot reveal a comprehensive history (Avise & Ollenberg 1997). Collecting more individuals per species is the other solution but not always taking sample especially in avian species is easy.

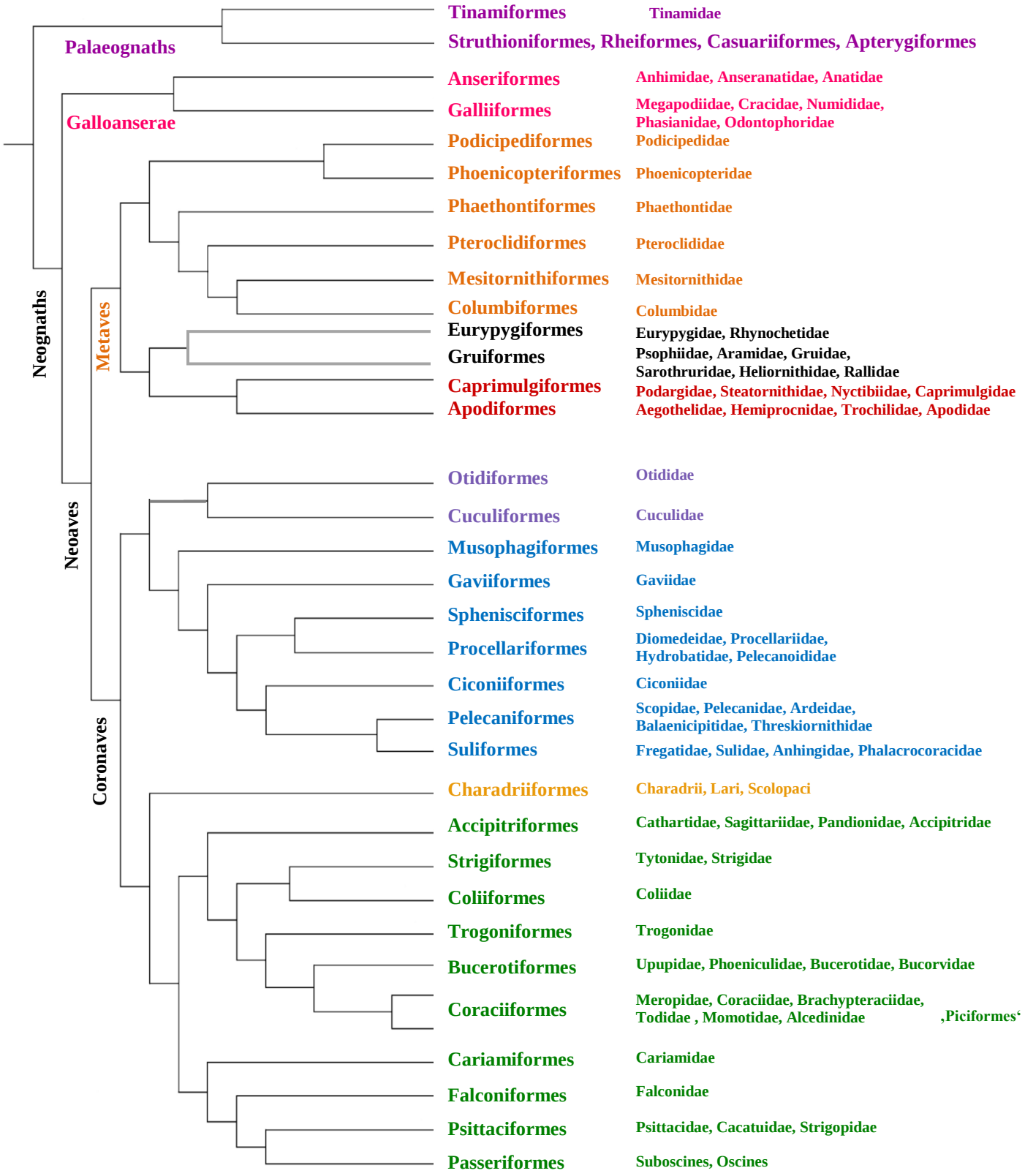

Fig. 1.2 Systematics and classification of birds (Wink 2014).

The term phylogeography was first introduced by Avise *et al*. (1987). Phylogeography study the relationships among individuals from multiple populations or geographical regions belonging to the population of a taxon, in order to find the population structure, Historical processes, demography, and degree of gene flow among breeding populations (Avise *et al.* 1987). The present patterns of diversity has emerged by both past evolutionary history and current population processes. For instance, low genetic diversity among the populations of a species can be due to a recent common ancestry or high contemporary gene flow. One of the most important factors in history affecting the structure of the present diversity between populations is the ice ages. During the last 2.5 million years (Fig. 1.3), the climatic fluctuations of the Pleistocene glaciations have forced species to adjust the distributional areas according to their adaptation capabilities. During cold periods, species moved to the southern refugia and during milder periods migrated northwards or expanded their distributional ranges without leaving the refugia (Blondel & Mourer-Chauviré 1998; Roy *et al.* 1996). Low levels of current diversification in the avian species of Northern Hemisphere have been explained as a result of population mixing during the Quaternary climatic fluctuations (Blondel & Mourer-Chauviré 1998; Klicka & Zink 1997; Webb & Bartlein 1992). Migration involves the exchange or transfer of genes and alleles in a population. The pattern of low genetic structure is expected for the avian species with high dispersal capacities such as long-distance migrants, for which geographical distances or landscape obstructions do not represent significant barriers to gene flow and have a homogenizing effect on the population structure (Grinnell 1922; Helbig 2003). Molecular studies can quantify genetic variation between the populations by measuring the number of haplotypes (*k*), haplotype diversity (*h*) and nucleotide diversity (π). Haplotype diversity is the frequency of haplotypes within a sample, in contrast, nucleotide diversity accounts for relationships among haplotypes (Nei 1987; Nei & Tajima 1981).

1.1.3 Molecular markers

There are numerous molecular techniques which allow the study of systematic of birds in more detail and precision than before (Wink 1998). Depending on the level of relatedness ('evolutionary window'), different molecular markers can be used to characterize and identify the individuals, populations, species or phylogenetic relationships (Table 1.1).

Avian mitochondrial DNA (Fig. 1.4) in various species contains different length from 16.6–16.8 kb (Zink & Dittmann 1991). It is haploid and uniparentally inherited, for exceptions see Zouros et al. (1994), and thus provides information only from the matrilineal

lineage compared to diploid nuclear genes with biparental transmission. Mitochondrial genes are inherited as one linkage group in the absence of recombination (Hayashi *et al.* 1985; Hoech *et al.* 1991; Zouros *et al.* 1994), therefore is less discriminating than nuclear DNA and all members of a maternal line have the same sequence of mtDNA. In mitochondrial genes, mutations occur frequently, on average 10–20 times faster than their nuclear parts (Firth *et al.* 2005), therefore these genes are suitable for resolving young evolutionary relationships whereas older relationships like ancestor nodes are better resolved with more conservative genes like nuclear loci (Lin & Danforth 2004; Zhang & Hewitt 1996); ideally, a combination of them is the best solution for solving the problems of a phylogenetic tree (Wink 1996; Wink *et al.* 1993). Even, when it is possible, combining the data with the other markers like DNA fingerprinting, will provide more possibility for investigating the genetic diversity (Wink 1994; Wink *et al.* 1990). Several DNA fingerprinting techniques are available for evaluating the genetic relationships (Wink *et al.* 1999). The most important of them are microsatellites / SSR: Simple Sequence Repeats / STR: Short Tandem Repeats (Tautz 1989; Wink & Dyrcz 1999), RAPD: Random Amplified Polymorphic DNA (Williams *et al.* 1990), RFLP: Restriction Fragment Length Polymorphism (Saiki *et al.* 1985), AFLP: Amplified Fragment Length Polymorphism (Vos *et al.* 1995) and ISSR: Inter Simple Sequence Repeat (Wink *et al.* 1998; Zietkiewciz *et al.* 1994). Main limitations of these techniques are low reproducibility of RAPD, high cost of AFLP and difficulties of designing primers for SSR. ISSR markers, which developed in our laboratory (Wink *et al.* 1998), overcome most of these limitations. They have advantages of low cost, high polymorphism and good reproducibility (Reddy *et al.* 2002). ISSR is based on inter tandem repeats of short DNA sequences. Due to the lack of evolutionary functional constraints in the non-functioning regions, these inter repeats are highly polymorphic in their sizes even among closely related genotypes (Wink 1998). The advantages and disadvantages of each technique have presented in Table 1.2.

Table 1.1 The most important methods of studying molecular evolution (Storch *et al.* 2013). For abbreviations see page vii.

method		DNA regions	question
DNA sequencing			
	sequence analysis	marker gene: mtDNA, cpDNA, nuclear gene, introns	phylogeny, taxonomy, phylogeography
	SNP-analysis	point mutations in any DNA regions	population genetics, identification of individuals, parentage analysis, phylogeography
	sequence analysis *next generation sequencing*	entire genome, exome or transcriptome	phylogeny, genome evolution, functional genomics
DNA fragment length analysis (fingerprints) mostly repetitive DNA			
	microsatellite analysis (*single locus*)	microsatellites (STR) of the nuclear genome	population genetics, identification of individuals, parentage analysis
	RFLP analysis (*multilocus*)	especial for nuclear genome	population genetics, gene mapping, hybridization
	ISSR analysis (*multilocus*)	especial for nuclear genome	phylogeny, population genetics, gene mapping, hybridization
	RAD marker (*restriction site associated DNA*)	especial for nuclear genome	gene mapping, population genetics, identification of individuals, parentage analysis
	DNA fingerprinting (*multilocus*)	satellite DNA (VNTR, STR)	parentage analysis, identification of individuals
	sexing (*single locus*)	sex chromosome	molecular sexing

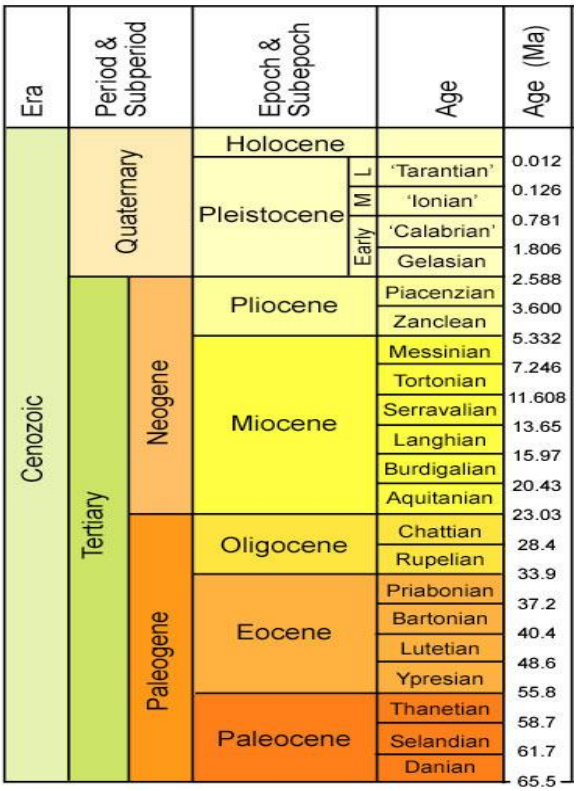

Fig. 1.3 The timescale for the Cenozoic Era (Cita *et al.* 2006).

Table 1.2 List of the most commonly used techniques in genetic studies (Kumar *et al.* 2009; Miah *et al.* 2013). (+) shows advantages and (-) shows disadvantages of the technique.

Technique	Advantages (+) / disadvantages (-)
RFLP	+ Species identification is possible + DNA sequence information is not necessary + Co-dominant - Requires large amounts of DNA template and high quality DNA - Cannot distinguish mixed DNA samples - Cannot distinguish between closely related species - Expensive - Time consuming
RAPD	+ Species identification is possible + Cheap + Fast + DNA sequence information is not necessary - Dominant - Reproducibility is a problem - Cannot distinguish mixed DNA samples - Variation of the same species occurs between different samples - DNA fingerprinting relies on quality and quantity of the sample - PCR condition and efficiency of primers affect band patterns
AFLP	+ Species identification is possible + DNA sequence information is not necessary + High polymorphism + Works with smaller RFLP fragments - Not reproducible - Needs very good primers - Cannot distinguish between closely related species
Microsatellites	+ Reproducibility + High polymorphism + Co-dominant - Difficulties of designing primers - Previous genetic information is necessary - Expensive
ISSR	+ Cheap + Reproducibility + High polymorphism + Species identification possible + Needs single primer - Many informative bands per reaction - Non-homology of similar sized fragments

Technique	Advantages (+) / disadvantages (-)
Sequencing	+ Species identification is possible + High specificity + High sensitivity + Reproducibility + Can analyze a wide range of species using universal primer - DNA sequence information is not necessary for primer design - Cannot separate mixtures

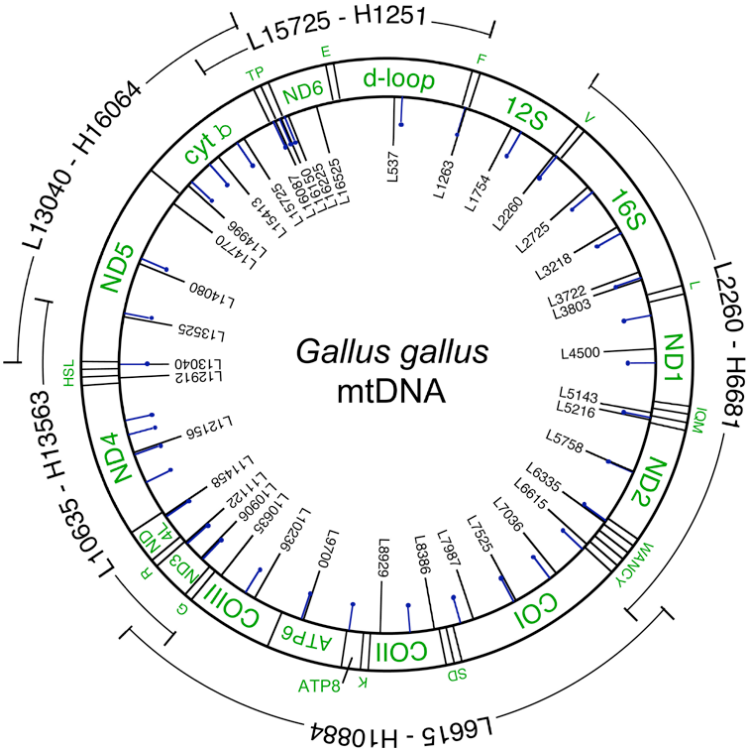

Fig. 1.4 Schematic diagram of the avian mitochondrial genome (Sorenson 2003).

1.1.4 Phylogenetic tree

Phylogenetic tree is a diagram which is composed of branches and nodes to illustrate the rate and pattern of changes occurring in DNA sequences, in order to reconstruct the evolutionary relationships between organisms. There are several methods for calculating phylogenetic trees (Wink 2006) but they can be classified into two main groups: distance methods and character methods. In distance methods, like Neighbor-Joining and UPGMA, the estimated genetic distance between pairs of taxa reflects the degree of relatedness. In contrast, character methods use the sequences directly rather than on pairwise distances. The most used distance models are Maximum Likelihood, Maximum Parsimony and Bayesian inference (Hall 2007).

For constructing a phylogenetic tree, evolutionary models are needed. Some of the most common evolutionary models are: (a) Tajima-Nei: calculating unequal rates of substitution between nucleotide pairs (Tajima & Nei 1984); (b) Tamura-Nei: using the base composition and the transition or transversion rate (Tamura & Nei 1993); (c) Kimura 2-parameter: calculating the branch lengths at two different rates based on the probability of transition and transversion mutations (Kimura 1980); (d) Hasegawa-Kishino-Yano (HKY): uses the different rate of transitions and transversions as well as unequal frequencies of the four nucleotides (Hasegawa *et al.* 1985) and (e) General Time Reversible (GTR): calculating the substitution rates to differ between each pair of nucleotides, in order to estimate the time of divergence and substitution rates between the sequence data (Tavaré 1986). JModelTest (Posada 2008) and Modeltest/MrModeltest (Posada & Crandall 1998) are the most popular programs for finding the best evolutionary model for the dataset.

1.1.5 Molecular clocks

Molecular dating is a technique in molecular biology that uses substitution rates of nucleotide or amino acid sequences to estimate the divergence time of taxa. The hypothesis of molecular clock was first introduced in the 1960s (Zuckerkandl & Pauling 1965). Time of diversification is an important key to understand the evolution of the species (Weir 2006), it is also using for estimation of the population demographic history (Hollatz *et al.* 2011; Vilaça & Santos 2010). The accuracy of the molecular clock strongly depends upon having an accurate calibration point. There are three common methods for calibrating the molecular clock. Fossils are one type of the sources to estimate substitution rates. They estimate the age of organisms using radioactive dating (Li & Makova 2001). However, due to limitations in the fossil record especially in Passeriformes, because of having small-bodied forms (Smith & Peterson 2002), other techniques like biogeological events are used to calibrate molecular clocks. Formation of islands, mountains and land bridges frequently used to infer rates of nucleotide sequence divergence. Formation of a mountain range, which split the geographic range of a species, provides the process of speciation. The formation of the Isthmus of Panama, which isolated the tropical western Atlantic and eastern Pacific oceans, is one of these important geological events (Weir *et al.* 2009). However, precise dating for dispersal events linked to most geological changes are still not clear (Wilke *et al.* 2009). Genetic approaches are the new techniques for estimating the time of divergence. Comparison of nucleotide data indicates the changes occur at predictable rates (Hedges & Kumar 2003; Li & Makova 2001). Assuming a consistent rate of change in DNA sequences or amino acids over

time allows their use as molecular clocks. For this purpose, Weir and Schluter (2008) analyzed 90 candidate avian clock calibrations obtained from both fossils and biogeographical events from 12 taxonomic orders of birds over a 12-million-year interval. They found an average molecular divergence of 2.1% per million years in the evolutionary rates of cytochrome *b* gene. Due to absence of suitable calibration points for many groups of birds; use of this consensus estimate of molecular rates for avian species is recommended by the authors. The relaxed clock model, which calculate various rates for different branches, and a strict clock model, which assumes that mutations occur at a single rate along all branches, are two used common models in the calibration (Drummond *et al.* 2006). Molecular dating can be used in both population genetics and phylogenetic studies. In population genetics, i.e. the divergence times within a species, a coalescent framework is employed to estimate the time to the most recent common ancestor (TMRCA); while in phylogenetic studies, genetic distance matrices between different taxa have been used for estimating the substitution rates (Wilke *et al.* 2009). The mitochondrial divergence rate has been used in this dissertation is based on the studies of Weir and Schluter (2008).

1.2 Introduction to the reed warblers

1.2.1 History of the systematic classification of the Acrocephalidae

The avian family Acrocephalidae (reed warblers and allies) has been classified in the superfamily Sylvioidea of the avian order Passeriformes and consists of 61 species (Gill & Donsker 2014). They widely distributed in diverse habitats of the old world (Fig. 1.5). The members of this family are mostly colorless and similar in body shape (Fig. 1.6) and are among the most monomorphic passerines which make them difficult to identify and classify (Leisler & Schulze-Hagen 2011). Therefore they are known for a long history of classification and successive revisions (Table 1.3), and it has continued to the present day (del Hoyo *et al.* 2006). The traditional classification was base of similarities in plumage, morphology, oology (Baker 1977; Cramp 1992; Shirihai *et al.* 1995; Svensson 1992; Voous 1977; Watson *et al.* 1986), ecology, behavior and vocalization (Dowsett-Lemaire & Dowsett 1987; Fiedler 2011; Leisler *et al.* 1989; Lemaire 1977; Svensson 2001, 2003). Sibley and Ahlquist (1990) by using the new technique of DNA-DNA hybridization for examining the phylogenetic relationships of avian, proposed subfamily Acrocephalinae (with 223 species, in 36 genera), of the family Sylviidae and superfamily Sylvioidea, with major genera *Acrocephalus*, *Hippolais*, *Cettia*, *Bradypterus*, *Locustella*, *Chloropeta*, *Sylvietta*,

Phylloscopus and *Eremomela*. Molecular methods of DNA sequences caused new changed in the classification. Short histories of the major revisions are as follows:

- Leisler *et al*. (1997) and Helbig and Seibold (1999) by comparing mitochondrial cytochrome *b* sequences removed genus *Locustella* from this group;
- Beresford *et al*. (2005) used two nuclear genes (exons RAG-1 and -2) and found *Acrocephalus* in a clade with *Xanthomixis*, *Thamnornis*, *Cincloramphus*, *Megalurus*, *Schoenicola* and *Bradypterus*;
- Alström *et al*. (2006) by using nuclear myoglobin and mitochondrial cytochrome *b* genes found a strongly supported clade consist of *Acrocephalus*, *Hippolais* and *Chloropeta*. For the first time they proposed the family Acrocephalidae for this group which has accepted till now.
- Johansson *et al*. (2008) by applying three nuclear introns (myoglobin intron 2, ornithine decarboxylase introns 6 and 7 and b-fibrinogen intron 5), introduced the genus *Nesillas* to this family.

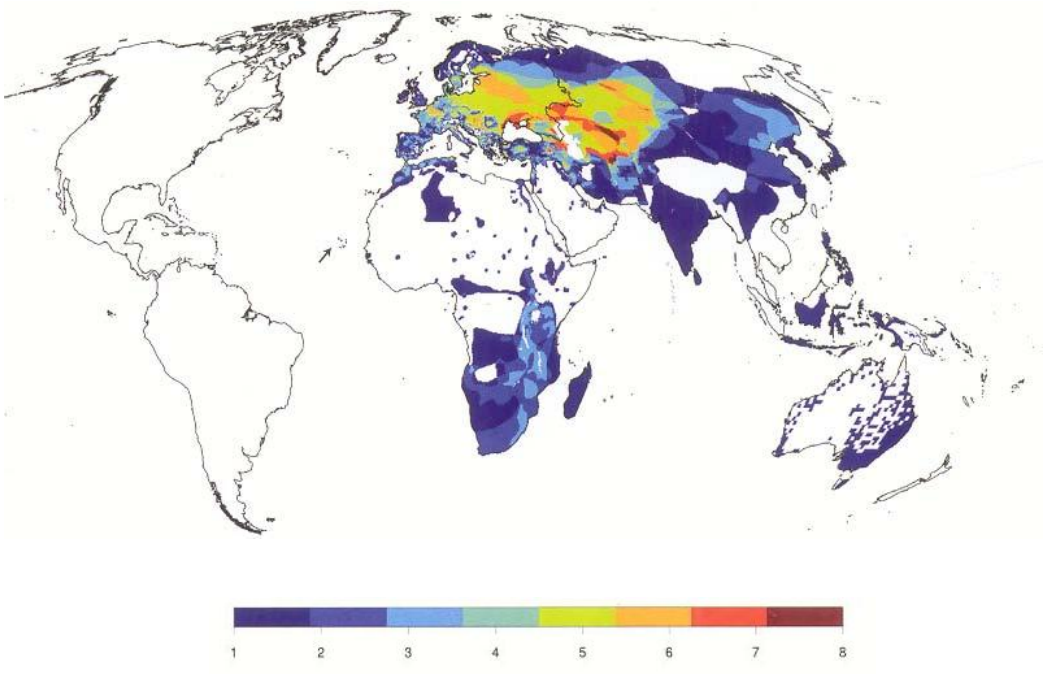

Fig. 1.5 Distribution map of the Acrocephalidae members. The scale related to their density. Derived from Leisler and Schulze-Hagen (2011).

In the case of relationships between the genera, *Acrocephalus*, *Hippolais* and *Chloropeta* have been split into several genera (Leisler *et al.* 1997) or subgenera (Helbig & Seibold 1999):

1. Genus *Acrocephalus*

Subgenus *Calamodus*

Subgenus *Notiocichla*

Subgenus *Acrocephalus*

Subgenus *Calamocichla*

Indeterminate subgenus (*Acrocephalus griseldis*)

Monotypic subgenus *Phragamaticola* (*Acrocephalus aedon*)

2. Genus *Hippolais*

Subgenus *Hippolais*

Subgenus *Iduna*

3. Genus *Chloropeta*

Fregin *et al*. (2009) revised the family Acrocephalidae based on one mitochondrial (*cyt b*) and three nuclear loci (*ODC, MYO* and *LDH*) as follow:

- **Genus *Acrocephalus:*** All members of the genus *Acrocephalus* except *A. aedon*;
- **Genus *Hippolais*:** Large *Hippolais*;
- **Genus *Iduna*:** Previous *Iduna* species (Table 1.3) plus two species of *Chloropeta (C. natalensis* and C. *similis) and A. aedon;*
- **Genus *Calamonastides***: Single species *Calamonastides gracilirostris;*
- **Genus *Nesillas***

Table 1.3 Overview of different classifications for the family Acrocephalidae (except *Nesillas*); / species not included in study. Derived and completed from Fregin *et al*. (2009).

Species name	Fregin *et al*. 2009	Helbig and Seibold 1999		Leisler *et al*. 1997		Sibley and Monroe 1990	Watson *et al*. 1986		Wolters 1982	
aedon	genus *Iduna*	subgenus *Phragamaticola*	genus *Acrocephalus*	/		genus *Acrocephalus*	subgenus *Phragamaticola*	genus *Acrocephalus*	genus *Phragamaticola*	
melanopogon	genus *Acrocephalus*	subgenus *Calamodus*		genus *Calamodus*			subgenus *Lusciniola*		subgenus *Lusciniola*	genus *Calamodus*
schoenobaenus							subgenus *Calamodus*		subgenus *Calamodus*	
paludicola									subgenus *Calamodyta*	
bistrigiceps									subgenus *Anteliocichla*	
tangorum		subgenus *Notiocichla*		genus *Notiocichla*			subgenus *Acrocephalus*		subgenus *Notiocichla*	genus *Acrocephalus*
concinens										
agricola										
scirpaceus										
baeticatus										
palustris										
dumetorum										
griseldis		Indeterminate subgenus		subgenus *Acrocephalus*	genus *Acrocephalus*				subgenus *Acrocephalus*	
vaughani		subgenus *Acrocephalus*								
arundinaceus										
orientalis										
stentoreus										
australis										
rufescens		subgenus *Calamocichla*		subgenus *Calamocichla*			subgenus *Calamocichla*		genus *Calamocichla*	
brevipennis										
newtoni										
gracilirostris										
sechellensis						genus *Bebromis*	genus *Bebromis*			
icterina	genus *Hippolais*	subgenus *Hippolais*	genus *Hippolais*	genus *Hippolais*		genus *Hippolais*	genus *Hippolais*		subgenus *Hippolais*	genus *Hippolais*
polyglotta										
languida										
olivetorum										
pallida	genus *Iduna*	subgenus *Iduna*		genus *Iduna*					subgenus *Iduna*	
opaca										
caligata										
rama										
gracilirostris	genus *Calamonastides*	genus *Chloropeta*		/		genus *Chloropeta*	genus *Chloropeta*		subgenus *Calamonastides*	gen. *Chlor.*
natalensis	genus *Iduna*	/							subgenus *Chloropeta*	
Similis										

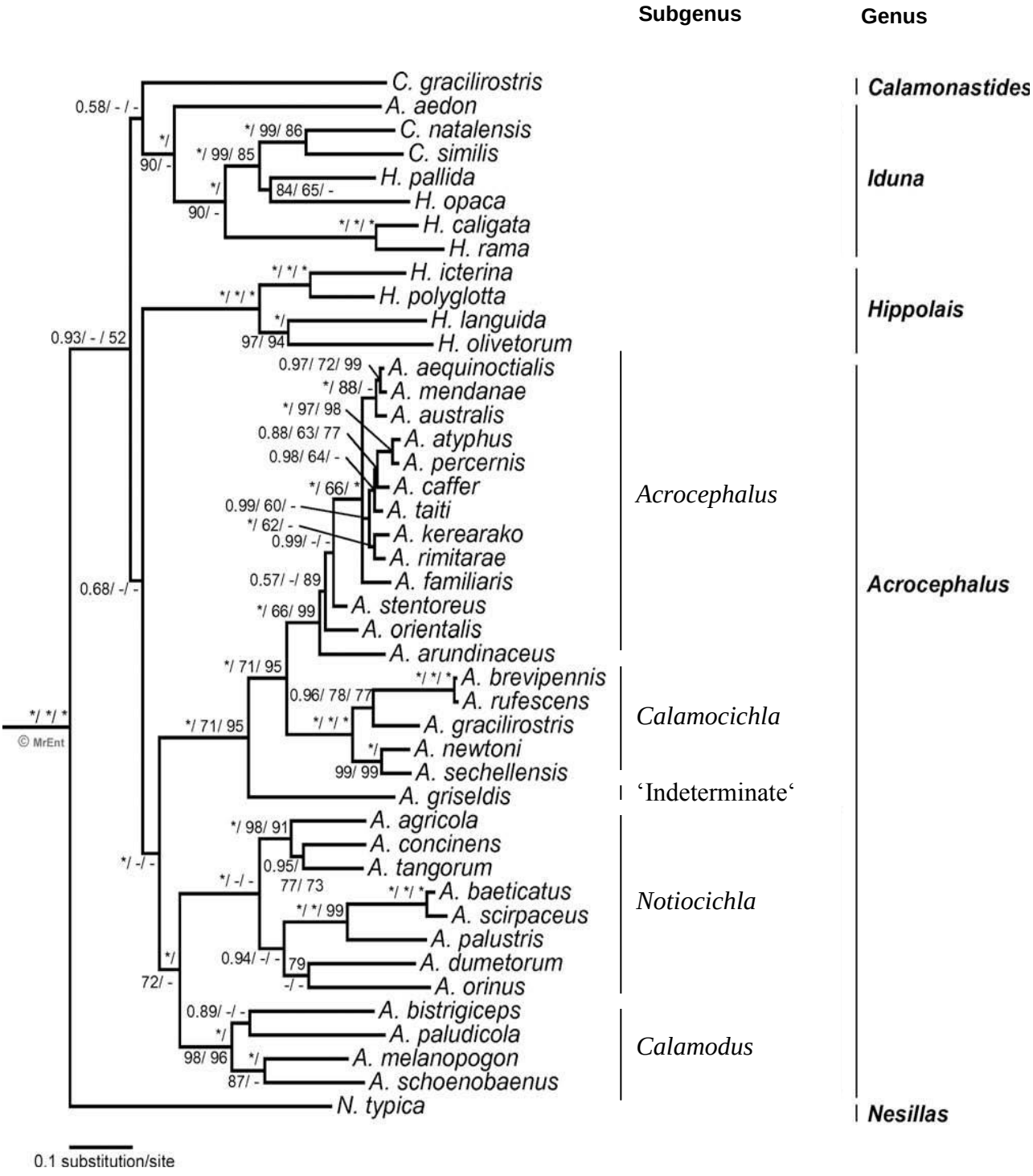

Fig. 1.6 Phylogenetic tree of Acrocephalidae, based on concatenated dataset of cytochrome *b*, *LDH*, *ODC* and myoglobin, analysed by Bayesian inference. Support values are Bayesian posterior probabilities, maximum likelihood bootstrap and parsimony bootstrap, respectively. Asterisks indicate posterior probability = 1.00 or bootstrap = 100%. Subgenera of the *Acrocephalus* as traditionally defined are indicated on the left column and the last taxonomy is in bold. Derived and completed from Fregin *et al*. (2009).

1.2.2 Characterization of the genera and subgenera of the family Acrocephalidae

Description of the specifications of each group with the list of the species (Gill & Donsker 2014), which has included in this research, is provided below:

1.2.2.1 Large reed warblers

The members of this group have long legs, bills, tails and broad wings. The song is deep and loud. They are distributed in Palaearctic region, Africa, Madagascar, Indomalaya, Australasia and Oceania (Leisler & Schulze-Hagen 2011). The large reed warblers divide into two subgenera of *Acrocephalus* and *Calamocichla*.

Subgenus *Acrocephalus* (Naumann, 1811; 13–20 cm; 15–31 g)

They are Eurasian to Polynesian large *Acrocephalus* with unstreaked plumage, heavy long bills and long pointed wings. Molecular data could support this clade but *A. vaughani* was not included (Fregin *et al.* 2009).

A. arundinaceus (Great reed warbler; Linnaeus, 1758): 2 ssp.
A. orientalis (Oriental reed warbler; Temminck and Schlegel, 1847)
A. stentoreus (Clamorous reed warbler; Hemprich and Ehrenberg, 1833): 10 ssp.
A. australis (Australian reed warbler; Gould, 1838): 3 ssp.
A. vaughani (Pitcairn reed warbler; Sharpe, 1900)

Subgenus *Calamocichla* (Sharpe, 1883; 13.5–18 cm; 14.5–23.9 g)

The species of this group are large Afrotropical and Malagasy reed warblers and the characteristics which separate them from the previous large taxa are some morphology features such as large feet, long graduated tails, round short wings and egg color; but song has a more important role in indication. They have short melodious (*sechellensis* and *gracilirostris*) or harsh (*newtoni*) songs and in general it is shorter, simpler and softer than subgenus *Acrocephalus* (Fiedler 2011). All the members of this group are sedentary species.

A. rufescens (Greater swamp warbler; Sharpe and Bouvier, 1877): 4 ssp.
A. brevipennis (Cape Verde warbler; Keulemans, 1866)
A. newtoni (Madagascan swamp warbler; Hartlaub, 1863)
A. gracilirostris (Lesser swamp warbler; Hartlaub, 1864): 6 spp.
A. sechellensis (Seychelles warbler; Oustalet, 1877)

Indeterminate subgenus

A. griseldis (Basra reed warbler; Hartlaub, 1891) is a large body (17–18 cm; 12–29 g) and unstreaked warbler close to the taxa of the subgenus *Calamocichla* and *Acrocephalus*; and traditionally classified with *arundinaceus* and *stentoreus* (Voous 1977), but genetically is highly distinct and held an isolated position in the *Acrocephalus* complex therefore treated as ‘’Indeterminate’’ by molecular studies of Leisler *et al*. (1997) and Helbig and Seibold (1999).

1.2.2.2 Small reed warblers

They have smaller body size and loner chattering songs than large reed warblers. They divide into two subgroups with clearly distinct plumage: the striped and unstreaked species.

Subgenus *Calamodus* (Kaup, 1829; 12–13 cm; 7–17.9 g): striped reed warblers plus unstreaked *A. bistrigiceps*

Species of this clade have striped plumage on the under- and/or upperparts, smaller size, slimer and finer bills and shorter and more rounded wings as compared to the group of small plain species. The eggs of these species are spotted and pale buff-olive, often completely obscuring ground-color. Because of using brief units, their songs are the fastest in the family (Leisler & Schulze-Hagen 2011). They have Palaearctic breeding distribution and except *A. melanopogon*, the other species are migrants. The molecular data strongly supported this clade but because of low bootstrap values, the relationships within this group were not received robust support in previous molecular studies. Due to differences in plumage, *A. bistrigiceps* was not included in this group by Voous (1977) but the molecular data confirmed its position in *Calamodus* instead of *Notiocichla* group.

A. melanopogon (Moustached warbler; Temminck, 1823): 3 ssp.
A. schoenobaenus (Sedge warbler; Linnaeus, 1758)
A. paludicola (Aquatic warbler; Vieillot, 1817)
A. bistrigiceps (Black-browed reed warbler; Swinhoe, 1860)

Subgenus *Notiocichla* (Oberholser, 1905; 12–14 cm; 7.7–19.7 g): unstreaked/small plain *Acrocephalus*

They have occupied a very wide distributed range in Palaearctic. These species have harsh and very complex songs. Although the inter-relationships among these taxa were not robustly supported in previous analyses, the clade received strong support.

A. concinens (Blunt-winged warbler; Swinhoe, 1870): 3 ssp.

A. agricola (Paddyfield warbler; Jerdon, 1845): 2 ssp.

A. scirpaceus (Eurasian reed warbler; Hermann, 1804): 3 ssp.

A. baeticatus (African reed warbler; Vieillot, 1817): 5 ssp.

A. palustris (Marsh warbler; Bechstein, 1798)

A. dumetorum (Blyth's reed warbler; Blyth, 1849)

A. tangorum (Manchurian reed warbler; La Touche, 1912)

1.2.2.3 *Hippolais* species (Tree warblers; Conrad von Baldenstein, 1827; 12–18 cm; 8–23 g)

Traditionally the separation between *Acrocephalus* and *Hippolais* was based on size, shape of the tail (rounded in *Acrocephalus*, square-ended in *Hippolais*), bill shape (flatter in *Hippolais*), relative length of under-tail coverts (longer in *Acrocephalus*), egg color (ground color whitish or olive in *Acrocephalus*, pinkish in *Hippolais*) song (harsh and musical with long notes in *Hippolais* (Maclean *et al.* 2003) but with many repetitions and fewer imitations in *Acrocephalus*) and habitat (*Hippolais* species, live in woodland or tall grass of drier habitats while most species of *Acrocephalus* occur in bushes of wet habitats (Cramp 1992; del Hoyo *et al.* 2006). The following four large *Hippolais* form this clade. Their song is not as harsh as the other species of the family and the units are the longest among all species.

H. icterina (Icterine warbler; Vieillot, 1817)

H. polyglotta (Melodious warbler; Vieillot, 1817)

H. languida (Upcher's warbler; Hemprich and Ehrenberg, 1833)

H. olivetorum (Olive-tree warbler; Strickland, 1837)

1.2.2.4 *Iduna* species (Bush and scrub warblers; Keyserling and Blasius, 1840; 11–15 cm; 7–17 g)

Previously they were classified within *Hippolais* but later the smaller *Hippolais* classified as *Iduna*. Molecular studies of Leisler *et al*. (1997) and Helbig and Seibold (1999) confirmed that *Hippolais* warblers formed two clades: clade I consisted of small *Hippolais* (*Iduna*) and clade II larger taxa (*Hippolais*). Clade I possess more brownish plumage and has a more *Acrocephalus*-like song, less pinkish and more whitish ground color of the eggs than those of the clade II. Fregin *et al*. (2009) got the same results. They included *Chloropeta natalensis* and *Chloropeta similis* in their analyses and placed them inside *Iduna*. Due to morphological similarities, *I. natalensis* and *I. similis* formerly classified within flycatchers (Yellow

flycatcher) or in *Chloropeta* genus (del Hoyo *et al.* 2006; Dickinson 2003; Sibley & Monroe 1990; Wolters 1982).

Moreover *A. aedon* was placed in this group (Fregin *et al.* 2009). *Iduna aedon* has intermediate similarities. It is a large warbler nearly as big as great reed warbler with few obvious distinctive plumage features. The forehead is rounded and the bill is short and pointed but its egg coloration (Schönwetter 1979), nest construction (Marova *et al.* 2005) and drier breeding habitat (del Hoyo *et al.* 2006) are similar to *Iduna*. However, it has many unique characters such as proportionally long tail with narrow feathers, thick bill and specially lack of supercilium, which is distinct in Acrocephalidae. Base on molecular data Helbig and Seibold (1999) could not find any close relationship between *aedon* and *Iduna* (*I. natalensis* and *I. similis* were not included in their studies) as well as the other species of Acrocephalidae. Therefore they agreed with its placement in the monotypic subgenus *Phragamaticola*. Fregin *et al*. (2009) classified it in *Iduna* group but base on molecular evidence of four loci, only nuclear gene of *ODC* could support this association, especially the reconstructed mitochondrial *cyt b* tree could not solve the position of *aedon*.

Iduna (Fregin *et al.* 2009)

They sing quickly, close to the small striped warblers but have very harsh sound (Marova *et al.* 2005), except aedon which cluster analysis of song characters by Leisler and Schulze-Hagen (2011) shows its closer relationship to *Notiocichla* group.

I. pallida (Eastern olivaceous warbler; Hemprich and Ehrenberg, 1833): 5 ssp.

I. opaca (Western olivaceous warbler; Cabanis, 1850)

I. caligata (Booted warbler; Lichtenstein, 1823)

I. rama (Sykes's warbler; Sykes, 1832)

I. natalensis (African yellow warbler; Smith, A, 1847): 4 ssp.

I. similis (Mountain yellow warbler; Richmond, 1897)

I. aedon (Thick-billed warbler; Pallas, 1776)

1.2.2.5 *Calamonastides gracilirostris* (Papyrus yellow warbler; Ogilvie-Grant, 1906) (13.5 cm; 12.5–16.2 g)

Based on yellow coloration, it was classified as *Chloropeta gracilirostris* with the other two *Chloropeta* species (*natalensis* and *similis*) but Grant and Mackworth-Praed (1940) noted that in a number of characters such as having a longer tail, larger feet and narrower bill than *Chloropeta* and also living in swamp habitat, it has more similarities to *Calamocichla* group,

so they proposed a separate subgenus, *Calamonastides*, for it. Previous molecular analyses could not support a close relationship between *gracilirostris* and *Calamocichla* group (Fregin *et al.* 2009; Helbig & Seibold 1999) or *Chloropeta* (Fregin *et al.* 2009) and therefore was placed in a monotypic genus (*Calamonastides*). The study of Maclean and his colleagues (2003) showed that their song is very distinct. They have the lowest-pitched songs of the family consist of different short songs which are repeated several times.

1.2.2.6 *Nesillas* (Brush warblers; Oberholser, 1899; 15–20 cm; 14–21 g)

They live in Malagasy region (Madagascar and Comoros Islands). Molecular studies of Johansson *et al*. (2008) showed that genus *Nesillas* is the sister group to all other groups of the family Acrocephalidae, which was confirmed by Fregin and her colleagues (2009). On the base of egg characteristics they were placed close to *Hippolais* (Meise 1976), although in their overall morphology they are closer to *Acrocephalus* species (Leisler & Schulze-Hagen 2011).

N. typica (Madagascar brush warbler; Hartlaub, 1860): 3 ssp.

N. lantzii (Lantz's brush warbler; Grandidier, A, 1867)

N. longicaudata (Anjouan brush warbler; Newton, E, 1877): Kennerley and Pearson (2010) treated it as a subspecies of Malagasy brush warble

N. brevicaudata (Grande comore brush warbler; Milne-Edwards and Oustalet, 1888)

N. mariae (Moheli brush warbler; Benson, 1960)

N. aldabranus (Aldabra brush warbler; Benson and Penny, 1968): extinct

1.3 Objectives of the thesis

In my doctoral dissertation I studied several aspects of the systematic of the reed warbles by using molecular analyses:

- At the species level (chapter 3.1), I attempt to resolve the phylogenetic relationships between the species of family Acrocephalidae using an alignment of a total of 6280 nt sequence data, by a combination of five nuclear (*ODC*, *MYO*, *LDH*, *FIB5* and *RAG-1*) and three mitochondrial genes (*cyt b*, *ND2* and *COI*) as well as ISSR fingerprinting. I compare the results with the three previous molecular studies: Leisler *et al*. (1997) and Helbig *et al*. (1999) base on *cyt b* and the last one (Fregin *et al.* 2009) based on one mitochondrial and three nuclear loci.

- At the population level, I carry out phylogeographic and population genetics analyses of *Acrocephalus scirpaceus* (chapter 3.2) and *Acrocephalus palustris* (chapter 3.3) with mitochondrial *COI* data for all individuals and mitochondrial *cyt b* sequence data from the selected individuals of the populations.

- In the final chapter of the thesis (chapter 4) I summarize the major results and compare the individual findings to each other.

2 Material and Methods

2.1 Sample material

As a source of DNA, blood, tissue or feather samples were collected from living birds and road kills. Blood samples were taken from the brachial vein of living birds with the help of a conventional syringe, and the animals were released immediately afterwards. All samples were stored in EDTA buffer (Table 2.3) or 70% ethanol and kept at –20°C until further processing.

In total, 402 individuals of *Acrocephalus scirpaceus*, 229 of *Acrocephalus palustris*, and 36 taxa of family Acrocephalidae (two individuals per taxon, in total 72 individuals) were analyzed. A further 142 *Acrocephalus paludicola*, 60 *Acrocephalus agricola* and 12 *Acrocephalus baeticatus* have also been sequenced for future research projects. List of samples with voucher numbers, GenBank accession numbers, and detailed information on localities is given in the particular chapters.

2.2 Equipment

All instruments that were used for laboratory analyses are listed in Table 2.1.

Table 2.1 Analytical instruments used in the present study.

Instruments	**Company**
Automated sequencer: ABI 310, ABI 3100	Applied Biosystems
Electrophoresis microcomputer power supply E452	Fröbel
Gel chambers for agarose gel	Heidelberg University
Laboratory scale	Sartorius
Microcentrifuge-Biofuge 13R	Heraeus
Microcentrifuge: Biofuge Fresco	Heraeus
PCR machines: TRIO-Thermoblock and T Gradient	Biometra
pH meter: Pipetman P2, P20, P100, P1000	Gilson
UV-transilluminator II-200-M [312nm]	Bachofer
Vortex: Reax 2000	Heidolph
Incubator	Heraeus
Photometer DU 640	Beckman

2.3 Solutions and chemicals

A list of chemicals, enzymes and other materials used in this study is given in Table 2.2 followed by a list of buffers and solutions in Table 2.3.

Table 2.2 Chemicals, enzymes and solutions used in this study.

Chemicals, enzymes and other materials	Company
Acetic acid	Merck
Acrylamide, Long Ranger	Lonza
Agarose	HYBAID-AGS
Ammonium acetate	Merck
Ammonium sulphate	Gerbu
Autoradiography film (XAR5)	Kodak
Big Dye Terminator kit	Applied Biosystems
Bovine serum albumin	Sigma
Bromophenol blue	Serva
Chloroform	Fluka
EDTA	Roth
Ethanol absolute	Merck and J.T Becker
Ethidium bromide	Serva
Formamide	Applied Biosystems
Guanidine thiocyanate	Roth
Isopropanol	Applichem
Nucleotides	Sigma
Phenol	Merck
Premix Terminator kit	Amersham- Bioscience
Proteinase K	Merck
Radioactive dATPs	Pharmacia
Reaction tubes (0.2, 0.5, 1.5, 2 ml)	Eppendorf
Sequagel Solution	Biozym
Silane	Sigma
Sodium acetate	Merck
Sodium dodecyl sulfate (SDS)	Applichem
Sterile filter, 0.22 µm	Sartorius
Taq DNA polymerase	Sigma
TEMED (N,N,N,N-Tetramethylendiamine)	Roth
Tris-HCl	Roth
ß-mercaptoethanol	Merck

Table 2.3 Buffers and solutions used in this study.

Stock solutions	Components
Agarose gel solution	1% agarose, 1 µg/ml ethidium bromide, in water
Ammonium acetate	4 M ammonium acetate, in water
Ammonium persulfate	10% solution in water
Chloroform/isoamyl alcohol	Chloroform/ isoamyl alcohol in ratio 24:1
EDTA buffer	10% EDTA, 0.5% NaF, 0.5% thymol, 1% Tris (pH 7.5)
Guanidine thiocyanate buffer	4 M guanidine thiocyanate, 0.1 M Tris-HCl, 1% ß-mercaptoethanol, pH 5
λ-PST I size standard	DNA cut with PST I restriction enzyme
Lysis buffer	25 mM EDTA, 75 mM NaCl, 10 mM Tris-HCl, pH 7.0
Nucleotide mix	2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP
PCR buffer (10X)	100 mM Tris, 500 mM KCl, 5% Triton X-100, 15 mM MgCl2
Phenol/chloroform	Phenol, chloroform, isoamyl alcohol in ratio 25:24:1
SDS solution	20% solution in water
Sodium acetate solution	3 M sodium acetate, acetic acid (pH 4.6)
Sodium chloride solution	Sodium chloride in water (saturated)
TAE buffer	40 mM Tris, 1 mM EDTA, acetic acid (pH 8.0)
TBA buffer (10X)	1 M Tris, 89 mM boric acid, 10 mM EDTA (pH 8.5)
TE buffer	10 mM Tris, 1 mM EDTA, hydrochloric acid (pH 8.0)

2.4 DNA extraction, amplification and sequencing

Total genomic DNA was extracted by an overnight incubation at 37 °C in lysis buffer [10 mmol/L Tris (pH 7.5), 25 mmol/L EDTA, 75 mmol/L NaCl, 1% SDS and 1 mg of Proteinase K (Merck, Darmstadt, Germany)] and standard phenol-chloroform procedures (Sambrook *et al.* 1989). The DNA was precipitated from the supernatant by adding 0.8 Vol.-% of ice-cold isopropanol. The extracted DNA was washed twice with 70% ethanol, dried and redissolved in TE buffer. DNA stock solutions were kept at 4°C until further analysis. In cases of very limited sample material and low DNA yield, the protein pellet was redigested by addition of guanidine isothiocyanate buffer to extract DNA that was trapped in the pellet. In this case, digestion was followed by extraction twice with phenol/chloroform, then once with chloroform/isoamyl alcohol and subsequent precipitation and washing of the DNA as explained above. To determine the approximate concentration and quality of the extracted DNA, 5 µl of each DNA solution were loaded onto a 1.4% agarose gel containing ethidium

bromide. DNA concentration was estimated by comparison of fluorescence intensities to samples of known DNA content.

I used several combinations of primers for amplification and sequencing (see Table 2.4). PCR amplifications were carried out in the following reaction mixture (total volume of 50 µL): 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris (pH 8.5), 50 mmol/L KCl, 100 µmol/L dNTPs, 0.2 units of *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng DNA, and 5 pmol of primers. PCR conditions consisted of an initial denaturation step at 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, annealing temperature for 1 min and 72 °C for 1 min; plus a final extension step at 72 °C for 10 min. Optimal annealing temperatures were measured by a gradient PCR in a Tgradient thermocycler (Biometra, Gottingen, Germany). Annealing temperatures for every gene is presented in table 2.4. Prior to further analysis, the success of the PCR was checked on 1 % agarose gels. PCR products were precipitated with 4 M NH_4Ac and ethanol (1 : 1 : 6) and centrifuged for 15 min (13,000 rpm). Sequencing was performed on an ABI 3730 automated capillary sequencer (Applied Biosystems, CA, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (performed by STARSEQ GmbH, Mainz, Germany). Sequencing primers correspond to the primers used in PCR amplifications.

2.5 Data preparation

Sequences were aligned with Clustal W (Thompson *et al.* 1994) available in BioEdit version 7.0.9.0 (Hall 1999) and all alignments were inspected and corrected visually. We translated the nucleotide sequences of mitochondrial genes to proteins using MEGA version 5.1 (Tamura *et al.* 2011) and found no stop-codons or indels, suggesting that we did not amplify nuclear pseudogenes (Allende *et al.* 2001). For the nuclear loci, heterozygous sites were coded as ambiguous.

Table 2.4 List of primers used in PCR amplifications and cycle sequencing. Forward primers are given in bold.

Gene / Annealing temp.	**Primer**	**Sequence (5′ to 3′)**	**Reference**
cyt b / 52°C	mtFNP	GGY TTA CAA GAC CAA TGT TT	(Fregin *et al.* 2009)
	mtF24	TTT GGT TTA CAA GAC CAA TGT TTT	(Fregin *et al.* 2009)
	L 14841	CCA TCC AAC ATC TCA GCA TGA TGA AA	(Kocher *et al.* 1989)
	H 15915	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	(Edwards & Wilson 1990)
	mtFSH	TAG TTG GCC AAT GAT GAT GAA TGG GTG TTC TAC TGG TT	(Dietzen *et al.* 2003)
	mtA1	CCC CCT ACC AAC ATC TCA GCA TGA TGA AAC TTC G	(Dietzen *et al.* 2003)
	L14990	CCA TCC AAC ATC TCA GCA TGA TGA AA	(Kocher *et al.* 1989)
	L14995	GGT TGT TTG AGC CTG ATT C	(Avise 1994)
	H16065	GGA GTC TTC AGT CTY TGG TTT ACA AGA C	(Hackett 1996)
	L14841	CCA TCC AAC ATC TCA GCA TGA TGA AA	(Kocher *et al.* 1989)
COI / 51.5°C	**PasserF1**	CCA ACC ACA AAG ACA TCG GAA CC	(Sheldon *et al.* 2009)
	PasserR1	GTA AAC TTC TGG GTG ACC AAA GAA TC	(Sheldon *et al.* 2009)
	ExtF	ACG CTT TAA CAC TCA GCC ATC TTA CC	(Johnsen *et al.* 2010)
	BirdF1	TTC TCC AAC CAC AAA GAC ATT GGC AC	(Johnsen *et al.* 2010)
	birdR2	ACG TGG GAG ATA ATT CCA AAT CCT G	(Johnsen *et al.* 2010)
ND2 / 50°C	**L5216**	GGC CCA TAC CCC GRA AAT G	(Sorenson *et al.* 1999)
	H5766	GGA TGA GAA GGC TAG GAT TTT KCG	(Sorenson *et al.* 1999)
	L5758	GGC TGA ATR GGM CTN AAY CAR AC	(Sorenson *et al.* 1999)
	H6313	CTC TTA TTT AAG GCT TTG AAG GC	(Sorenson *et al.* 1999)
MYO / 56°C	**Myo 2**	GCC ACC AAG CAC AAG ATC CC	(Slade *et al.* 1993)
	Myo 3F	TTC AGC AAG GAC CTT GAT AAT GAC TT	(Heslewood *et al.* 1998)
ODC / 56°C	**OD6**	GAC TCC AAA GCA GTT TGT CGT CTC AGT GT	(Allen & Omland 2003)
	OD8r	TCT TCA GAG CCA GGG AAG CCA CCA CCA AT	(Allen & Omland 2003)
LDH / 51.5°C	**b1**	GGA AGA CAA ACT AAA AGG AGA AAT GAT GGA	(Helbig *et al.* 2005)
	b4	GGG CTG TAT TTN ACR ATC TGA GG	(Helbig *et al.* 2005)
	P5	GCT TGC TCT GGT TGA YGT TAT GG	(Fregin *et al.* 2009)
	P6	CAC ATT CCT CTG CAC YAG GTT GAG	(Fregin *et al.* 2009)
FIB5 / 51.5°C	**Fib5**	CGC CAT ACA GAG TAT ACT GTG ACA T	(Marini & Hackett 2002)
	Fib6	GCC ATC CTG GCG ATT CTG AA	(Marini & Hackett 2002)
RAG-1 / 53°C	**R17**	CCC TCC TGC TGG TAT CCT TGC TT	(Groth & Barrowclough 1999)
	R22	GAA TGT TCT CAG GAT GCC TCC CAT	(Groth & Barrowclough 1999)
	R50	CTG ATC TGG TAA CCC CAG TGA AAT CC	(Irestedt 2001)
	R51	GAC CCT CTT TCT GCT ATG AGG GGG C	(Irestedt 2001)

2.6 Data analysis

2.6.1 Population genetics

Molecular diversity parameters such as number of haplotypes (*H*), number of polymorphic or segregating sites (*S)*, haplotype diversity (*Hd*), average number of pairwise differences (*K*), nucleotide diversity (π), Tajima's *D* (Tajima 1989), Fu's F_S neutrality tests (Fu 1997) and gene flow (*Nm* based on F_{ST}) among populations (Hudson *et al.* 1992) were estimated in Arlequin version 3.5.1.2 (Excoffier & Lischer 2010) and DnaSP version 5.1 (Librado & Rozas 2009). Significance was determined based on 1000 coalescent simulations. Diversity indices were not calculated for populations with very small sample sizes ($N < 4$). The degree of geographical structuring among populations evaluated with Analysis of molecular variance (AMOVA). AMOVA transforms the data into an analysis of variance format and estimates the variance components and *F*-statistic. AMOVA is implemented in the program Arlequin version 3.5.1.2 (Excoffier & Lischer 2010). Mean pairwise *p*-distances (Nei 1987) within and among lineages were calculated by MEGA version 5.1 (Tamura *et al.* 2011). Median-joining network analyses were conducted with Network version 4.6.1.1 (Bandelt *et al.* 1999) in order to show genealogical relationships among individuals or different haplotypes. Networks like phylogenetic trees use DNA sequence variations but they show better the relationship between large sample sizes with small genetic distances between individuals or haplotypes.

2.6.2 Reconstruction of phylogenetic trees

Two methods of Bayesian inference (BI) and Maximum Likelihood (ML) were used to construct the phylogenetic trees. Maximum Likelihood was performed in MEGA version 5.2 (Tamura *et al.* 2011). Best evolutionary models that fit our data were selected according to a Bayesian Inference Criteria (BIC) in JModelTest version 2.1.4 (Posada 2008). BI was carried out with two independent runs of 10 million generations and trees were sampled every 2000 generations, with the first 10 % of samples discarded as "burn-in". ML node support was assessed through 1000 bootstrap replicates. Bayesian inference of phylogeny was carried out with the program MrBAYES 3.1.2 (Ronquist & Huelsenbeck 2003). This program generates a posterior probability distribution using Markov chain Monte Carlo (MCMC) analysis under the appropriate substitution model (Bollback 2002). The analysis is run for 10 – 30 million generations (depends on the locus) with every 2000 generations being sampled and the first 10% of samples discarded as "burn-in". Trees visualized with FigTree version 1.4.1. Posterior probabilities ≥ 0.95 and bootstrap values $\geq 85\%$ were regarded as high support following (Erixon *et al.* 2003). For rooting the trees, I used outgroups, which is the most

common used approach if no fossil data are available. The selected taxa as outgroup have considerable influence on the topology of the tree (Smith 1994) and it is possible that different roots give different hypotheses of basal relationships (Johansson & Ericson 2004). Therefore, it is crucial to include more than one taxon and to choose outgroup taxa as closely related as possible (sister taxa) to the ingroup.

2.6.3 Historical demography

To assess the demographic history of a population, pairwise mismatch distributions (the frequency distribution of the numbers of differences between all pairwise haplotype comparisons in a population) are very useful. This distribution is multimodal in populations at demographic equilibrium, but unimodal in populations that have passed through a recent demographic expansion (Slatkin and Hudson 1991, Rogers and Harpending 1992). Mismatch distributions (Rogers & Harpending 1992) and the time of population expansion ($\tau = 2\ \mu T$) were estimated in Arlequin version 3.5.1.2 (Excoffier & Lischer 2010) with 1000 bootstrap replicates and compared statistically against models of sudden population expansion (100 replicates) using the sum of squared deviations test. I conducted the R2 test (Ramos-Onsins & Rozas 2002) in DnaSP version 5.1 (Librado & Rozas 2009). BEAST version 1.7.5 (Drummond & Rambaut 2007a) was also employed to model the demographic changes of populations using the Bayesian skyline analysis (Drummond *et al.* 2005) with a chain length of 100 million generations sampled every 2000. Effective sample size (ESS) values of at least 200 were evaluated with TRACER 1.5 (Rambaut & Drummond 2007). After discarding the first trees with a 'burn-in' equivalent to 10%, maximum clade credibility trees were reconstructed using TreeAnnotator version 1.7.5 (http://beast.bio.ed.ac.uk/TreeAnnotator) and visualized with FigTree version 1.4.1 (http://beast.bio.ed.ac.uk/FigTree).

2.6.4 Molecular dating

Molecular evolutionary rates and the time of the most common ancestor (TMRCA) were estimated using a Bayesian coalescent approach implemented in the BEAST version 1.7.5 (Drummond & Rambaut 2007a). Several coalescent tree priors were employed: constant size (Kingman 1982), exponential, logistic and expansion growth (Griffiths & Tavaré 1994). Assuming a population expansion model, strict molecular clock and a mutation rate uniform distribution ranging from 0.01105 to 0.02500 substitutions/site/lineage/million years or a fixed standard universal clock with a mutational rate of 2.1 % per million years or 0.0105 for mitochondrial genes (Neto *et al.* 2012; Weir & Schluter 2008). We conducted an analysis

consisting of 20 million generations sampled at intervals of 1000. The Yule process (Gernhard 2008; Yule 1925) with 10 million generations, sampling every 1000 trees and 10% as "burn-in" was utilized for accounting speciation. Stationarity was assessed in TRACER 1.5 (Rambaut & Drummond 2007).

2.7 Genomic fingerprinting by Inter-Simple Sequence Repeats (ISSR)

Inter-simple sequence repeat (ISSR) markers constitute another PCR-based technique, useful for genome studies. This technique provides a rapid, highly reproducible and inexpensive way of targeting structural changes at the genomic level (Gonzalez *et al*. 2008; and references therein). In birds, this technique has been used for population genetic studies and has proved to be a powerful molecular marker to investigate intraspecific differentiation and phylogenetic relationships among closely related taxa (Gonzales and Wink 2010).

Twenty five ISSR primers were initially evaluated to identify the species variability within the family Acrocephalidae. Out of 25 screened primers, MW_4 $(GACA)_4$ and $(GGTA)_4$ were chosen because banding patterns could be identified unambiguously. ISSR-PCR was performed with 60 ng of template DNA in 25 µL reaction volumes containing 10 pmol of the 5´-anchored microsatellite repeat primer, 0.1 mM each of dGTP, dCTP and dTTP, 0.045 mM dATP, 1 µCi (α-^{33}P)-dATP (Perkin Elmer, LAS, Rodgau, Germany), 0.6 units of *Taq* DNA polymerase (Bioron) and 2.5 µL of 10 × amplification buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$). DNA amplifications were performed in a thermocycler Tgradient (Biometra, Goettingen, Germany). Following the initial 5 min denaturation step at 94 °C, the program consisted of 38 cycles of 50 s at 94 °C, 40 s at 50 °C, 2 min at 72 °C and 10 min at 72 °C for final elongation. Amplified products were mixed with 8 µL bromophenol blue and run on a high-resolution denaturing polyacrylamide gels 6 % (0.2 mm) for 4 h at 65 W (gel length 40 cm) containing 1 × TBE buffer. The gels were dried and exposed for 2–5 days (depend on levels of radioactivity) to X-ray hyperfilms (Kodak, Taufkirchen, Germany) and subsequently developed. Gel electrophoresis for every primer were performed several times to get the best resolution of the results. Finally, ISSR-PCR bands were coded with 1 = band present and 0 = band absent. Clustering and Neighbor joining tree reconstructions were conducted with Jaccard coefficient and 1000 bootstrap replicates in FAMD 1.31 (Schlüter & Harris 2006) and visualized using TreeView version 1.6.6 (Page 1996).

3 Results

3.1 A Re-evaluation of Phylogenetic Relationships within Reed Warblers (Aves: Acrocephalidae) Based on Eight Molecular Loci and ISSR Profiles

3.1.1 Abstract

The members of the avian family Acrocephalidae (reed warblers and allies) are among the most monomorphic passerines that makes them difficult to identify in the field. Reed warblers have seen a long history of different classifications and successive revisions. In this study, we evaluated the phylogenetic relationships among 35 species of reed warblers and allies based on DNA sequences from five nuclear loci [myoglobin intron 2, introns 6 and 7 of *ODC*, *LDH* intron 3, intron 5 of b-fibrinogen and *RAG-1* nuclear exon], three mitochondrial genes [*cyt b*, *ND2* and *COI*] and ISSR genomic fingerprinting. We included previously published data and obtained nucleotide sequences from at least two individuals per taxon so that all loci were available for all species. We improved the resolution of phylogenetic relationships among many species, but despite the use of 6280 nucleotides, some deep-level relationships remain enigmatic. Lack of nodal support at some branches may be the result of rapid radiation in the history of the family. The last common ancestor of this family could be dated for the Middle Miocene (12.5 million years ago, approximately). In agreement with previous phylogenetic studies, we recovered the major clades recognized within this family: *Acrocephalus*, *Iduna* (except *I. aedon*), *Hippolais*, *Nesillas* and *Calamonastides*. We accept the current taxonomic position of *Calamonastides gracilirostris* as a monotypic genus in our phylogenetic reconstructions. Although the inclusion of *natalensis* and *similis* within *Iduna* was not robustly supported, we cannot reject a close relationship of these species with *Iduna*. Phylogenetic analyses based on mitochondrial genes and ISSR profiles did not support the position of *I. aedon* within *Iduna* or *Acrocephalus*. Therefore, we prefer to resurrect the former genus name *Phragamaticola* for this species which would agree with the phylogenetic data and would not create paraphyletic clades.

3.1.2 Introduction

The avian family Acrocephalidae (reed warblers and allies) comprises 61 species (Gill & Donsker 2014) in the Palaearctic, Africa and Australasia. Most of the species share a similar plumage pattern which represent identification challenges for ornithologists (del Hoyo *et al.*

2006; Leisler & Schulze-Hagen 2011). Despite numerous phylogenetic studies within this family (Dickinson 2003; Helbig & Seibold 1999; Leisler *et al.* 1997), the phylogenetic relationships remain partly speculative at the genus level. In a recent near complete phylogeny of the Acrocephalidae, based on nucleotide sequences of the mitochondrial cytochrome *b* and three nuclear genes (Fregin *et al.* 2009), five genera have been circumscribed: *Acrocephalus, Iduna, Hippolais, Nesillas* and *Calamonastides*. These authors considered *Chloropeta natalensis*, *C. similis* and *Acrocephalus/Phragamaticola aedon* being part of the genus *Iduna* (i.e. together with species *pallida*, *opaca*, *caligata* and *rama*). It had been formerly suggested to place *A. aedon* in its previous monotypic genus *Phragamaticola* which was accepted by Kennerley and Pearson (2010). Furthermore, *Chloropeta gracilirostris* is now treated as belonging to the genus *Calamonastides* as previously proposed by Wolters (1982). Recent phylogenetic studies enabled to classify most taxa with confidence but the phylogenetic relationships among a few groups as well as some species have remained elusive (Fregin *et al.* 2009).

Here, we evaluated the phylogenetic relationships within the Acrocephalidae based on DNA sequences from eight loci: five nuclear and three mitochondrial genes. Our alignment consists of 6280 nucleotides and DNA sequences from all loci were available for all species considered in this study. Sequences from previously published papers were combined with our data set and re-analyzed using standard phylogenetic reconstruction methods. Additionally, we made use of Inter Simple Sequence Repeat (ISSR) PCR for genomic fingerprinting as a complementary genetic marker since it has been successfully used in avian genetic studies (Fernandes *et al.* 2013; Gonzalez & Wink 2010; Gonzalez *et al.* 2008).

3.1.3 Methods

Sampling, amplification and sequencing

We included 35 species of the family Acrocephalidae in our multi-locus phylogenetic analyses. These taxa comprise two individuals per taxon and two subspecies of *A. scirpaceus* (Table 3.1.1). Based on previous phylogenetic studies (Fregin *et al.* 2009, 2012), four species, i.e. *Pnoepyga pusilla, Locustella fluviatilis, Bradypterus baboecala* and *Megalurus palustris* were used as outgroups.

DNA was extracted from blood or tissue using a standard phenol chloroform protocols (Sambrook *et al.* 1989). Eight genetic markers were sequenced and used for phylogenetic reconstruction: three mitochondrial genes, i.e., cytochrome *b* (*cyt b*; 1043 nt), NADH dehydrogenase subunit 2 (*ND2*; 1077 nt) and cytochrome *c* oxidase subunit I (*COI*;

662 nt); and five nuclear genes, i.e. myoglobin intron 2 (*MYO*; 703 nt), ornithine decarboxylase, introns 6 and 7 (*ODC*; 776 nt), lactate dehydrogenase intron 3 (*LDH*; 513 nt), the fifth intron of beta fibrinogen (*FIB5*; 562 nt) and a single exon of the recombination activating gene 1, (*RAG-1*; 944 nt; Table 3.1.2). Primer pairs used for amplification were: *cyt b*: L14990/H16065 (Hackett 1996; Kocher *et al.* 1989); *ND2*: L5758/H5766 and L5216/H6313 (Sorenson *et al.* 1999); *COI*: PasserF1/PasserR1 and ExtF/BirdR2 (Johnsen *et al.* 2010; Sheldon *et al.* 2009); *MYO*: Myo2 (Slade *et al.* 1993)/Myo3F (Heslewood *et al.* 1998); *ODC*: OD6/OD8r (Allen & Omland 2003); *LDH*: b1/b4 and P5/P6 (Helbig *et al.* 2005); *FIB5*: Fib5/Fib6 (Marini & Hackett 2002) and *RAG-1*: R17/R22 (Groth & Barrowclough 1999) and R50/R51 (Irestedt 2001). In our analyses, we included sequences available in GenBank and the new sequences generated in this study were deposited in GenBank (see Table 3.1.1).

PCR amplifications were carried out in the following reaction mixture (total volume of 50 µL): 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris (pH 8.5), 50 mmol/L KCl, 100 µmol/L dNTPs, 0.2 units of *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng DNA, and 5 pmol of primers. PCR conditions consisted of an initial denaturation step at 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, annealing temperature for 1 min and 72 °C; plus a final extension step at 72 °C for 10 min. Optimal annealing temperatures were measured by a gradient PCR in a Tgradient thermocycler (Biometra, Gottingen, Germany). Annealing temperatures were: 50 °C (*ND2*), 51.5 °C (*COI*, *LDH* and *FIB5*), 52 °C (*cyt b*), 53 °C (*RAG-1*) and 56 °C (*MYO* and *ODC*). PCR products were precipitated with 4 M NH_4Ac and ethanol (1 : 1 : 6) and centrifuged for 15 min (13,000 rpm). Sequencing was performed on an ABI 3730 automated capillary sequencer (Applied Biosystems, CA, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (performed by STARSEQ GmbH, Mainz, Germany). Sequencing primers correspond to the primers used in PCR amplifications.

ISSR genomic fingerprinting

ISSR–PCR was performed with 60 ng of template DNA in 25 µL reaction volumes containing 10 pmol of the 5´-anchored microsatellite repeat primer [MW_4 $(GACA)_4$ or $(GGTA)_4$], 0.1 mM each of dGTP, dCTP and dTTP, 0.045 mM dATP, 1 µCi (α-^{33}P)-dATP (Perkin Elmer, LAS, Rodgau, Germany), 0.6 units of *Taq* DNA polymerase (Bioron) and 2.5 µL of 10 × amplification buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$). DNA amplifications were performed in a thermocycler Tgradient (Biometra, Goettingen,

Germany). Following the initial 5 min denaturation step at 94 °C, the program consisted of 38 cycles of 50 s at 94 °C, 40 s at 50 °C, 2 min at 72 °C and 10 min at 72 °C for final elongation. Amplified products were mixed with 8 µL bromophenol blue and run on a high-resolution denaturing polyacrylamide gels 6 % (0.2 mm) for 4 h at 65 W (gel length 40 cm) containing 1 × TBE buffer. The gels were dried and exposed for 2–5 days (depend on levels of radioactivity) to X-ray hyperfilms (Kodak, Taufkirchen, Germany) and subsequently developed. Gel electrophoresis and PCR reactions were performed twice for each sample to verify the repeatability of the results.

Alignment and phylogenetic analyses

Alignment was performed using Clustal W (Thompson *et al.* 1994) available in BioEdit version 7.0.9.0 (Hall 1999). All alignments were inspected and corrected visually. For nuclear loci, heterozygous sites were coded as ambiguous. The concatenated alignment consisted of 6280 nucleotides. Bayesian inference (BI) phylogenetic analyses of mitochondrial and nuclear genes were conducted in MrBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) and Maximum Likelihood (ML) analyses in MEGA version 5.2 (Tamura *et al.* 2011). Best evolutionary models that fit our data were selected according to a Bayesian Inference Criteria (BIC) in JModelTest version 2.1.4 (Posada 2008). BI were carried out with two independent runs of 10,000,000 generations each were conducted and trees were sampled every 2000 generations, with the first 10 % of samples discarded as "burn-in". ML node support was assessed through 1000 bootstrap replicates. Phylogenetic analyses were independently conducted on every single gene and three main partitions: mitochondrial genes (*cyt b*, *ND2* and *COI*), nuclear genes (*MYO*, *ODC*, *LDH*, *FIB5* and *RAG-1*), and all genes combined in a single matrix.

Molecular clock based on mtDNA

Molecular clock analyses were conducted with three mitochondrial genes, i.e. *cyt b*, *ND2* and *COI*. We made use of the standard universal clock with a mutational rate of 2.1 % per million years or 0.01105 substitutions/site/lineage/million years (Weir & Schluter 2008), a relaxed lognormal clock model, Yule process, 10 million generations, sampling every 1000 trees and 10 % as "burn-in". These analyses were performed in BEAST version 1.7.5 (Drummond & Rambaut 2007a). Stationarity was assessed in TRACER 1.5 1 (Rambaut & Drummond 2007).

Table 3.1.1 Studied taxa in alphabetical order, locality, collector, IPMB (Institute of Pharmacy and Molecular Biotechnology) numbers and GenBank accession numbers for every gene. Sequences that are new to this study are in bold. Asterisks indicate the sequences have previously done in IPMB lab (Leisler *et al.* 1997).

Taxon	**Locality**	**Collector**	**IPMB**	***COI***	***cyt b***	***ND2***	***FIB5***	***LDH***	***MYO***	***ODC***	***RAG-1***
Acrocephalus agricola	Russia	A. Poluda	60251	**KJ453121**	AJ004247*	**KJ453197**	**KJ453263**	FJ883055	FJ883108	FJ883127	**KJ453456**
	Russia	A. Poluda	60252	**KJ453122**	AJ004245*	**KJ453198**	**KJ453262**	**KJ453329**	**KJ453373**	**KJ453414**	**KJ453457**
Acrocephalus arundinaceus	Kazakhstan	G. Nikolaus	47571	FR847226	FJ883022	AB621334	**KJ453264**	FJ883056	FJ883098	**KJ453415**	**KJ453460**
	Crete	D. Ristow	25724	**KJ453123**	AJ004253*	GQ242092	**KJ453265**	**KJ453331**	DQ008530	FJ883128	**KJ453461**
Acrocephalus australis	West Australia	K. Schulze-Hagen	6544	**KJ453124**	AJ004306*	**KJ453200**	**KJ453267**	**KJ453330**	**KJ453374**	**KJ453416**	**KJ453459**
	West Australia	K. Schulze-Hagen	6543	**KJ453125**	AJ004786	**KJ453199**	**KJ453266**	FJ883057	FJ883097	FJ883129	**KJ453458**
Acrocephalus baeticatus	Namibia	U. Franke-Bryson	58926	**KJ453126**	**KF547904**	**KJ453201**	**KJ453268**	FJ883058	FJ883103	**KJ453417**	**KJ453462**
	Namibia	U. Franke-Bryson	58927	KF467519	**KF547905**	**KJ453202**	**KJ453269**	**KJ453333**	**KJ453375**	**KJ453418**	**KJ453463**
Acrocephalus bistrigiceps	Siberia	J. Martens	6629	**KJ453127**	FJ883025	**KJ453203**	**KJ453270**	FJ883059	**KJ453376**	**KJ453419**	**KJ453464**
	Thailand	G. Nikolaus	6659	**KJ453128**	**KJ453182**	AY136601	**KJ453271**	**KJ453334**	FJ883109	FJ883131	**KJ453465**
Acrocephalus brevipennis	Cape Verde	W. Fiedler	2285	**KJ453129**	FJ883026	**KJ453205**	**KJ453273**	FJ883060	FJ883099	FJ883132	**KJ453467**
	Cape Verde	W. Fiedler	2288	**KJ453130**	AJ004259*	**KJ453204**	**KJ453272**	**KJ453335**	**KJ453377**	FJ883132	**KJ453466**
Acrocephalus concinens	Thailand	S. Rumzey	212	**KJ453131**	FJ883027	**KJ453206**	**KJ453274**	FJ883061	**KJ453379**	**KJ453421**	**KJ453468**
	Thailand	P. Rorind	213	**KJ453132**	**KJ453183**	**KJ453207**	**KJ453275**	**KJ453332**	**KJ453378**	**KJ453420**	**KJ453469**
Acrocephalus dumetorum	Kazakhstan	G. Nikolaus	3134	**KJ453133**	AJ004263*	**KJ453208**	EF626749	FJ883062	AY887682	FJ883134	FJ358146
	Kazakhstan	G. Nikolaus	6648	**KJ453134**	AJ004264*	**KJ453209**	**KJ453276**	**KJ453336**	FJ883105	EF625338	**KJ453483**
Acrocephalus gracilirostris	South Africa	G. Nikolaus	6646	**KJ453135**	FJ883029	**KJ453210**	**KJ453277**	**KJ453337**	FJ883095	**KJ453422**	**KJ453470**
	Kenya	G. Nikolaus	6668	**KJ453136**	AJ004266*	**KJ453211**	**KJ453278**	**KJ453338**	**KJ453380**	FJ883135	**KJ453471**
Acrocephalus griseldis	Kenya	G. Nikolaus	46384	**KJ453138**	AJ004779	**KJ453212**	**KJ453279**	FJ883064	FJ883092	**KJ453423**	**KJ453472**
	Kenya	46391	46391	**KJ453137**	AJ004271*	**KJ453213**	**KJ453280**	**KJ453339**	**KJ453381**	FJ883136	**KJ453473**
Acrocephalus melanopogon	Austria	B. Leisler	6510	**KJ453139**	AJ004767	**KJ453214**	**KJ453281**	FJ883066	**KJ453382**	**KJ453424**	**KJ453474**
	Austria	B. Leisler	6509	**KJ453140**	**KJ453184**	**KJ453215**	**KJ453282**	**KJ453340**	**KJ453383**	**KJ453425**	**KJ453475**
Acrocephalus newtoni	Madagascar	F. Woog	44294	**KJ453141**	AJ004283*	**KJ453216**	**KJ453283**	**KJ453341**	FJ883123	**KJ453426**	AY319972
	Madagascar	F. Woog	6611	**KJ453142**	AJ004780	**KJ453217**	**KJ453284**	FJ883067	**KJ453384**	FJ883139	**KJ453476**
Acrocephalus orientalis	Philippines	J. Martens	6676	JF499074	AB159187	**KJ453218**	**KJ453285**	FJ883068	FJ883102	**KJ453427**	**KJ453477**
	Philippines	J. Martens	6675	**KJ453143**	AJ004289*	**KJ453219**	**KJ453286**	**KJ453342**	**KJ453385**	**KJ453428**	**KJ453478**
Acrocephalus paludicola	Lithuania	B. Giessing	26894	**KJ453144**	AJ004768	**KJ453220**	**KJ453287**	FJ883069	**KJ453387**	**KJ453429**	**KJ453479**
	Lithuania	B. Giessing	26893	**KJ453145**	AJ004292*	**KJ453221**	**KJ453288**	**KJ453343**	FJ883112	FJ883141	**KJ453480**
Acrocephalus palustris	Germany	H.-H. Witt	52642	**KJ453146**	EU861031	**KJ453222**	**KJ453289**	FJ883070	**KJ453388**	**KJ453430**	**KJ453481**
	Crete	D. Ristow	19601	**KJ453147**	AJ004774	**KJ453223**	**KJ453290**	**KJ453344**	FJ883106	FJ883142	**KJ453482**
Acrocephalus rufescens	Kenya	G. Nikolaus	6672	**KJ453148**	AJ004296*	**KJ453224**	**KJ453291**	FJ883071	FJ883124	**KJ453431**	**KJ453484**
	Kenya	G. Nikolaus	6673	**KJ453149**	AJ004295*	**KJ453225**	**KJ453292**	**KJ453345**	**KJ453386**	FJ883143	**KJ453485**
Acrocephalus schoenobaenus	Israel	P.Heidrich	12562	**KJ453150**	FJ883038	**KJ453226**	**KJ453293**	FJ883072	FJ883107	**KJ453432**	**KJ453486**
	Israel	P.Heidrich	12557	**KJ453151**	AJ004244*	**KJ453227**	**KJ453294**	**KJ453346**	**KJ453389**	FJ883144	**KJ453487**

Taxon	**Locality**		**IPMB**	***COI***	***cyt b***	***ND2***	***FIB5***	***LDH***	***MYO***	***ODC***	***RAG-1***
Acrocephalus s. scirpaceus	Germany	H.-H. Witt	54327	**KJ453152**	**KF547878**	**KJ453229**	**KJ453302**	**KJ453348**	**KJ453393**	**KJ453436**	**KJ453491**
	Germany	H.-H. Witt	54355	**KJ453153**	**KF547875**	**KJ453228**	**KJ453303**	**KJ453347**	**KJ453392**	**KJ453435**	**KJ453490**
Acrocephalus scirpaceus fuscus	Saudi Arabia	G. Nikolaus	47548	**KJ453154**	**KF547898**	**KJ453230**	**KJ453300**	**KJ453349**	**KJ453390**	**KJ453433**	**KJ453488**
	Kazakhstan	G. Nikolaus	47555	**KJ453155**	**KF547895**	**KJ453231**	**KJ453301**	**KJ453350**	**KJ453391**	**KJ453434**	**KJ453489**
Acrocephalus sechellensis	Seychelles	U. Querner	6612	**KJ453156**	AJ004284*	**KJ453232**	**KJ453295**	**KJ453351**	**KJ453398**	**KJ453437**	**KJ453492**
	Seychelles	U. Querner	-	-	AJ004781	-	-	FJ883074	FJ883122	FJ883146	-
Acrocephalus stentoreus	Oman	G. Nikolaus	6561	FJ465293	FJ883031	**KJ453233**	**KJ453296**	FJ883065	FJ883100	FJ883137	**KJ453495**
	Somalia	G. Nikolaus	46956	**KJ453157**	AJ004307*	**KJ453234**	**KJ453297**	**KJ453352**	**KJ453396**	**KJ453438**	**KJ453496**
Acrocephalus tangorum	Thailand	S. Rumzey	6662	**KJ453158**	FJ883041	**KJ453235**	**KJ453305**	FJ883075	**KJ453397**	**KJ453439**	**KJ453497**
	Thailand	P. Rorind	6661	**KJ453159**	AJ004777	**KJ453236**	**KJ453304**	**KJ453353**	FJ883110	FJ883147	**KJ453498**
Acrocephalus vaughani	Henderson Island	De L. Brooke	6631	**KJ453161**	**KJ453185**	**KJ453237**	**KJ453299**	**KJ453354**	**KJ453394**	**KJ453440**	**KJ453493**
	Henderson Island	De L. Brooke	6633	**KJ453160**	**KJ453186**	**KJ453238**	**KJ453298**	**KJ453355**	**KJ453395**	**KJ453441**	**KJ453494**
Bradypterus baboecala	South Africa	G. Nikolaus	-	-	FJ883053	AY382344	-	FJ883090	DQ008525	FJ883162	JX236420
Calamonastides gracilirostris	Kenya	G. Nikolaus	47573	**KJ453162**	FJ883043	**KJ453239**	**KJ453306**	FJ883077	FJ883113	FJ883149	**KJ453499**
	Kenya	G. Nikolaus	47574	**KJ453163**	AJ004765	**KJ453240**	**KJ453307**	**KJ453356**	**KJ453399**	**KJ453442**	**KJ453500**
Hippolais icterina	Germany	H.-H. Witt	54494	**KJ453164**	DQ008479	GQ242091	EU680651	**KJ453367**	FJ883120	GQ242150	**KJ453516**
	Crete	D. Ristow	11487	**KJ453165**	FJ883046	FJ178357	**KJ453325**	FJ883078	DQ008531	EU680731	**KJ453517**
Hippolais languida	Kenya	G. Nikolaus	47564	**KJ453166**	FJ883047	**KJ453242**	**KJ453308**	FJ883079	**KJ453402**	FJ883154	**KJ453502**
	Kenya	G. Nikolaus	47565	**KJ453167**	AJ004794	**KJ453241**	**KJ453309**	**KJ453357**	**KJ453403**	**KJ453443**	**KJ453501**
Hippolais olivetorum	Kenya	G. Nikolaus	64439	JQ175054	AJ004795	**KJ453243**	**KJ453310**	FJ883080	FJ883121	FJ883155	**KJ453503**
	Kenya	G. Nikolaus	47566	JQ175053	**KJ453189**	**KJ453244**	**KJ453311**	**KJ453361**	**KJ453404**	**KJ453444**	**KJ453504**
Hippolais polyglotta	Senegal	H.-H. Witt	10325	**KJ453169**	AJ004797	**KJ453245**	**KJ453312**	FJ883081	FJ883115	**KJ453445**	**KJ453505**
	Senegal	H.-H. Witt	6708	**KJ453168**	**KJ453196**	**KJ453246**	**KJ453313**	**KJ453362**	**KJ453405**	FJ883157	**KJ453506**
Iduna aedon	China	J. Dierschke	61111	JQ173900	AJ004778	**KJ453247**	**KJ453314**	**KJ453358**	FJ883104	FJ883126	**KJ453507**
	India	J. B. Kristensen	62313	**KJ453170**	AF094623	**KJ453248**	**KJ453315**	**KJ453359**	DQ125965	**KJ453446**	**KJ453508**
Iduna caligata	Kazakhstan	K. Schulze-Hagen	6585	GQ481975	FJ883044	**KJ453250**	**KJ453317**	**KJ453363**	FJ883116	FJ883151	**KJ453512**
	Western Siberia	B.Giessing	17076	GQ481974	AJ004793	**KJ453249**	**KJ453316**	FJ883084	**KJ453406**	**KJ453447**	**KJ453511**
Iduna natalensis	Uganda	J. Garcia-Moreno	61113	**KJ453171**	AF094620	**KJ453252**	**KJ453319**	**KJ453369**	**KJ453408**	FJ883150	**KJ453519**
	Tanzania	J. B. Kristensen	62314	**KJ453172**	DQ008523	**KJ453251**	**KJ453318**	**KJ453368**	FJ883114	**KJ453448**	**KJ453518**
Iduna opaca	Guinea	Ch. Meibauer	47562	**KJ453173**	**KJ453195**	**KJ453253**	**KJ453320**	**KJ453364**	**KJ453407**	**KJ453449**	**KJ453513**
	Senegal	-	-	-	FJ883049	-	-	FJ883086	FJ883118	FJ883156	FJ358146
Iduna pallida	Kenya	Ch. Meibauer	47556	**KJ453175**	**KJ453187**	**KJ453254**	**KJ453321**	FJ883085	**KJ453400**	**KJ453450**	**KJ453509**
	Saudi Arabia	Ch. Meibauer	47560	**KJ453174**	**KJ453188**	**KJ453255**	**KJ453322**	**KJ453360**	**KJ453401**	**KJ453451**	**KJ453510**
Iduna rama	Kazachstan	J. Scheider	61109	**KJ453176**	**KJ453190**	**KJ453256**	**KJ453323**	**KJ453366**	**KJ453409**	**KJ453452**	**KJ453514**
	Afghanistan	C. Bamian	61110	**KJ453177**	**KJ453191**	**KJ453257**	**KJ453324**	**KJ453365**	**KJ453410**	**KJ453453**	**KJ453515**
Iduna similis	Kenya	J. B. Kristensen	62316	**KJ453178**	FJ899738	**KJ453258**	**KJ453326**	FJ883083	FJ883125	FJ883159	**KJ453520**
	Kenya	J. B. Kristensen	62317	**KJ453179**	**KJ453192**	**KJ453259**	**KJ453327**	**KJ453370**	**KJ453411**	**KJ453454**	**KJ453521**
Locustella fluviatilis	Kenya	D. J. Pearson	6700	JQ175275	HQ608847	GQ242093	GQ242048	HQ706203	DQ008527	GQ242152	**KJ453524**
Megalurus palustris	-	-	-	JF957022	DQ008477	JN614731	EU680661	FJ883089	DQ008529	FJ883161	AY319988
Nesillas typica	Madagascar	F. Woog	44295	**KJ453180**	**KJ453193**	**KJ453260**	EU680665	**KJ453371**	**KJ453412**	**KJ453455**	**KJ453522**
	Madagascar	F. Woog	44297	**KJ453181**	**KJ453194**	**KJ453261**	**KJ453328**	**KJ453372**	**KJ453413**	EU680744	**KJ453523**
Pnoepyga pusilla	-	-	-	JQ175914	JX518496	JX518511	JN826121	-	FJ357960	JX518521	FJ358125

ISSR analyses

ISSR-PCR bands were coded with 1 = band present and 0 = band absent (Table 3.1.3). Clustering and Neighbor joining tree reconstructions were conducted with Jaccard coefficient and 1000 bootstrap replicates in FAMD 1.31 (Schlüter & Harris 2006). The tree was visualized using TreeView version 1.6.6 (Page 1996).

3.1.4 Results

Phylogenetic inferences

Our mitochondrial dataset for 72 individuals comprised 2782 characters. We translated the nucleotide sequences to amino acid sequences using MEGA and did not find either stop codons nor indels, suggesting that nuclear pseudogenes were not amplified (Allende *et al.* 2001). We also obtained sequences of five nuclear genes for all species (Table 3.1.1). Including indels, the concatenated mitochondrial and nuclear alignment comprised 6280 sites. Details are presented in Table 3.1.2. From all reconstructed trees only five reconstructions are shown in this publication: combined mitochondrial genes (Fig. 3.1.1.), combined nuclear genes (Fig. 3.1.2), combined mitochondrial and nuclear genes (Fig. 3.1.3) and a chronogram based on mitochondrial genes (Fig. 3.1.4). Finally, the Neighbor-joining tree from ISSR fingerprints is illustrated in Fig. 3.1.6. All reconstructed trees (Figs. 3.1.1–3.1.4) reveal six major clades in agreement with Leisler *et al*. (1997), Helbig and Seibold (1999) and Fregin *et al*. (2009). Genus *Acrocephalus* with (1) subgenus *Acrocephalus*: large reed warblers (Helbig & Seibold 1999), (2) subgenus *Calamocichla*: Afrotropical/Malagasy reed warblers (Leisler *et al.* 1997), (3) subgenus *Calamodus*: streaked reed warblers plus unstreaked *A. bistrigiceps* (Leisler *et al.* 1997) and (4) subgenus *Notiocichla*: small unstreaked reed warblers (Leisler *et al.* 1997). The former genus *Hippolais* had been split into two clades: (5) *Hippolais* (Helbig & Seibold 1999) and (6) *Iduna* (Fregin *et al.* 2009) which exclude *I. aedon* (formerly *Acrocephalus aedon*). The subgenera *Acrocephalus* and *Calamocichla* cluster as sister groups in all reconstructed trees with high support in the mitochondrial (Fig. 3.1.1), nuclear (Fig. 3.1.2) and combined dataset (Fig. 3.1.3); and *Acrocephalus griseldis* clusters as a sister to the aforementioned clades. *Calamodus* is basal to the cluster of *Acrocephalus*/*Calamocichla* and *Notiocichla* is basal to all other reed warblers, suggesting that the ancestral reed warbler was small and unstreaked. The *Hippolais* clade (according to Fregin *et al*., 2009) was well supported in all analyses. Within *Iduna*, a sister relationship was found between *I. natalensis* and *I. similis* (previously placed in the genus *Chloropeta*; Sibley and Monroe, 1990), and *I. rama* and *I. caligata* (Figs. 3.1.1–3.1.3). *Iduna aedon* (Fregin *et al.* 2009), also regarded as

Phragamaticola aedon (Helbig & Seibold 1999) or *Acrocephalus aedon*, can be found in differing positions in Figs. 3.1.1–3.1.3. Mostly, *aedon* clusters as a sister clade to *Calamonastides gracilirostris* with a poor bootstrap support. *Nesillas typica* from Madagascar was basal to all these clades; however, this association was only supported in BI with high bootstrap values.

Our chronogram based on a coalescent multi-locus analysis of three mitochondrial genes revealed that most of the speciation events might have occurred in the Middle Miocene to Early Pleistocene (12.5–0.90 million years ago; Fig. 3.1.4).

Table 3.1.2 Number of nucleotides and polymorphic sites (without outgroups) for each partition and combined data set.

Gene	Nucleotides	PI sites (percentage)
cyt b	1043	421 (40.36 %)
ND2	1077	506 (46.98 %)
COI	662	237 (35.80 %)
MYO	697	78 (11.19 %)
ODC	774	107 (13.82 %)
LDH	492	93 (18.90 %)
FIB5	562	97 (17.26 %)
RAG-1	944	44 (4.66 %)
Mitochondrial loci	2782	1164 (41.84 %)
Nuclear loci	3498	419 (11.98 %)
Combined dataset	6280	1583 (25.21 %)

ISSR

ISSR-PCR fingerprints obtained with the primers $(GACA)_4$ and $(GGTA)_4$ from 22 taxa of Acrocephalidae (two individuals per taxon with the exception of *Iduna opaca*) are shown in Fig. 3.1.5. Profiles were more diverse with the $(GGTA)_4$ primer than $(GACA)_4$; however, a series of group-specific bands were obtained. In total, 45 clearly identifiable bands were available for analysis. The ISSR tree is congruent with our phylogenetic analyses based on nucleotide sequences. The major clades within the genus *Acrocephalus* (the subgenus *Calamocichla* was not included) and the distinction between *Hippolais* and *Iduna* are also detected by ISSR analysis. In agreement with the sequence data, *Iduna aedon* did not cluster within *Iduna* and *Calamonastides gracilirostris* clustered as an isolated clade (Fig. 3.1.6).

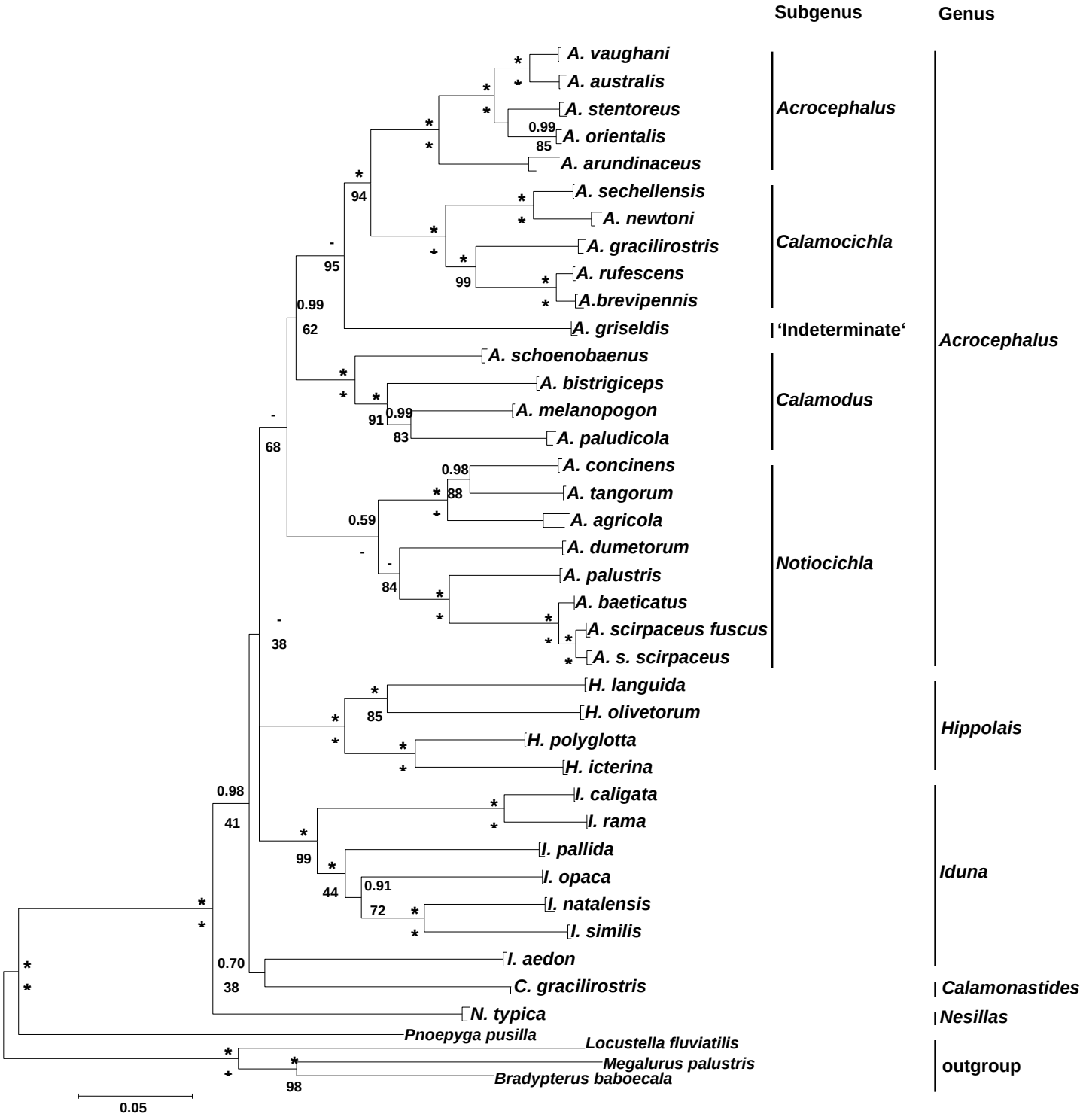

Fig. 3.1.1 Bayesian inference tree based on concatenated mitochondrial *cyt b*, *ND2* and *COI*. BI posterior probabilities and ML bootstrap values indicated above and below the branches, respectively. Asterisks indicate posterior probability = 1.00 or bootstrap = 100%. Terminal branches contain two individuals per species. Species names and genera at the right column follow Fregin *et al*. (2009) and subgenera within *Acrocephalus* are indicated on the left side. The small scale bar at the bottom (*N*=0.05) indicates the numbers of nucleotide substitutions per site.

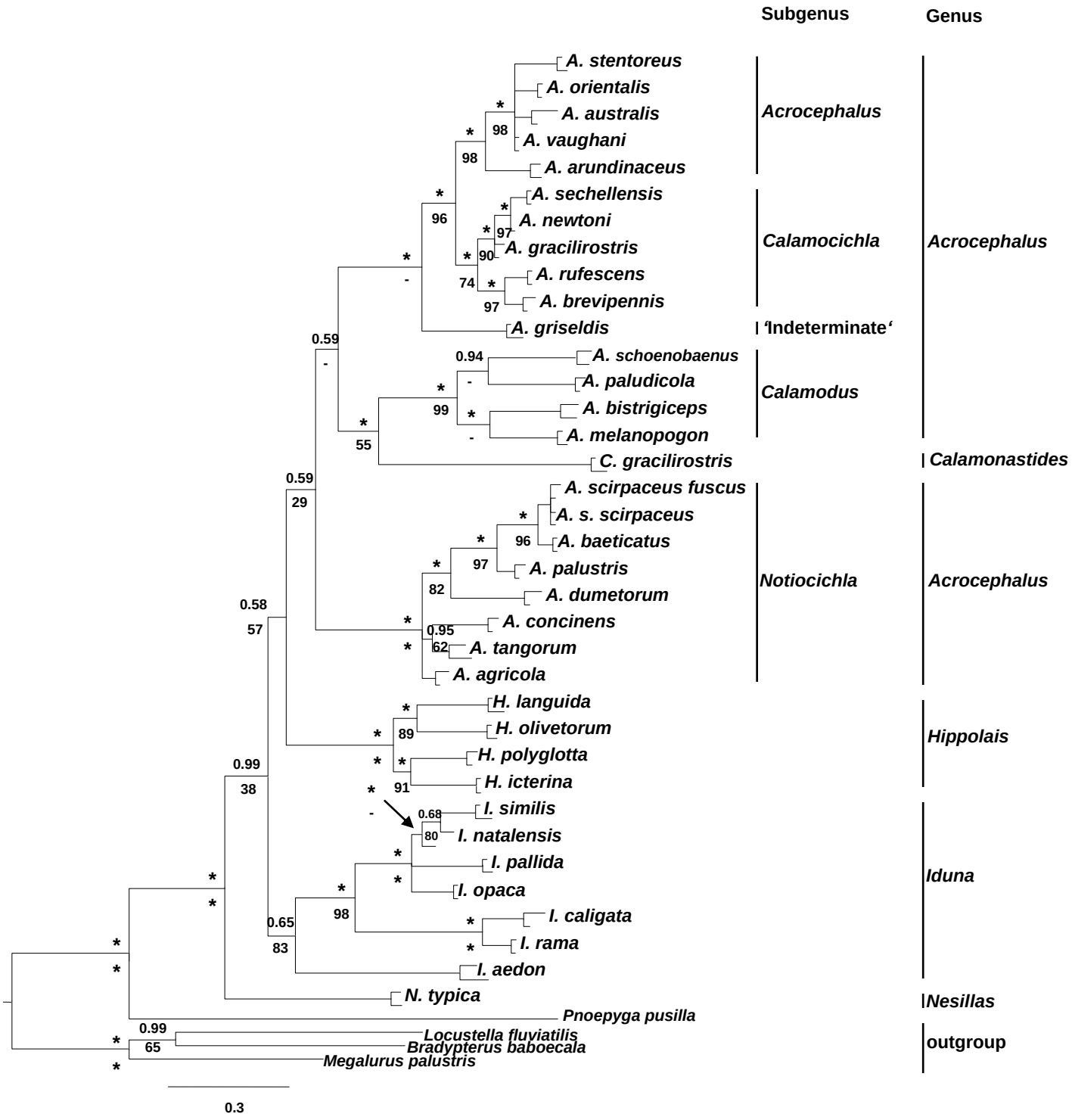

Fig. 3.1.2 Bayesian inference tree based on nuclear genes concatenated (*MYO, ODC*, *LDH*, *FIB5* and *RAG-1*). Support values as in Fig. 3.1.1. Terminal branches contain two individuals per species. Species names and genera on the right column follow Fregin *et al*. (2009) and subgenera within *Acrocephalus* are shown on the left column. The small scale bar at the bottom (*N*=0.3) indicates the numbers of nucleotide substitutions per site.

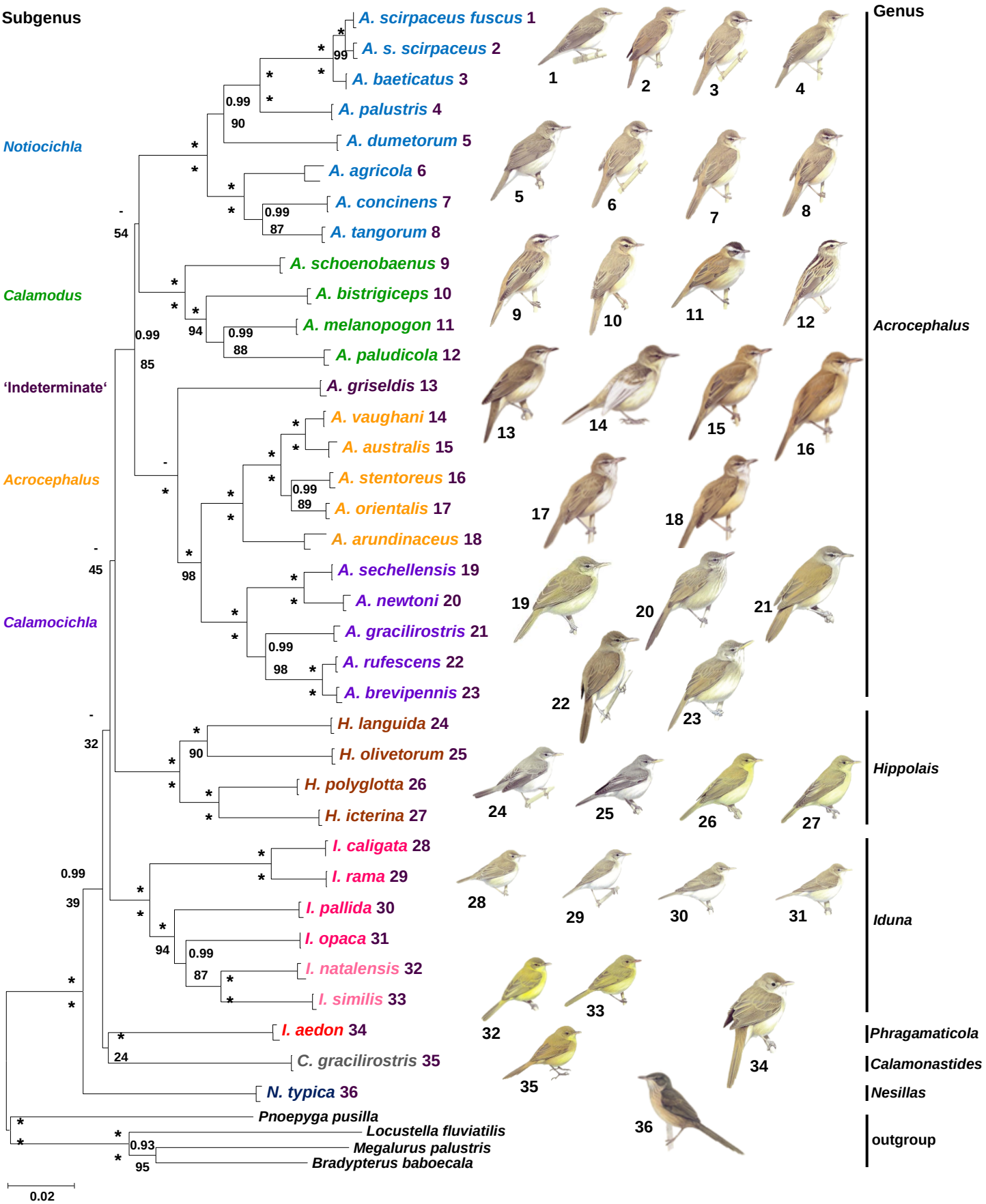

Fig. 3.1.3 Phylogenetic tree based on combined mitochondrial and nuclear dataset, analysed by Bayesian inference. Support values marked as in Fig. 3.1.1. Species names follow Fregin *et al.* (2009), left side indicates subgenera within Acrocephalus and genera suggested by the authors are on the right. The small scale bar at the bottom (*N*=0.02) indicates the numbers of nucleotide substitutions per site. Photos from del Hoyo *et al.* (2006).

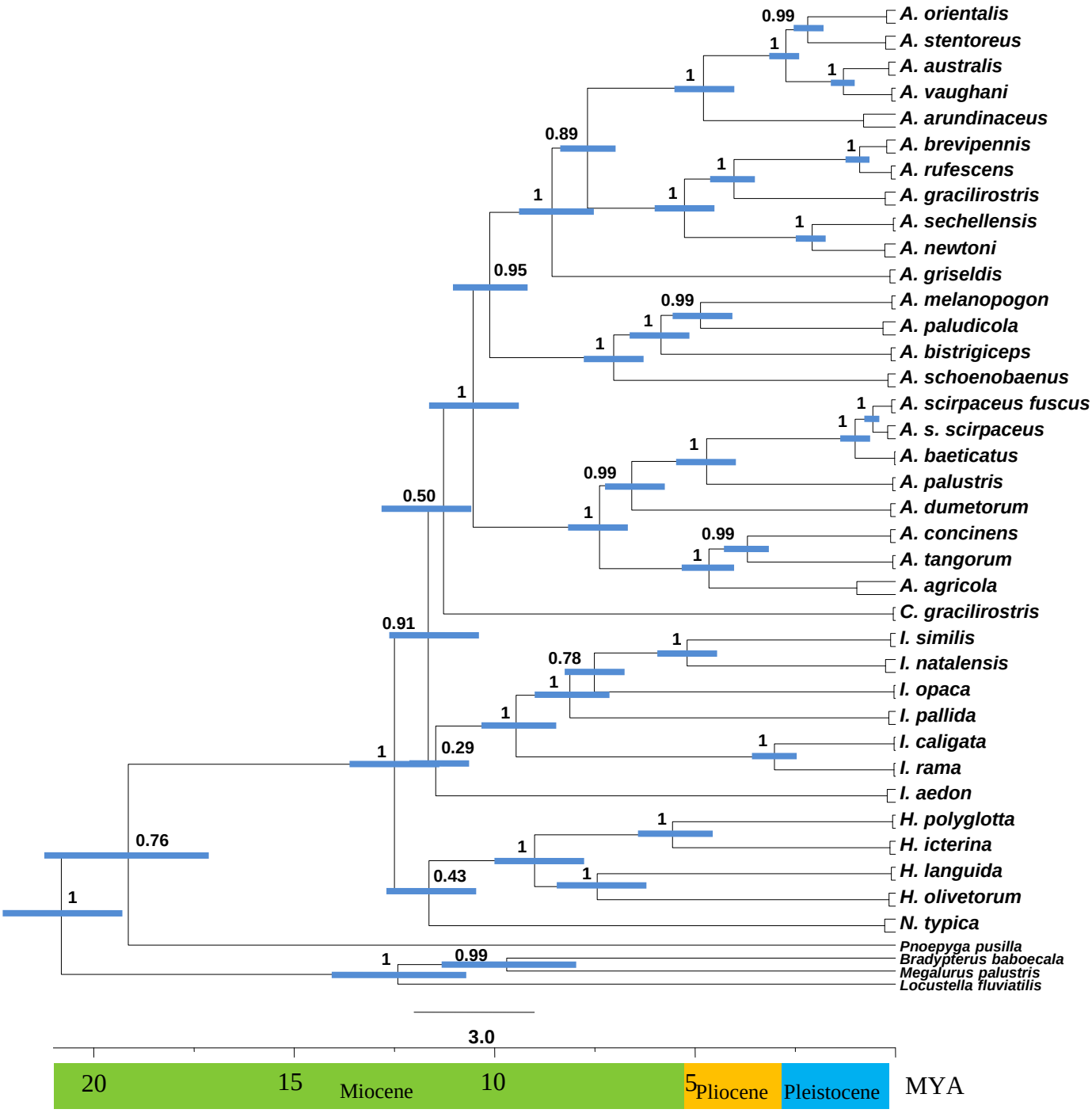

Fig. 3.1.4 Chronogram of Acrocephalidae reconstructed based on three mitochondrial genes and assuming a relaxed molecular clock with a mutational rate of 2.1% per million years. Blue bars represent 95% highest posterior density intervals for the node ages. Posterior probabilities are indicated at the nodes. Species names follow Fregin *et al*. (2009). The small scale bar at the bottom (*N*=0.3) indicates the numbers of nucleotide substitutions per site.

3.1.5 Discussion

The clade of the subgenus *Acrocephalus*, which contains a large assemble of large reed warblers (*A. vaughani*, *A. australis*, *A. stentoreus*, *A. orientalis* and *A. arundinaceus*) from Palearctic-Australasia, is strongly supported by all our phylogenetic reconstructions. Both Ml and BI supported the sister relationship between *vaughani* and *australis* (PP = 1.0, ML bootstrap = 100%); in agreement with Leisler *et al*. (1997), the only phylogenetic study that considered *vaughani*. The sister relationship of *stentoreus* and *orientalis*, which was not supported strongly in previous studies, received high (PP = 0.99) to moderate (ML = 86%) support in our analyses, with *Acrocephalus arundinaceus* as a sister group. In agreement with previous molecular studies the large African reed warblers (subgenus *Calamocichla*) form consistently a monophyletic group together with subgenus *Acrocephalus*. *Calamocichla* consists of two well-defined groups: one clade contains *A. newtoni*/*A. sechellensis* from Madagascar and the Seychelles and the other includes *A. gracilirostris* as sister to the *A. rufescens*/*A. brevipennis* clade. The other larger reed warbler, *A. griseldis*, occupies a basal position in relation to other large reed warblers (subgenera *Acrocephalus* and *Calamocichla*). Due to this particular phylogenetic position, it had been nominated as ‘‘Indeterminate” subgenus by Helbig and Seibold (1999). Four striped reed warbler species of the monophyletic subgenus *Calamodus: A. melanopogon, A. paludicola, A. bistrigiceps,* and *A. schoenobaenus* formed a very well supported clade. *A. bistrigiceps* as sister taxon to the *melanopogon*/*paludicola* pair; and *schoenobaenus* is basal of all of them. These relationships were well supported in trees except the nuclear gene tree. Fregin *et al*. (2009) found different relationships: In the combined nuclear tree and complete concatenated dataset *bistrigiceps* clustered as a sister of *paludicola*, and *melanopogon* the sister of *schoenobaenus* but without high bootstrap support.

The subgenus *Notiocichla* consists of two groups: in one clade *A. baeticatus* is the sister species of the *A. scirpaceus* complex, followed by *A. palustris* and *A. dumetorum* which occupies a basal position within this group. The second group comprises the *A. concinens*/*A. tangorum* clade and the sister *A. agricola*. All these relationships were well resolved in our analyses while previous studies did not provide strong evidence for these associations. Fregin *et al*. (2009) included *A. orinus* and found a sister relationship with *dumetorum*. In most reconstructions reed warblers of the subgenus *Notiocichla* clustered (albeit without strong bootstrap support) at the base of the *Acrocephalus* complex whereas *Calamodus* took an intermediate position (Figs. 3.1.1–3.1.4). The new genus *Hippolais* consists of two well-supported sister clades: 1) *languida* and *olivetorum*, and 2) *polyglotta* and *icterina*. BI and

ML trees based on our combined data set show *Hippolais* in a basal position in relation to the genus *Acrocephalus*, supporting the consensual phylogeny of Dickinson (2003) and Fregin *et al*. (2009).

The newly formed genus *Iduna* splits into the following sister groups with high bootstrap support in all analyses: *caligata/rama* and *natalensis/similis*. *Iduna pallida* and *I. opaca* cluster between these two clades but closer to *natalensis*/*similis* than to *caligata/rama* clade. The association between *Chloropeta natalensis/C. similis* (del Hoyo *et al.* 2006; Dickinson 2003; Sibley & Monroe 1990; Wolters 1982) and warblers of the genus *Iduna* (Fregin *et al.* 2009), was not supported by high bootstrap values. However, these two groups share ISSR bands (see yellow arrows on Fig. 3.1.5); but several other distinct bands distinguish these former *Chloropeta* species from other members of the genus *Iduna* (red arrows on lanes 15–20 in both profiles of Fig. 3.1.5), indicating a long period of genetic isolation.

The placement of *Iduna aedon* (Fregin *et al.* 2009) within the *Iduna* clade was only supported by analyses based on nuclear genes, with a posterior probability of 0.65 and bootstrap support of 83%. Among the individual gene trees, only *ODC* (PP = 1; ML = 91%) and *LDH* (ML = 64%) trees showed the relationship and none of the mitochondrial genes supported this association. The ISSR genomic fingerprint clustering analyses reveal that *aedon* is not a member of *Iduna* (Fig. 3.1.6) and, due to low bootstrap support, the position of this taxon remains uncertain. The ISSR profiles (Fig. 3.1.5) of *aedon* show considerable differences compared with all other species in agreement with diverging morphological characters (Leisler & Schulze-Hagen 2011). Therefore, we suggest to place this taxon in a monotypic genus *Phragamaticola aedon* as suggested earlier (Helbig & Seibold 1999).

The position of *Calamonastides gracilirostris* (Fregin *et al.* 2009; Grant & Mackworth-Praed 1940) remains uncertain. The ML analyses based on both mitochondrial and combined datasets revealed a monophyletic group (but poorly supported) containing *C. gracilirostris* and *I. aedon*. However, this species clustered with *Calamocichla* in our BI tree based on nuclear genes and BEAST analyses where it received high bootstrap support. This relationship has been previously proposed by Grant and Mackworth-Praed (1940), based on morphological similarities.

3.1.6 Conclusions

We used nucleotide sequences from eight loci, a number of markers close to the optimal amount of genes suggested by (Maddison & Knowles 2006), and ISSR fingerprints, in order

to reconstruct a phylogeny for 35 species of Acrocephalidae. Despite the use of more than 6000 nucleotides, some relationships and several deep nodes in our phylogenetic trees were not well-supported. The ISSR fingerprinting evidence did not resolve the basal polytomy in the phylogeny of Acrocephalidae; however, it supports several clades retrieved by DNA sequence analyses.

We accept the current taxonomic position of *Calamonastides gracilirostris* as a monotypic genus, due to the unresolved position in the tree. Although the phylogenetic position of *Iduna natalensis* and *I. similis*, proposed by Fregin *et al*. (2009), was not strongly supported in our analyses, we do not reject the inclusion of these taxa within *Iduna*. We strongly recommend the exclusion of *I. aedon* from *Iduna* and we propose to resurrect the previous status for this species: the monotypic genus *Phragamaticola.*

Estimated divergence times suggest that the Old World warblers shared a common ancestor approximately 12.5 million years ago in the Middle Miocene. This relative young evolutionary time (Beresford *et al.* 2005) of this groups and a subsequent rapid radiation make the systematics of this diverse family a challenge (Helbig & Seibold 1999).

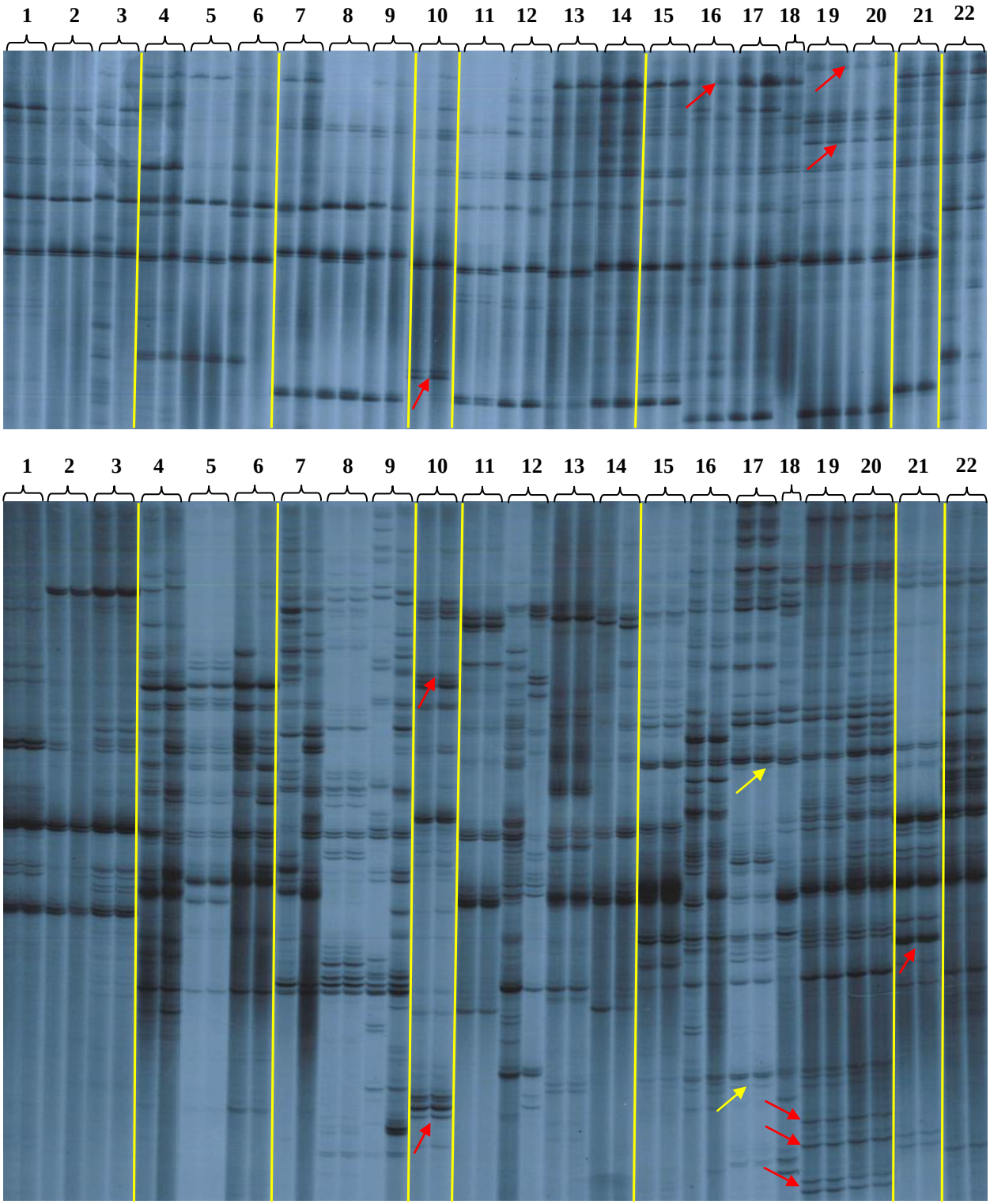

Fig. 3.1.5 ISSR profiles using the primers $(GACA)_4$ (top) and $(GGTA)_4$ (bottom). Numbers correspond to the following groups and species: 1–3 subgenus *Acrocephalus* (*A. arundinaceus*, *A. orientalis*, *A. australis*); 4–6 *Calamodus* (*A. schoenobaenus*, *A. melanopogon*, *A. paludicola*); 7–9 *Notiocichla* (*A. agricola*, *A. scirpaceus*, *A. palustris*); 10 *I. aedon*; 11–14 *Hippolais* (*H. languida*, *H. olivetorum*, *H. polyglotta*, *H. icterina*); 15–18 *Iduna* (*I. rama*, *I. caligata*, *I. pallida*, *I. opaca*, *I. natalensis*, *I. similis*); 19 *Calamonastides gracilirostris*; and 20 *Nesillas typica*. Yellow arrows indicate shared bands and red ones point to the distinct bands.

Table 3.1.3 Matrix of 45 ISSR bands produced by the primers $(GACA)_4$ and $(GGTA)_4$. 1 = band present, 0 = band absent. Species numbers as in Fig. 3.1.5.

Bands	Species numbers																																										
	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18	19		20		21		22	
1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	0	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1
2	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
3	1	1	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
9	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
11	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
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17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
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24	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
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29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
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33	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
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35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
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37	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
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40	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1

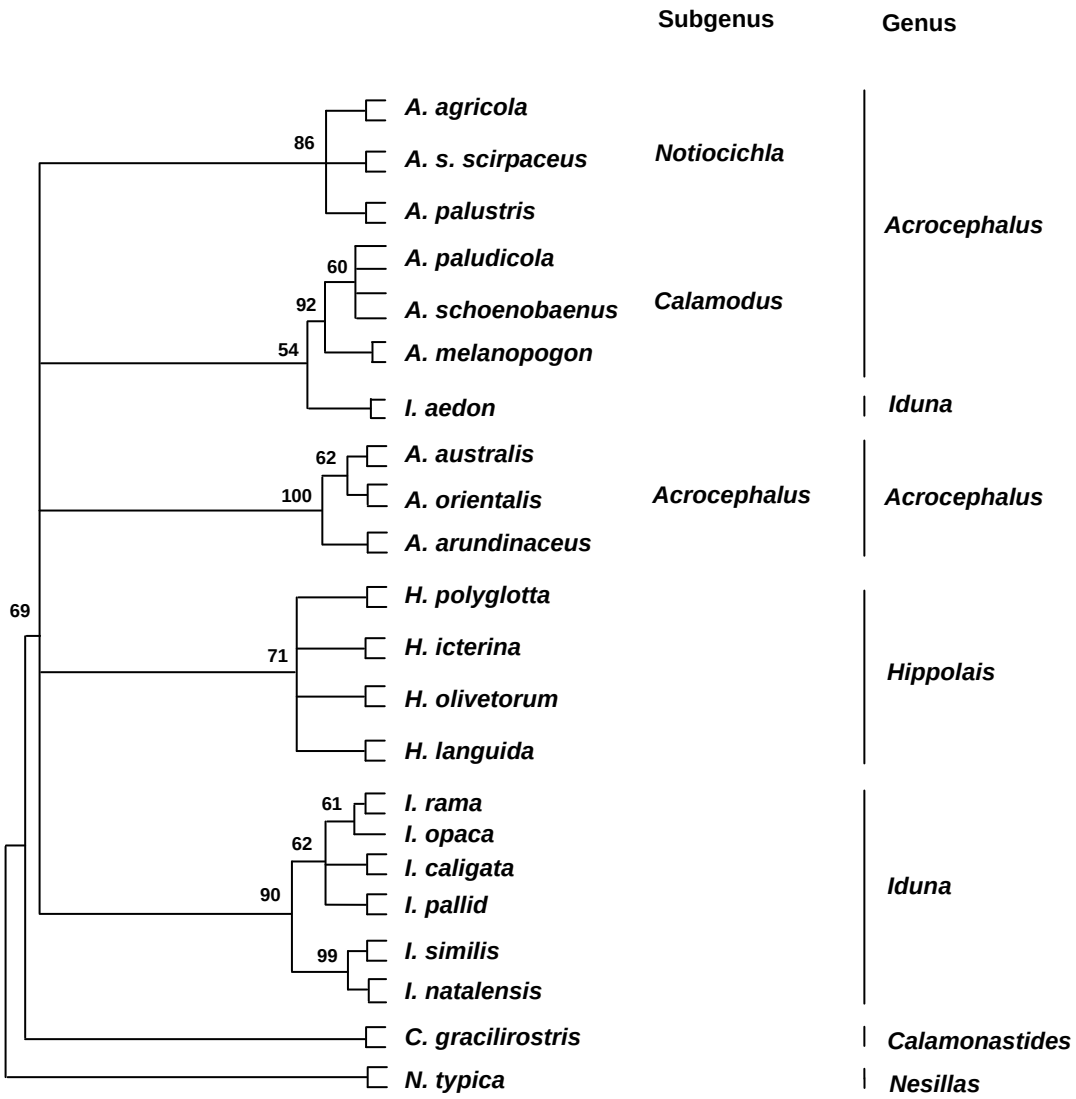

Fig. 3.1.6 Neighbor-joining tree of 22 selected taxa of Acrocephalidae, reconstructed using 45 polymorphic ISSR bands (presented in Table 3.1.3), Jaccard coefficient of similarity and 1000 bootstrap replicates. Species names and genera on the right column follow Fregin *et al*. (2009) and subgenera within *Acrocephalus* are indicated on the left column.

3.2 Mitochondrial Phylogeography of the Eurasian Reed Warbler (*Acrocephalus scirpaceus*) and a First Genetic Report of *A. s. fuscus* in Central Europe

3.2.1 Abstract

Phylogeography and evolutionary history of the long distance migratory Eurasian reed warbler *Acrocephalus scirpaceus* were analyzed using the sequence data from the mitochondrial DNA *COI* gene of 405 individuals and the sequences of *cyt b* form a subset of individuals of *COI* phylogeny in 20 breeding, migrating and wintering populations over its widespread geographic range. Median-joining network and Bayesian phylogenetic analyses recovered three lineages which corresponded to three subspecies of *A. scirpaceus* and started to diverge since approximately 400,000 years ago: one spanning Asia (*A. s. fuscus*); one encompassing Europe and Northern Africa (*A. s. scirpaceus*); and a third, including Eastern Africa and South-western Asia (*A. s. avicenniae*). Phylogenetic analyses suggest that the third clade has the basal position and diverged from its sister species African reed warbler *A. baeticatus* ca. 600,000 years ago. This primitive subspecies (*avicenniae*) may have survived in one of the African refugia, likely low forest refugium of the Ethiopian Highlands during the Last Glacial Maximum. Furthermore, the results of the sequences of 348 samples from Germany, which were captured for ringing, showed that one bird was genetically assigned to the subspecies *fuscus*. As *fuscus* is an Asian subspecies, this is the first genetic evidence of it in Central Europe. According to our DNA sequences, 6.8% of Eurasian reed warblers were misidentified as the wrong species; *A. palustris* was the most misidentified specimen. At the subspecies level, from 22 *fuscus* identified by genetic analyses only six had been correctly named by the ringers.

3.2.2 Introduction

Genus *Acrocephalus* (reed warbler) belongs to the family Acrocephalidae with 37 species and 67 subspecies (Fregin *et al.* 2009). Considering morphology and plumage characteristics, the reed warblers are among the most monomorphic groups of Passeriformes (Leisler & Schulze-Hagen 2011). The Eurasian reed warbler *Acrocephalus scirpaceus* is a small, indistinct, plain and unstreaked warbler. Three subspecies are presently recognised in this large range and long-distance migrant warbler based on differences in feather colour and geographical breeding ranges (Cramp 1992; Dowsett-Lemaire & Dowsett 1987; Pearson *et al.* 2002): *Acrocephalus scirpaceus scirpaceus* (hereafter *scirpaceus*) breeds almost all over Europe,

Northern Africa (Stępniewska & Ożarowska 2012), Western Russia and W Asia Minor. *A. s. fuscus* (hereafter *fuscus*) occupies E Asia Minor, Cyprus and Levant, E to SE Kazakhstan and extreme NW China (W Xinjiang), N and E of Iran, and NW Afghanistan (Hering *et al.* 2009). The third subspecies, *A. s. avicenniae* (hereafter *avicenniae*), breeds in the Red Sea mangroves of Eritrea, Saudi Arabia, Ethiopia, Northern Somalia, Sudan and Yemen (Hering *et al.* 2011; Kennerley & Pearson 2010). In the non-breeding season, *A. scirpaceus* winters in the Sub-Saharan Africa (Cramp 1992; Kralj *et al.* 2010). Israel is a stopover site and all subspecies are common transients, passing through during spring and autumn migration (Merom *et al.* 2000). However, *fuscus* also commonly breeds in Israel between April and June (Hovel 1987; Merom *et al.* 1999).

The plumages of Eurasian reed warbler subspecies and closely related species such as African reed warbler *A. baeticatus*, marsh warbler *A. palustris*, Blyth's reed warbler *A. dumetorum* and paddyfield warbler *A. agricola* are very similar, making them hard or impossible to separate in the field (Dement'ev & Gladkov 1968; Harrap & Quinn 1989; Harris *et al.* 1995), even in the hand, identification can be challenging (Pearson *et al.* 2002; Svensson 1992; Yosef & Chernetsov 2005). Because of morphological similarity, this group of warblers has been the subject of some taxonomic debates. For instance, *A. dumetorum* had been suggested to be a subspecies of *A. scirpaceus* (Devillers & Dowsett-Lemaire 1978; Dowsett-Lemaire & Dowsett 1987; Hall & Moreau 1970); and by comparing habitat, morphology and calls of *A. scirpaceus* and *A. baeticatus*, researchers concluded that these two species are synonymous (Dowsett-Lemaire & Dowsett 1987; Sibley & Monroe 1990, 1993). On the other hand *fuscus* has been considered as a full species, A. *fuscus*, 'Caspian reed warbler' (Sangster *et al.* 1998). In addition of defined a subspecies of *A. scirpaceus* (Helbig & Seibold 1999), *avicenniae* has also been considered to be a subspecies of *A. baeticatus* (Ash *et al.* 1989; Urban *et al.* 1997) or a separate taxon namely the mangrove reed warbler *A. avicenniae* (Sangster *et al.* 1998), but Kennerley and Pearson (2010) treated it as a subspecies of *A. scirpaceus*.

Pearson *et al*. (2002) discussed the identification keys of scirpaceus and fuscus and compared them with *avicenniae* and A. palustris (Pearson *et al.* 2002). In order to distinguish among these taxa, they used combinations of morphological and biometrical characters, distribution range, migration patterns, moult and vocalizations. Careful attention to the wing formula, colouration and other characteristics is needed to separate these taxa. Nevertheless, most keys only refer to adults in fresh plumage (Wolfe *et al.* 2010) and sometimes the distinction is controversial. In recent years, there have been several reports of fuscus from

Britain (Pearson *et al.* 2002), but they remained equivocal due to suspected identification mistakes.

Complementing the traditional taxonomic methods, molecular techniques have proved to be essential for providing valuable information on species limitation and identification. The previous molecular analysis of A. *scirpaceus* by Leisler *et al.*, 1997 and Helbig & Seibold 1999 were based on nucleotide sequences of the cytochrome-b (*cyt b*) gene of a limited data set. Recently, based on 10 microsatellite loci, Procházka *et al.* (2011) investigated the population structure and levels of gene flow of two subspecies of *scirpaceus* and *fuscus* across Europe.. Barrier analysis showed some limitations of gene flow that corresponds to range limits among two subspecies and also among Iberian and the other European populations which support the role of Iberian Peninsula as one of the Pleistocene glacial refugia in Europe.

Here we reconstruct the evolutionary history and population structure of the Eurasian reed warbler complex by comparing nucleotide sequences of two mitochondrial loci, the mitochondrial cytochrome-c oxidase I gene (*COI*) for 405 individuals and *cyt b* for 42 selected individuals from a subset of *COI* phylogeny, from a range-wide sampling. Sequences from previously published papers were combined with our data set and re-analyzed using the last phylogenetic reconstruction methods. Additionally, we made use of BEAST analysis to assess the timing of diversification and demographic changes. Phylogeographic analyses in species with broad distribution, on the scale of continents, are very useful since different populations have not experienced the same evolutionary phenomenon throughout their extensive range, consequently distinct populations containing genetic information of historical events that have differentially influenced them (Adams *et al.* 2006). The objectives of the present study are: (i) to identify clades and test whether their mtDNA sequences are geographically structured; (ii) to confirm the degree of differentiation across the distribution of the species; (iii) to determine the divergence time between the subspecies and the sister species; (iv) to draw the demographic history of the populations including possible past expansions; and (v) to infer the role of the Pleistocene glaciations in driving lineage differentiation.

3.2.3 Methods

Sampling, amplification and sequencing

Blood and tissue samples of 398 *A. scirpaceus* plus two *A. baeticatus* (outgroup) were collected during breeding, migration and wintering (Nigeria and Kenya) season (Table 3.2.1

& Fig. 3.2.1). We amplified and sequenced 633 base pairs (bp) of *COI*. Seven *COI* sequences available in GenBank from Sweden, Russia, Norway and Iran were also included in the analyses (Table 3.2.1). Furthermore we sequenced 830 nt of the *cyt b* from a subset of 32 individuals in order to confirm phylogenetic structure. Ten *cyt b* sequences available in GenBank (Leisler *et al.* 1997), were also added to the analyses. Detailed information of sampling sites, GenBank accession numbers, collectors and voucher numbers are provided in Table 3.2.2.

Table 3.2.1 Localities, number of samples (*N*) of *A. scirpaceus* collected at various sites and collectors.

Code	**Sample site**	***N***	**Collector**
Tre	Treysa (Hesse, Germany)	184	H.-H. Witt
Saa	Saarland (Germany)	139	R. Klein, F. Feß, L. Hayo
Fra	Franconia (Germany)	25	B. Leisler
Isr	Israel	27	P. Heidrich, (Singh *et al.* 2008)
Sau	Saudi Arabia	10	G. Nikolaus, (Helbig & Seibold 1999)
Kaz	Kazakhstan	5	G. Nikolaus, (Helbig & Seibold 1999)
Swe	Sweden	3	(Johnsen *et al.* 2010)
Can	Canary Islands	2	C. Dietzen
Rus	Russia	2	(Kerr *et al.* 2009)
Ukr	Ukraine	2	G. Nikolaus, (Leisler *et al.*, 1997)
Aus	Austria	2	(Leisler *et al.*, 1997)
Eth	Ethiopia	2	(Leisler *et al.*, 1997)
Bul	Bulgaria	1	(Fregin *et al.* 2012)
Swi	Switzerland	1	U. Schneppat
Nor	Norway	1	(Johnsen *et al.* 2010)
Aze	Azerbaijan	1	G. Nikolaus
Ira	Iran	1	(Aliabadian *et al.* 2009)
Tun	Tunisia	1	D. Ristow
Nig	Nigeria	1	G. Nikolaus
Ken	Kenya	1	G. Nikolaus
Total	**20**	**411**	

Total genomic DNA was extracted by an overnight incubation at 37 °C in lysis buffer [10 mmol/L Tris (pH 7.5), 25 mmol/L EDTA, 75 mmol/L NaCl, 1% SDS and 1 mg of Proteinase K (Merck, Darmstadt, Germany)] and standard phenol-chloroform procedures (Sambrook *et al.* 1989). Primer pairs were L-14990/H-16065 (Weir & Schluter 2004) and A1/Fr (Dietzen *et al.* 2003; Leisler *et al.* 1997) for *cyt b*; and PasserF1/PasserR1 and

ExtF/BirdR2 (Johnsen *et al.* 2010; Sheldon *et al.* 2009) for *COI*. PCR reactions were performed in 50µL reaction volumes containing 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris (pH 8.5), 50 mmol/L KCl, 100 µmol/L dNTPs, 0.2 units of *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng DNA and 5 pmol of primers. Optimal annealing temperature was found by gradient PCR in a Tgradient thermocycler (Biometra, Gottingen, Germany). A negative control was run side by side of each PCR. Thermal cycling was performed as follows: (1) an initial denaturation at 94 °C for 5 min; (2) 35 cycles (1 min at 94 °C, 1 min at 51.5 °C and 1 min at 72 °C); and (3) a final 10 min extension at 72 °C. PCR products were cleaned by centrifugation for 30 min at 16 060 *g*; precipitated with 4 mol/L NH_4Ac and ethanol (1 : 1 : 6) and washed with 300 µL 70% ethanol. PCR products were then diluted in 15 µL of distilled water. The PCR product size was verified by agarose electrophoresis (1.4%). Sequencing was performed in an ABI 3730 automated capillary sequencer (Applied Biosystems, CA, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (carried out by STARSEQ GmbH, Mainz, Germany). Sequencing primers correspond to the same primers used for PCR amplifications.

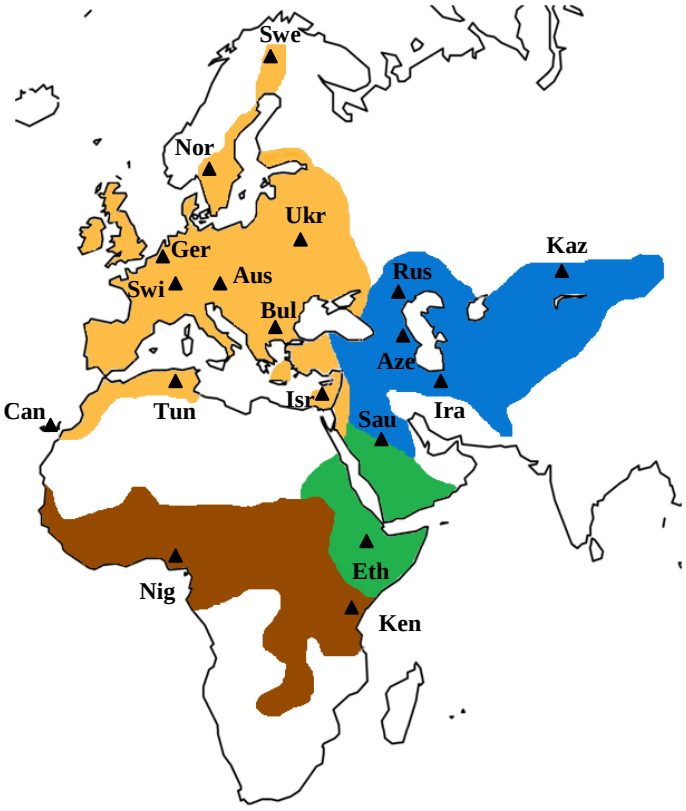

Fig. 3.2.1 Approximate location of the sampling sites of *A. scirpaceus*. Blue (*fuscus*), orange (*scirpaceus*) and green (*avicenniae*) represent the breeding and brown represents wintering distribution of *A. scirpaceus* (del Hoyo *et al.* 2006). The names of localities are abbreviated as in Table 3.2.1. Germany includes Treysa, Saarland and Franconia.

Table 3.2.2 Taxa, IPMB (Institute of Pharmacy and Molecular Biotechnology) numbers, *COI* haplotypes (H), origins, collectors and GenBank accession numbers included in this study. Data are organised according to their Haplotype (H) number. The individuals who share the same haplotype and locality are documented by *N* under Locality.

Taxon	IPMB	H	Country	Locality (*N*)	Collector	*COI*	*cyt b*
A. scirpaceus scirpaceus	52285	1	Germany	Treysa (130)	H. –H. Witt	KF467455	–
A. scirpaceus scirpaceus	2508	1	Germany	Saarland	P. Bauer	–	KF547872
A. scirpaceus scirpaceus	6651	1	Switzerland	–	U. Schneppart	–	–
A. scirpaceus scirpaceus	12567	1	Israel	Eilat	P. Heidrich	–	–
A. scirpaceus scirpaceus	12572	1	Israel	Hazewa	P. Heidrich	–	–
A. scirpaceus scirpaceus	12576	1	Israel	Hazewa	P. Heidrich	–	KF547882
A. scirpaceus scirpaceus	12578	1	Israel	Hazewa	P. Heidrich	–	–
A. scirpaceus scirpaceus	12580	1	Israel	Hazewa	P. Heidrich	–	–
A. scirpaceus scirpaceus	12583	1	Israel	Hazewa	P. Heidrich	–	KF547881
A. scirpaceus scirpaceus	12584	1	Israel	Hazewa	P. Heidrich	–	–
A. scirpaceus scirpaceus	12588	1	Israel	Hazewa	P. Heidrich	–	–
A. scirpaceus scirpaceus	12760	1	Israel	Hazewa	P. Heidrich	–	KF547883
A. scirpaceus scirpaceus	12761	1	Israel	Hazewa	P. Heidrich	–	–
A. scirpaceus scirpaceus	12763	1	Israel	Hazewa	P. Heidrich	–	KF547880
A. scirpaceus scirpaceus	30767	1	Canary Islands	Fuerteventura	C. Dietzen	–	KF547889
A. scirpaceus scirpaceus	30768	1	Canary Islands	Fuerteventura	C. Dietzen	–	KF547890
A. scirpaceus scirpaceus	31907	1	Germany	Franconia (19)	B. Leisler	–	–
A. scirpaceus scirpaceus	47545	1	Ukraine	Black sea	G. Nikolaus	–	KF547879
A. scirpaceus scirpaceus	52381	1	Germany	Treysa	H. –H. Witt	–	KF547873
A. scirpaceus scirpaceus	54355	1	Germany	Treysa	H. –H. Witt	–	KF547875
A. scirpaceus scirpaceus	57018	1	Germany	Saarland (81)	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	54327	1	Germany	Treysa	H. –H. Witt	–	KF547878
A. scirpaceus scirpaceus	56875	1	Germany	Saarland	R. Klein, F. Feß, L. Hayo	–	KF547876
A. scirpaceus scirpaceus	56876	1	Germany	Saarland	R. Klein, F. Feß, L. Hayo	–	KF547877
A. scirpaceus scirpaceus	–	1	Sweden	Bredaryd	(Johnsen *et al.* 2010)	GU571696	–
A. scirpaceus scirpaceus	–	1	Sweden	Huddinge	(Johnsen *et al.* 2010)	GU571697	–
A. scirpaceus scirpaceus	–	1	Sweden	Örebro	(Johnsen *et al.* 2010)	GU571698	–
A. scirpaceus scirpaceus	52347	2	Germany	Treysa (9)	H. –H. Witt	KF467456	–
A. scirpaceus scirpaceus	12577	3	Israel	Hazewa	P. Heidrich	KF467457	KF547884
A. scirpaceus scirpaceus	12568	3	Israel	Eilat	P. Heidrich	–	KF547887
A. scirpaceus scirpaceus	12582	3	Israel	Hazewa	P. Heidrich	–	KF547885
A. scirpaceus scirpaceus	12585	3	Israel	Hazewa	P. Heidrich	–	KF547886
A. scirpaceus scirpaceus	47544	3	Nigeria	ObuduBoje	G. Nikolaus	–	KF547888
A. scirpaceus scirpaceus	54271	3	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	56999	3	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	52280	4	Germany	Treysa (3)	H. –H. Witt	KF467458	–
A. scirpaceus scirpaceus	31922	4	Germany	Franconia	B. Leisler	–	–
A. scirpaceus scirpaceus	57015	4	Germany	Saarland (3)	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	54338	5	Germany	Treysa (2)	H. –H. Witt	KF467459	–
A. scirpaceus scirpaceus	6498	5	Germany	Franconia	B. Leisler	–	–
A. scirpaceus scirpaceus	56883	5	Germany	Saarland	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	–	5	Russia	Astrakhan	(Kerr *et al.* 2009)	GQ481283	–
A. scirpaceus scirpaceus	–	5	Norway	Trøgstad	(Johnsen *et al.* 2010)	GU571220	–
A. scirpaceus scirpaceus	57024	6	Germany	Saarland (4)	R. Klein, F. Feß, L. Hayo	KF467460	–
A. scirpaceus scirpaceus	54514	6	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	54333	7	Germany	Treysa (4)	H. –H. Witt	KF467461	–
A. scirpaceus scirpaceus	56937	7	Germany	Saarland	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	54343	8	Germany	Treysa (5)	H. –H. Witt	KF467462	–
A. scirpaceus scirpaceus	54335	9	Germany	Treysa (5)	H. –H. Witt	KF467463	–

Taxon	IPMB	H	Country	Locality (*N*)	Collector	*COI*	*cyt b*
A. scirpaceus scirpaceus	56932	10	Germany	Saarland (3)	R. Klein, F. Feß, L. Hayo	KF467464	–
A. scirpaceus scirpaceus	60696	10	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	56895	11	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467465	–
A. scirpaceus scirpaceus	60636	11	Germany	Treysa (3)	H. –H. Witt	–	–
A. scirpaceus scirpaceus	12579	12	Israel	Hazewa (2)	P. Heidrich	KF467466	–
A. scirpaceus scirpaceus	54329	12	Germany	Treysa (2)	H. –H. Witt	–	–
A. scirpaceus scirpaceus	31934	13	Germany	Franconia (3)	B. Leisler	KF467467	–
A. scirpaceus scirpaceus	56909	14	Germany	Saarland (3)	R. Klein, F. Feß, L. Hayo	KF467468	–
A. scirpaceus scirpaceus	56955	15	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467469	–
A. scirpaceus scirpaceus	60671	15	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	56977	16	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467470	–
A. scirpaceus scirpaceus	63364	16	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	12589	17	Israel	Hazewa	P. Heidrich	KF467471	–
A. scirpaceus scirpaceus	52593	17	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	56903	17	Germany	Saarland	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	57007	18	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467472	–
A. scirpaceus scirpaceus	31917	18	Germany	Franconia	B. Leisler	–	–
A. scirpaceus scirpaceus	56991	19	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467473	–
A. scirpaceus scirpaceus	56933	20	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467474	–
A. scirpaceus scirpaceus	56918	21	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467475	–
A. scirpaceus scirpaceus	56921	22	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467476	–
A. scirpaceus scirpaceus	56984	23	Germany	Saarland (2)	R. Klein, F. Feß, L. Hayo	KF467477	–
A. scirpaceus scirpaceus	56924	24	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467478	–
A. scirpaceus scirpaceus	60661	24	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	57016	25	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467479	–
A. scirpaceus scirpaceus	63265	25	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	12573	26	Israel	Hazewa	P. Heidrich	KF467480	–
A. scirpaceus scirpaceus	57012	26	Germany	Saarland	R. Klein, F. Feß, L. Hayo	–	–
A. scirpaceus scirpaceus	12762	27	Israel	Hazewa	P. Heidrich	KF467481	–
A. scirpaceus scirpaceus	60613	27	Germany	Treysa	H. –H. Witt	–	–
A. scirpaceus scirpaceus	60780	28	Germany	Treysa	H. –H. Witt	KF467482	–
A. scirpaceus scirpaceus	56972	29	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467483	–
A. scirpaceus scirpaceus	45966	30	Germany	Treysa	H. –H. Witt	KF467484	–
A. scirpaceus scirpaceus	60708	31	Germany	Treysa	H. –H. Witt	KF467485	–
A. scirpaceus scirpaceus	57039	32	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467486	–
A. scirpaceus scirpaceus	56885	33	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467487	–
A. scirpaceus scirpaceus	56893	34	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467488	–
A. scirpaceus scirpaceus	56992	35	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467489	–
A. scirpaceus scirpaceus	60629	36	Germany	Treysa	H. –H. Witt	KF467490	–
A. scirpaceus scirpaceus	60778	37	Germany	Treysa	H. –H. Witt	KF467491	–
A. scirpaceus scirpaceus	57020	38	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467492	–
A. scirpaceus scirpaceus	56962	39	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467493	–
A. scirpaceus scirpaceus	60639	40	Germany	Treysa	H. –H. Witt	KF467494	–
A. scirpaceus scirpaceus	56944	41	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467495	–
A. scirpaceus scirpaceus	56898	42	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467496	–
A. scirpaceus scirpaceus	57010	43	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467497	–
A. scirpaceus scirpaceus	56961	44	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467498	–
A. scirpaceus scirpaceus	57009	45	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467499	–
A. scirpaceus scirpaceus	60617	46	Germany	Treysa	H. –H. Witt	KF467500	–
A. scirpaceus scirpaceus	60645	47	Germany	Treysa	H. –H. Witt	KF467501	–
A. scirpaceus scirpaceus	57001	48	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467502	–
A. scirpaceus scirpaceus	56870	49	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467503	–
A. scirpaceus scirpaceus	56983	50	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467504	–
A. scirpaceus scirpaceus	52675	51	Germany	Treysa	H. –H. Witt	KF467505	–

Taxon	IPMB	H	Country	Locality (*N*)	Collector	*COI*	*cyt b*
A. scirpaceus scirpaceus	52636	52	Germany	Treysa	H. –H. Witt	KF467506	–
A. scirpaceus scirpaceus	56879	53	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467507	–
A. scirpaceus scirpaceus	57028	54	Germany	Saarland	R. Klein, F. Feß, L. Hayo	KF467508	–
A. scirpaceus scirpaceus	52626	55	Germany	Treysa	H. –H. Witt	KF467509	–
A. scirpaceus scirpaceus	12581	56	Israel	Hazewa	P. Heidrich	KF467510	–
A. scirpaceus scirpaceus	12575	57	Israel	Hazewa	P. Heidrich	KF467511	–
A. scirpaceus scirpaceus	32987	58	Tunisia	Galite	D. Ristow	KF467512	–
A. scirpaceus fuscus	6608	59	Kazakhstan	Lake Alakol	G. Nikolaus	KF467513	–
A. scirpaceus fuscus	53547	59	Azerbaijan	–	G. Nikolaus	–	–
A. scirpaceus fuscus	12587	59	Israel	Hazewa	P. Heidrich	–	KF547893
A. scirpaceus fuscus	12755	59	Israel	NeotHakikar	P. Heidrich	–	KF547894
A. scirpaceus fuscus	47546	59	Saudi Arabia	Riyadh	G. Nikolaus	–	KF547896
A. scirpaceus fuscus	47549	59	Saudi Arabia	Riyadh	G. Nikolaus	–	KF547899
A. scirpaceus fuscus	47550	59	Saudi Arabia	Riyadh (2)	G. Nikolaus	–	–
A. scirpaceus fuscus	47554	59	Saudi Arabia	Riyadh	G. Nikolaus	–	KF547902
A. scirpaceus fuscus	46407	60	Kenya	Ngulia	G. Nikolaus	KF467514	–
A. scirpaceus fuscus	47548	60	Saudi Arabia	Riyadh	G. Nikolaus	–	KF547898
A. scirpaceus fuscus	47555	60	Kazakhstan	Lake Alakol (2)	G. Nikolaus	–	KF547895
A. scirpaceus fuscus	60649	61	Germany	Treysa	H. –H. Witt	KF467515	KF584230
A. scirpaceus fuscus	47547	61	Saudi Arabia	Riyadh	G. Nikolaus	–	KF547897
A. scirpaceus fuscus	12574	62	Israel	Hazewa	P. Heidrich	KF467516	KF547891
A. scirpaceus fuscus	12586	62	Israel	Hazewa	P. Heidrich	–	KF547892
A. scirpaceus fuscus	47551	63	Saudi Arabia	Riyadh	G. Nikolaus	KF467517	KF547900
A. scirpaceus fuscus	47553	63	Saudi Arabia	Riyadh	G. Nikolaus	–	KF547901
A. scirpaceus fuscus	6623	64	Kazakhstan	Lake Alakol	G. Nikolaus	KF467518	–
A. scirpaceus fuscus	–	65	Russia	Astrakhan	(Kerr *et al.* 2009)	GQ481282	–
A. scirpaceus fuscus	–	66	Iran	–	(Aliabadian *et al.* 2009)	FJ465292	–
A. scirpaceus avicenniae	6652	67	Ethiopia	–	G. Nikolaus		–
A. scirpaceus avicenniae	6653	67	Ethiopia	–	G. Nikolaus		–
A. scirpaceus avicenniae	6654	68	Saudi Arabia	–	G. Nikolaus		–
A. scirpaceus scirpaceus	52390	–	Germany	Treysa	H. –H. Witt	–	KF547874
A. scirpaceus scirpaceus	–	–	Bulgaria	–	(Fregin *et al.* 2012)	–	JN574450
A. scirpaceus scirpaceus	–	–	Austria	Neusiedler See	(Leisler *et al.* 1997)	–	Aj004301
A. scirpaceus scirpaceus	–	–	Ukraine	–	(Leisler *et al.* 1997)	–	Aj004302
A. scirpaceus scirpaceus	–	–	Austria	Neusiedler See	(Leisler *et al.* 1997)	–	Aj004303
A. scirpaceus scirpaceus	–	–	Israel	MaozHaim	(Singh *et al.* 2008)	–	AM889139
A. scirpaceus scirpaceus	–	–	–	–	(Helbig *et al.* 1995)	–	Z73483
A. scirpaceus fuscus	–	–	Kazakhstan	–	(Helbig & Seibold 1999)	–	AJ004771
A. scirpaceus avicenniae	–	–	Saudi Arabia	–	(Helbig & Seibold 1999)	–	AJ004770
A. scirpaceus avicenniae	–	–	Ethiopia	–	(Leisler *et al.* 1997)	–	AJ004237
A. scirpaceus avicenniae	–	–	Ethiopia	–	(Leisler *et al.* 1997)	–	AJ004238
A. baeticatus	58927	outgroup	Namibia	–	U. Franke-Bryson	KF467519	KF547905
A. baeticatus	58924	outgroup	Namibia	–	U. Franke-Bryson	KF467520	–
A. baeticatus	58926	outgroup	Namibia	–	U. Franke-Bryson	–	KF547904

Phylogenetic and population genetic analyses

Sequences were aligned with CLUSTAL W (Thompson *et al.* 1994) available in BIOEDIT version 7.0.9.0 (Hall 1999) and double-checked by eye. The best model of molecular substitutions was explored by using JModelTest (Posada 2008). Phylogenetic relationships were implemented in the BEAST version 1.8.0 (Drummond & Rambaut 2007b) and rooted with *A. baeticatus* (Fregin *et al.* 2009). We made the analyses using GTR+ Γ, *birth-death* model as priors, strict molecular clock and a mutation rate uniform distribution ranging from 0.01105 to 0.02500 substitutions/site/lineage/million years (Neto *et al.* 2012; Weir & Schluter 2008). Molecular evolutionary rates (of combined *COI* and *cyt b* loci) calculated using the same analyses including a codon position partitioned nucleotide substitution model. We conducted the analyses consisting of 20,000,000 generations sampled every 1,000th generation (Gernhard 2008). BEAST was also employed to model the demographic changes of major clades using the Bayesian Skyline Plot (Drummond *et al.* 2005) with a chain length of 60,000,000 generations sampled every 2,000th generation. Effective sample size (ESS) values of at least 200 individuals were evaluated with Tracer version 1.5 (http://beast.bio.ed.ac.uk/Tracer). After discarding the first trees with a 'burn-in' equivalent to 10%, maximum clade credibility trees were reconstructed with TREEANNOTATOR 1.8.0 (http://beast.bio.ed.ac.uk/TreeAnnotator) and visualized with FIGTREE version 1.4.1 (http://beast.bio.ed.ac.uk/FigTree).

Median-joining network analyses were conducted with NETWORK version 4.6.1.1 (Bandelt *et al.* 1999). Molecular diversity parameters, Tajima's *D* (Tajima 1989), Fu's F_S neutrality tests (Fu 1997), mismatch distributions (Rogers & Harpending 1992) and the time of population expansion ($\tau = 2\ \mu T$) were estimated in ARLEQUIN version 3.5.1.2 (Excoffier & Lischer 2010) with 1,000 bootstrap replicates and compared statistically against models of sudden population expansion (100 replicates) using the sum of squared deviations test. We conducted the R2 test (Ramos-Onsins & Rozas 2002) in DnaSP version 5.1 (Librado & Rozas 2009). Diversity indices were not calculated for populations with very small sample sizes ($N < 4$). We measured the genetic variation among and within subspecies (Φ_{ST}), and degree of geographical structuring among populations (F_{ST}) with AMOVA (Excoffier *et al.* 1992). The significance of the variance components was tested using 1,000 permutations. Mean pairwise *p*-distances (Nei 1987) within and among lineages were calculated by MEGA v. 5.1.

3.2.4 Results

Population structure and genetic diversity in A. scirpaceus complex

We analyzed 633 nt of *COI* from 405 Eurasian reed warblers. The sequenced region contained 61 variable sites defining 68 different haplotypes: 58 haplotypes for *scirpaceus* (*N* = 380), eight haplotypes for *fuscus* (*N* = 22) and two haplotypes for *avicenniae*. Thirty five haplotypes were restricted to single individuals and the most common haplotype (*N*1) was detected in 62% (*N* = 253) of the samples (Table 3.2.3). The genetic diversity (*Hd*) was high in every population, ranging from *Hd* = 0.42 (Franconia) to *Hd* = 1.00 (Kazakhstan). In contrast, the nucleotide diversity (π) was relatively low in each population. Overall diversity was similar among populations while gene diversity and nucleotide diversity were both lowest in Franconia and highest in Kazakhstan; after discarding Israel because of being stopover site for both subspecies during migration (Table 3.2.4). According to AMOVA, the overall genetic variation among subspecies was much larger than the variation within subspecies (*COI*: 81.85% vs. 18.15%; *cyt b*: 70.74% vs. 29.26%; Table 3.2.5). Pairwise Φ_{ST} value between subspecies was also highly significant (*COI*: 0.82, *cyt b*: 0.71; $P < 0.001$). The highest pairwise F_{ST} value, calculated by *COI* sequences, was found between *fuscus* in Saudi Arabia and *scirpaceus* in Franconia (0.481) while the lowest value was between *fuscus* samples from Kazakhstan and Saudi Arabia ($F_{ST} = –0.02$; Table 3.2.6).

The phylogenetic trees base on 68 haplotypes of *COI* (Fig. 3.2.2), *cyt b* sequences of selected individuals from a subset of *COI* phylogeny (not shown) and concatenated loci (Fig. 3.2.3) as well as haplotype network (Fig. 3.2.4), identified three distinctive clades/clusters representing the three subspecies [*p*-distance = 1.1% (*scirpaceus/fuscus*), 1.4% (*scirpaceus/avicenniae*) and 1.8% (*fuscus/avicenniae*)].

Field morphological identification errors

Mistakes in identifying birds in the field may only be detectable using molecular methods. DNA sequence data can discover and reduce greatly these types of errors. Field identifications were validated using the mitochondrial *COI* in order to assess delimitation mistakes. In the present study, we sequenced all samples which were identified as *A. scirpaceus* by collectors and were available in the data set of IPMB (Institute of Pharmacy and Molecular Biotechnology, *N* = 398, Table 3.2.2). After alignment, we determined that 27 specimens (6.8% of samples) were not *A. scirpaceus*. The results of the nucleotide blast confirmed that they belong to the other species mostly *A. palustris*; lower error rates were observed for *A. dumetorum and A. melanopogon* (Table 3.2.7). These misidentified species were deleted from

Table 3.2.3 Variable sites among *A. scirpaceus* haplotypes (*COI*). Numbers between parentheses indicate the number of samples in each area.

H	*N*	Alignment position	Sample sites and subspecies
		11 1111222222 2233333333 3333444444 4444444555 5555555566 6 2335667802 5666244457 7901234567 7889011334 4556789000 2233568901 2 9256251015 8145725812 5321987081 4098437140 9157424036 1736731081 6	
1	252	GGAGCCGGTG GGTCAGAGAG GCTTTAATAG GAATCAAATG CACGGCAGAT TCAGCCAGAC T	Tre(130),Saa(85),Fra(19),Isr(11), Swe(3),Can(2),Swi(1),Ukr(1)
2	9	....A.....	Tre(9)
3	8	....G.... ....C.	Isr(4),Saa(2),Tre(1),Nig(1)
4	7	....C	Tre(3),Saa(3),Fra(1)
5	6	 C	Tre(2),Fra(1),Saa(1),Nor(1),Rus(1)
6	5	....C.	Saa(4),Tre(1)
7	5	....T.... .	Tre(4),Saa(1)
8	5	..G..... .A..... ....T....	Tre(5)
9	5	.... .G..... ....A.. .	Tre(5)
10	4	....G.....	Saa(3),Tre(1)
11	4	.......A..	Tre(3),Saa(1)
12	4	A.....	Tre(2),Isr(2)
13	3	...A.....	Fra(3)
14	3	....C. ....G.	Saa(3)
15	3	....A	Saa(2),Tre(1)
16	3	....G...	Saa(2),Tre(1)
17	3	....C.....	Saa(1),Tre(1),Isr(1)
18	2	....T....	Fra(1),Saa(1)
19	2	....G.	Saa(2)
20	2	....G....	Saa(2)
21	2	....C.....	Saa(2)
22	2	.... .T...G.... ....C. ....T.... .	Saa(2)
23	2	....T..... A.....G... ....A	Saa(2)
24	2	....A.. ....G... .	Saa(1),Tre(1)
25	2	.... A.....	Saa(1),Tre(1)
26	2	.......C.	Saa(1),Isr(1)
27	2	....C.....	Tre(1),Isr(1)
28	1	....G...	Tre(1)
29	1	.... A.....	Saa(1) ***scirpaceus***
30	1	....A....	Tre(1)
31	1	.........A .....A....	Tre(1)
32	1	....G...	Saa(1)
33	1	....C..... ....G.. ....A..	Saa(1)
34	1	....C..... ....T.....	Saa(1)
35	1	....C..... ....T.....	Saa(1)
36	1	....C.....	Tre(1)
37	1	....C......A	Tre(1)
38	1	....A	Saa(1)
39	1	....C	Saa(1)
40	1	....A.....	Tre(1)
41	1	....A.....	Saa(1)
42	1	.........A	Saa(1)
43	1	....G.	Saa(1)
44	1	....T..... .	Saa(1)
45	1	.... T.....	Saa(1)
46	1	.... .T..... .	Tre(1)
47	1	....T .	Tre(1)
48	1	......A...	Saa(1)
49	1	.A.....	Saa(1)
50	1	..... .A..... ....C.	Saa(1)
51	1	..... .AC.....	Tre(1)
52	1	.....T.... ....G.....	Tre(1)
53	1	....T.... ....G. .	Saa(1)
54	1	.... .T...G.... ....C.	Saa(1)
55	1	.... .G..... ....A.. C	Tre(1)
56	1	....C ....T..... .	Isr(1)
57	1	....C.....A	Isr(1)
58	1	.... C..... .	Tun(1)
59	9	....C.. ....A..... ...A...A.. .	Sau(5),Isr(2),Kaz(1),Aze(1)
60	3	....C.. ....A...A.. ...A...A.. .	Ken(1),Sau(1),Kaz(1)
61	3	..... .A..... ....C.. ....A..... ...A...A.. .	Tre(1),Sau(1),Kaz(1)
62	2	....G..... ....C.. ....A...A.. ...A...A.. .	Isr(2)
63	2	....A..... ....C.A ....A..... ...A...A.. .	Sau(2) ***fuscus***
64	1	....C.. ...C..... ...A...A.. ...A...A.. .	Kaz(1)
65	1	....A ....A..... ...A...A.. C	Rus(1)
66	1	....C.A ....A..... ...A...A.. C	Ira(1)
67	2	....T..... .A.......A .T..... ....A .C.A..... ....G. .	Eth(2) ***avicenniae***
68	1	....T..... .A..... .T..... ..G.....A .C.A..... ....G. .	Sau(1)
Total	405	61 variable sites	18 populations

the further analyses. Mistake rates of identification between subspecies *scirpaceus* and *fuscus* were much higher, mostly in Israel where they occur together. From 22 detected *fuscus* by genetic analyses; only six were correctly identified in the field.

Demographic analyses and molecular dating

Negative and statistically significant Tajima's *D* and Fu's F_S values show sudden population expansions with the strongest evidence detected in German populations (Table 3.2.4). Neutrality tests were negative and highly significant for *scirpaceus* and showed demographic expansion. Additionally, the Bayesian Skyline Plot (BSP) and mismatch distribution analyses (Fig. 3.2.5) support population growth in this subspecies. The BSP indicates that this clade experienced expansions especially in the last 10,000 years. Pairwise difference distributions were not significantly different ($P > 0.05$) from expected distribution, supporting unimodal curves that fit a sudden expansion model. Neutrality tests, mismatch distribution and BSP analyses done for *fuscus* also reveal that the population size had a gradual expansion during the last 35,000 years but it was slighter than that of *scirpaceus* (Table 3.2.4, Fig. 3.2.5). Due to small sample size of *avicenniae* ($N = 3$), we did not calculate the demographic changes of this subspecies. Estimated divergence times based on concatenated dataset (Fig. 3.2.3) are as follows: between *A. scirpaceus* and the sister species *A. baeticatus* 611,100 years ago (95% Highest Posterior Density: 321,400–983,500); between *avicenniae* and *fuscus*/*scirpaceus* 415,200 years ago (95% HPD: 230,000–672,800) and between *scirpaceus* and *fuscus* 342,300 years ago (95% HPD: 181,900–562,400).

3.2.5 Discussion

Field identification errors in reed warblers

The identification of birds, especially of warblers, is often difficult because specific distinctive morphological characteristics may not be visible at the time of observation, particularly for co-occurring taxa and species similar in appearance. Subspecies identification can be even more difficult due to higher morphological similarities and sometimes is impossible without capturing the birds and having experience in identifying them by a series of morphological measurements (Pearson *et al.* 2002; Walton 2012), thus some individuals may be wrongly identified. DNA sequencing provides a powerful tool for improving identification. Among our sequenced samples we detected 6.8% of misidentifications, especially between *A. scirpaceus* and *A. palustris*. At the subspecies level, *fuscus* was correctly identified from *scirpaceus* in less than 27%.

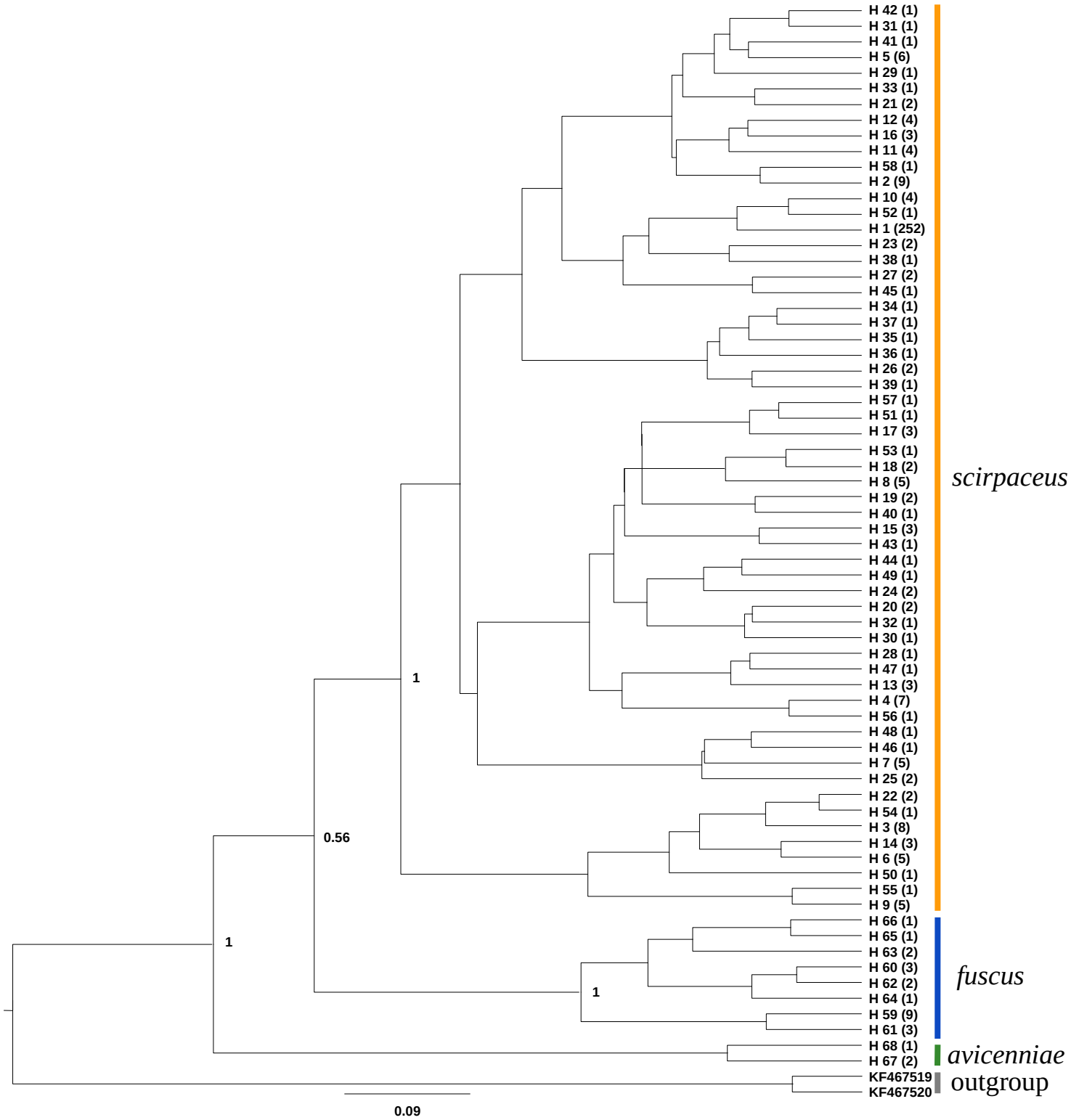

Figure 3.2.2 Bayesian phylogeny of 68 *COI* haplotypes of *A. scirpaceus* and its sister species *A. baeticatus* as outgroup, implemented in BEAST with strict clock (uniform distribution 0.01105 – 0.02500 substitutions/site/lineage/million years) and *birth-death* model as priors. Numbers between parentheses indicate the number of individuals sharing the corresponding haplotype. The Bayesian posterior probabilities were given for deeper nodes and the small scale bar at the bottom (*N*=0.09) indicates the numbers of nucleotide substitutions per site.

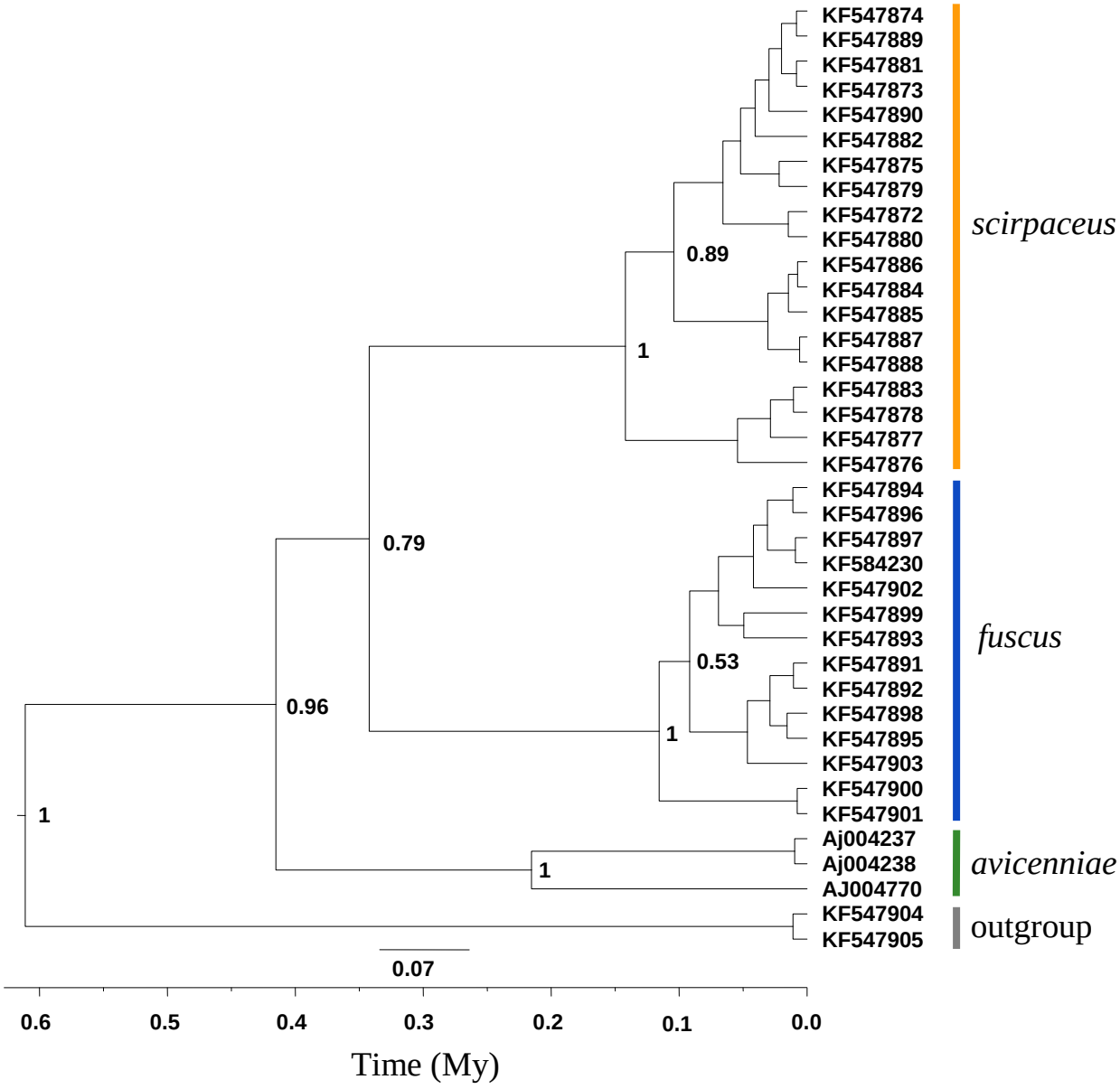

Figure 3.2.3 Chronogram of *A. scirpaceus* and *A. baeticatus* as outgroup. Analysis are based on Bayesian inference of a concatenated dataset (*COI* and *cyt b*) implemented in BEAST using strict clock (uniform distribution 0.01105 – 0.02500 substitutions/site/lineage/million years), *birth-death* model as priors and codon position partitioned nucleotide substitution model. GenBank numbers indicate the *cyt b* sequences (see Table 3.2.2 for the corresponding *COI* sequences). The Bayesian posterior probabilities were given for deeper nodes, branch lengths are in millions of years ago and the small scale bar at the bottom (*N*=0.07) indicates the numbers of nucleotide substitutions per site.

First record of fuscus in Europe based on molecular evidence

The Asian subspecies, *fuscus*, breeds in Southwestern Siberia, Caucasus and Northern Caspian, Iran, Kazakhstan, Cyprus and Levant, Central and Eastern Asia Minor and Northern Middle East (del Hoyo *et al.* 2006), migrates through the Eastern Mediterranean and winters in Sub-Saharan Africa (Dowsett-Lemaire & Dowsett 1987). The birds start their autumn migration from late July onwards, but mostly they migrate in August. The first birds arrive in Africa in late September, the main arrival is in late October, but lots of birds arrive also in late January. Departure from wintering areas begins in late March and they have reached their breeding grounds by the end of May (Pearson *et al.* 2002).

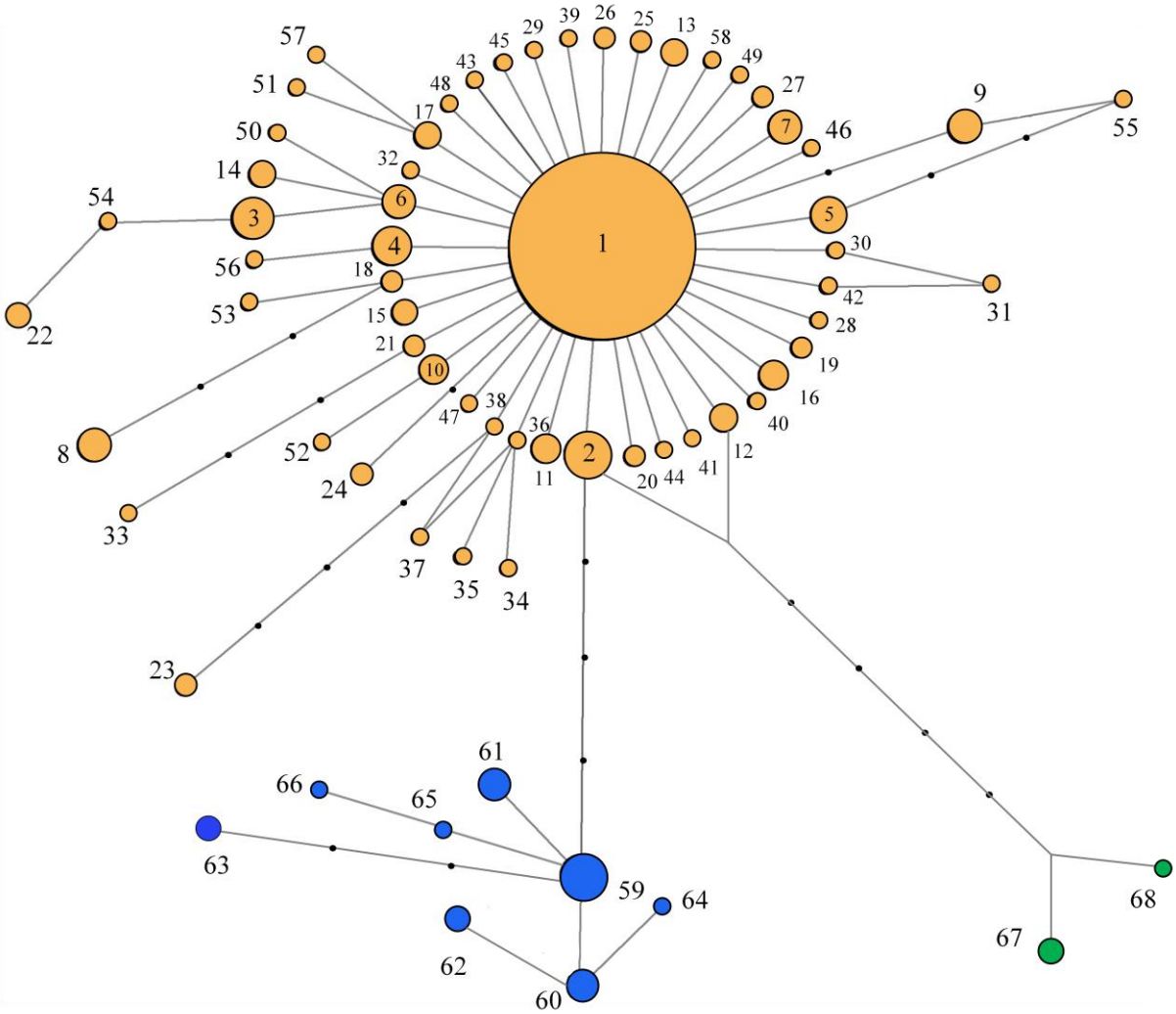

Fig. 3.2.4 Minimum-spanning network (*scirpaceus*: orange, *fuscus*: blue and *avicenniae*: green) among 68 *COI* haplotypes (see Table 3.2.3). Size of circles represents the relative frequency of given haplotypes and each line between black nodes indicates a single mutational step.

Table 3.2.2 Genetic diversity indices for mitochondrial *COI* and *cyt b* loci within different populations and subspecies of *Acrocephalus scirpaceus.*

Locus	Locality	*N*	*H*	*S*	*Hd*	*K*	*π*	Fu's *Fs*	Tajima's *D*	R2	*τ*
COI	Germany (*scirpaceus*)	347	55	54	0.54±0.03	0.92±0.64	0.001±0.001	–28.89***	–2.55***	0.01**	0.41
	Treysa (*scirpaceus*)	184	30	32	0.50±0.05	0.86±0.61	0.001±0.001	–30.50***	–2.42***	0.01**	0.50
	Saarland (*scirpaceus*)	139	38	41	0.62±0.05	1.13±0.74	0.001±0.001	–29.92***	–2.57***	0.01***	0.52
	Franconia (*scirpaceus*)	25	5	4	0.42±0.12	0.46±0.42	0.001±0.001	–2.95**	–1.53*	0.09 ns	0.55
	Israel (*scirpaceus*)	22	8	9	0.73±0.09	1.42±0.90	0.002±0.002	–3.22*	–1.42 ns	0.08**	1.87
	Israel (*fuscus*)	4	2	2	0.67±0.20	1.33±1.02	0.002±0.002	1.53 ns	1.89 ns	0.33 ns	2.29
	Saudi Arabia (*fuscus*)	9	4	4	0.70±0.15	1.22±0.85	0.002±0.001	–0.63 ns	–0.69 ns	0.14*	1.73
	Kazakhstan (*fuscus*)	4	4	3	1.00±0.18	1.67±1.22	0.003±0.002	–2.18*	0.17 ns	0.20*	1.83
	scirpaceus	380	58	56	0.56±0.03	0.95±0.65	0.001±0.001	–29.02***	–2.53***	0.01**	0.68
	fuscus	22	8	7	0.81±0.07	1.53±0.95	0.002±0.002	–2.95*	–0.65 ns	0.11 ns	1.66
	avicenniae	3	2	2	0.67±0.31	1.33±1.09	0.002±0.002	1.06 ns	–	0.47 ns	2.29
	Population	405	68	61	0.61±0.03	1.51±0.91	0.002±0.002	–27.66***	–2.39***	0.01**	0.11
cyt b	*scirpaceus*	25	16	20	0.95±0.02	3.83±1.99	0.005±0.003	-6.28**	-1.003 ns	0.08*	4.42
	fuscus	14	9	11	0.91±0.06	2.53±1.45	0.003±0.002	-3.82**	-1.06 ns	0.09*	2.69
	avicenniae	3	2	11	0.83±0.22	7.00±4.17	0.008±0.006	2.04 ns	1.67 ns	0.28 ns	11.30
	Population	42	28	45	0.97±0.01	8.29±3.92	0.01±0.005	-9.22**	-0.71 ns	0.08 ns	12.93

Note: diversity indices were not calculated for populations with very small sample sizes ($N < 3$).
ns = non-significant; *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$
N = sample size,
H = number of haplotypes,
S = number of polymorphic (segregating) sites,
Hd = haplotype diversity,
K = average number of pairwise differences,
π = nucleotide diversity,
R2 = Ramos-Onsins and Rozas (2002) test,
τ = time of population expansion ($\tau = 2\ \mu T$).

This subspecies has been recorded several times by bird watchers in England (Dunn 2001; Neal 1996; Shaw *et al.* 2000), very far from its expected range. However, the presence of *fuscus* in Western Europe has not been unequivocal due to difficulties in distinguishing it from closely related taxa. Sequence data analyses showed that out of 348 Eurasian reed

warblers captured in Germany for ringing (mostly migrant birds), one sample clustered in the *fuscus* group (haplotype 61; see Table 3.2.3). The corresponding blood sample was collected in Treysa, northern Hesse (Germany) by H.-H. Witt on May 15, 2010. We extracted, amplified and sequenced this sample twice and employed another mitochondrial marker (*cyt b*). The variable sites clearly show this individual was a member of the *fuscus* clade (bird KF584230, Table 3.2.8). Therefore, our study provides the first safe identification of *fuscus* in Central Europe.

Table 3.2.5 Analysis of molecular variance (AMOVA) for *COI* haplotypes among different localities (a) and for *COI* (b) and *cyt b* (c) haplotypes among three subspecies (c).

a: among all populations (*COI*)						
Source of variation	d.f.	SS	Variance	% var.	Φ-stat.	P
Among populations	12	146.48	0.11	17.03	0.17	< 0.001
Within populations	1533	846.43	0.55	82.97		
Total	1545	992.91	0.66			
b: *scirpaceus* vs. *fuscus* vs. *avicenniae* (*COI*)						
Source of variation	d.f.	SS	Variance	% var.	Φ-stat.	P
Between populations	2	106.03	2.22	81.85	0.82	< 0.001
Within populations	402	198.18	0.49	18.15		
Total	404	304.22	2.72			
c: *scirpaceus* vs. *fuscus* vs. *avicenniae* (*cyt b*)						
Source of variation	d.f.	SS	Variance	% var.	Φ-stat.	P
Among Populations	2	99.63	4.32	70.74	0.71	< 0.001
Within Populations	39	69.68	1.79	29.26		
Total	41	169.31	6.11			

Table 3.2.6 Pairwise estimated values of F_{ST} among six populations of *A. scirpaceus* based on *COI*.

	Treysa	Saarland	Franconia	Israel	Saudi Arabia	Kazakhstan
Treysa	–					
Saarland	0.01*	–				
Franconia	0.003	0.01	–			
Israel	0.09***	0.04*	0.09**	–		
Saudi Arabia	0.45***	0.35***	0.48***	0.21***	–	
Kazakhstan	0.38**	0.27**	0.42**	0.11	–0.02	–

*** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$

Long-distance migratory birds are regularly reported by birders far outside their normal range (Dudley *et al.* 2006; Pfeifer *et al.* 2007; Thorup *et al.* 2012), but the causes of this, despite many studies, are not well understood. The occurrence of such rarities can be due to bad weather condition (Baker 1977; Kaufman 1977), misorientation (Alerstam 1991; Arvin 1992; Patten & Marantz 1996; Thorup 1998; Van Impe & Derasse 1994), nocturnal migration (Alerstam 1991), and inexperienced migrants (Newton 2008). Rarities are frequent among species having small body sizes (Wikelski *et al.* 2007), long wing lengths (Alonso & Arizaga 2013; Cramp & Perrins 1994) or big populations (Pfeifer *et al.* 2007; Stake 2012; Veit 1997). Pfeifer and his colleagues studied 38 species of migratory birds to understand why large numbers of vagrants from Asia have been reported in Europe. They found that this is due to reverse migration, i.e. flying the same distance but in the wrong direction (Pfeifer *et al.* 2007). This can explain several thousand reports of Asian leaf-warbler species and Asian thrush species in Europe. Reverse migration has also reported in *scirpaceus* leaving Europe (Åkesson *et al.* 2001, 2002). Mirror-image misorientation (DeSante 1983; Diamond 1982), is another mechanisms, can cause vagrancy in migratory birds. Mirror-image misorientation involves confusion of right and left along the mirror-image of the correct direction (e.g. west instead of east, southwest instead of southeast) and it is responsible for the occurrences of the numerous species of vagrant wood warblers in California (DeSante 1973).

Table 3.2.7 The name and number (*N*) of 27 misidentified species with *A. scirpaceus* in the field.

Identified by collectors in the field	**Identified by DNA sequencing**	**Origin**
Acrocephalus scirpaceus fuscus (*N* = 3)	*Acrocephalus dumetorum*	Kazakhstan, Lake Alakol
Acrocephalus scirpaceus fuscus	*Acrocephalus melanopogon*	Kazakhstan, Lake Alakol
Acrocephalus scirpaceus scirpaceus	*Acrocephalus melanopogon*	Kazakhstan, Lake Alakol
Acrocephalus scirpaceus scirpaceus	*Acrocephalus arundinaceus*	Germany, Franconia
Acrocephalus scirpaceus fuscus	*Acrocephalus agricola*	Kazakhstan, Lake Alakol
Acrocephalus scirpaceus fuscus	*Locustella naevia*	Kazakhstan, Lake Alakol
Acrocephalus scirpaceus fuscus	*Locustella certhiola*	Kazakhstan, Lake Alakol
Acrocephalus scirpaceus fuscus	*Acrocephalus schoenobaenus*	Kazakhstan, Lake Alakol
Acrocephalus palustris (*N* = 13)	*Acrocephalus scirpaceus scirpaceus*	Germany, Treysa
Acrocephalus palustris	*Acrocephalus scirpaceus scirpaceus*	Germany, Saarland
Acrocephalus palustris (*N* = 2)	*Acrocephalus scirpaceus scirpaceus*	Ukraine
Acrocephalus palustris	*Acrocephalus scirpaceus scirpaceus*	Kenya, Ngulia

For the occurrence of *fuscus* in Germany in mid May, two hypotheses are possible: the first and more plausible one is the consequence of mirror-image misorientation from wintering areas (Africa) to North West instead of North East. The second explanation is north westerly mirror-image misorientation (instead of South West) starting from breeding grounds, maybe last summer. This pattern has been reported in vagrants of Common Crossbills *Loxia curvirostra.* They usually remain at a site for only a short time (i.e. some months), but sometimes they stay for longer period (possibly for more than one year) before departing (Alonso & Arizaga 2013). We cannot rule out that our *fuscus* individual might be a hybrid between *fuscus* and *scirpaceus* that may even breed in Germany.

Pleistocene and evolutionary history

The Pleistocene climatic cycles with subsequent glacial and interglacial periods have had a profound impact on the distribution and evolution of avian species (Avise 2000; Hewitt 2000). Isolation in different glacial refugia leads to genetic divergence between populations and over a long time period produces distinct lineages. Using genetic analytical tools considerably have increased our understanding of these processes (Schmitt 2007). The phylogeographic history of the *A. scirpaceus* reveals a deep genetic differentiation of three major mitochondrial evolutionary lineages, which have been isolated for a sufficient period of time (c. 400,000 years) into three different glacial refugia during the Middle Pleistocene glaciations: one spanning Asia (*fuscus*); one encompassing Europe and Northern Africa (*scirpaceus*); and a third, including Eastern Africa and South-western Asia (*avicenniae*).

Gene flow analysis on two subspecies of *scirpaceus* and *fuscus* across Europe by Procházka *et al.* (2011) identified three barriers of nuclear gene flow that corresponds to a pattern of Pleistocene differentiation in an Eastern European/Middle East refuge, a Central/Western European refuge and an Iberian refuge. Two mtDNA data in the present study strongly corroborate an Eastern European/Middle East Pleistocene biogeographical division in *scirpaceus*/*fuscus*. This pattern is commonly found in the other passerine birds.

Analysing the mitochondrial DNA sequence data of a close species, great reed warbler *Acrocephalus arundinaceus,* across Eurasian breeding range of the species also showed two major clades of European and Middle East populations, which correspond to two subspecies of *A. a. arundinaceus* and *A. a. zarudnyi*, respectively; and suggest their independent postglacial expansions from two isolated refugia (Hansson *et al.* 2008). Chiffchaffs (Helbig *et al.* 1996), the subalpine warbler (Brambilla *et al.* 2006), and flycatchers (Hogner *et al.* 2012;

Lehtonen *et al.* 2009) as well as many different avian species (Avise & Walker 1998) showed similar distribution and phylogeographical patterns.

Analyses revealed *avicenniae* as the basal clade, diverged from its sister species African reed warbler *A. baeticatus* (Fregin *et al.* 2009) ca. 600,000 years ago. This primitive subspecies may have survived in one of the African refugia, likely low forest refugium of the Ethiopian Highlands during the Last Glacial Maximum (Levinsky *et al.* 2013). This pattern is quite similar to the origin of the Old World Sparrows (Allende *et al.* 2001).

Table 3.2.8 Variable sites among cytochrome-b sequences of *A. scirpaceus.*

Subspecies	H	Alignment position	Samples
		111122 2223333344 4444555555 6666677777 77788 459357813 3682345703 3699155679 1345923345 67802 6459891294 6424624065 8834025114 8084035871 81912	Localities and GenBank Numbers
scirpaceus	A	GTGCCGAAAC CGACAAAATC AGTCTTAACA ACTGTGGTCT GCGGA	Saarland-KF547872
	B	....C.	Israel-KF547880
	C	....T..... ....C.	Israel-KF547881
	C	....T..... ....C.	Canary Islands-KF547889
	C	....T..... ....C.	Treysa-KF547873
	C	....T..... ....C.	Treysa-KF547874
	D	....T..... ....C. ....A..	Canary Islands-KF547890
	E	....T..... ....C. ....C.C.....	Israel-KF547882
	F	....T..... ....C. ....A....	Ukraine-KF547879
	G	....T..... ..G.....C. ....A.A....	Treysa-KF547875
	H	....T....T ....C. .....C.... ......A...	Israel-KF547883
	H	....T....T ....C. .....C.... ......A...	Treysa-KF547878
	I	.C..T....T ....C. .....C.... ...A..A...	Saarland-KF547876
	J	....T....T ....C. ....CC.... ......A...	Saarland-KF547877
	K	....T..... TA......C. .... .A....A...	Austria-Aj004301
	K	....T..... TA......C. .... .A....A...	Ukraine-Aj004302
	K	....T..... TA......C. .... .A....A...	Austria-Aj004303
	L	....T....T ....C. ....A...	Israel-AM889139
	M	....T....T ....C. ....A. ......A...	???-Z73483
	N	....T..... .....G..C. ....A...	Israel-KF547884
	N	....T..... .....G..C. ....A...	Israel-KF547885
	N	....T..... .....G..C. ....A...	Israel-KF547886
	O	....T..... .....GG.C. ....A...	Israel-KF547887
	O	....T..... .....GG.C. ....A...	Nigeria-KF547888
	P	....T..... .....GGCC. ....A...	Bulgaria-JN574450
fuscus	Q	....TA..GT ..G.....C. ....C ......A.T. A..AG	Treysa- KF584230
	Q	....TA..GT ..G.....C. ....C ......A.T. A..AG	Kazakhstan-KF547894
	Q	....TA..GT ..G.....C. ....C ......A.T. A..AG	Saudi Arabia-KF547896
	Q	....TA..GT ..G.....C. ....C ......A.T. A..AG	Saudi Arabia-KF547897
	R	...TTA..GT ..G.....C. ....C ......A.TC A..AG	Saudi Arabia-KF547899
	S	....TA..GT ..G.....C. G........C ....C.A.T. A..AG	Israel-KF547893
	T	....TA..GT ..G.....C. ....C ....C.ACT. A..AG	Israel-KF547892
	U	....TA..GT ..G.....C. ....C ......ACT. A..AG	Israel-KF547891
	U	....TA..GT ..G.....C. ....C ......ACT. A..AG	kazakhstan-KF547895
	V	..A.TA..GT ..G...G.C. ....C ......A.T. A..AG	Saudi Arabia-KF547903
	W	....TA..GT ..G...G.C. ....C ......ACT. A..AG	Saudi Arabia-KF547898
	X	....TA..GT ..G.G.G.C. ....C ......ACT. A..AG	Kazakhstan-AJ004771
	Y	....TA..GT ..GT....C. ....G.C .....AA.T. A..AG	Saudi Arabia-KF547900
	Y	....TA..GT ..GT....C. ....G.C .....AA.T. A..AG	Saudi Arabia-KF547901
	Z	..A.TA..GT ..G.....C. ....C ......A.T. A..AG	Saudi Arabia-KF547902
avicenniae	Z2	....T...GT ..G.....CT .A....G..C G..A..A.T.	Saudi Arabia-AJ004770
	Z3	A...T.GCGT ..G.....C. ..CT.....C ...A..A.T. AA...	Ethiopia-Aj004237
	Z3	A...T.GCGT ..G.....C. ..CT.....C ...A..A.T. AA...	Ethiopia-Aj004238
Total	28	45 variable sites	42 samples

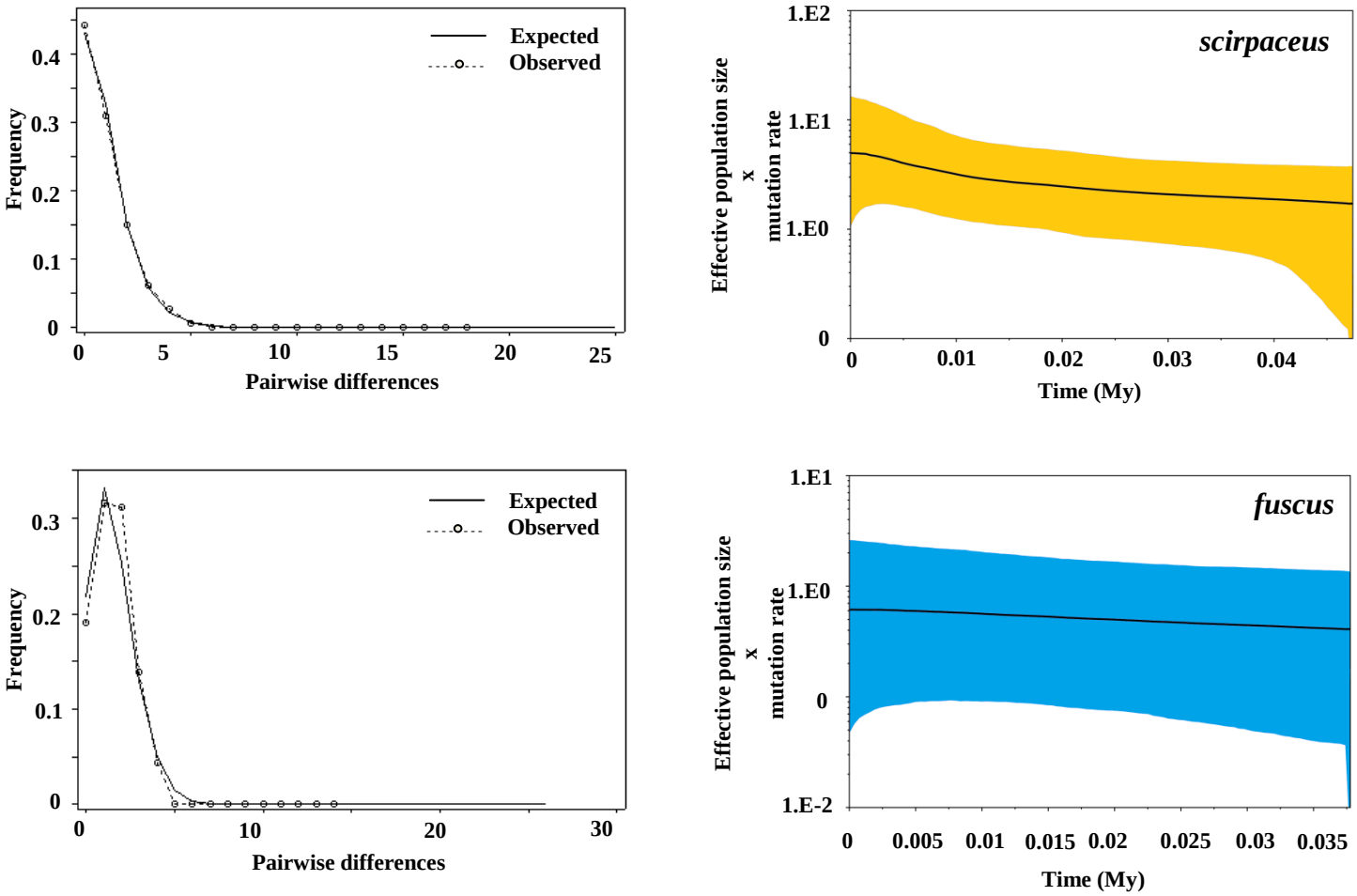

Figure 3.2.5 Mismatch distribution and Bayesian Skyline Plot based on *COI* gene for two subspecies of *A. scirpaceus*. Expected (solid line) and observed (broken line) mismatch distribution under population expansion model and a BSP representing the historical demographic trend of mitochondrial lineages of *scirpaceus* (above) and *fuscus* (below) populations. The median estimates are shown as thick solid lines, and the grey areas are the 95% highest posterior density (HPD) limits. The comparison with the expected distributions for sudden population growth is neither significantly different for *scirpaceus* (Sum of Squared Deviations = 0.00004, P = 0.94; τ = 0.678), nor for *fuscus* (SSD = 0.0028, P = 0.57; τ = 1.658).

Furthermore, the high levels of haplotype diversity and low levels of nucleotide diversity (Table 3.2.4) indicate survival in glacial refugia and demographic expansions of small and isolated populations. Star-like networks also suggest rapid demographic and recent population expansions from single sources (Rogers & Harpending 1992; Slatkin & Hudson 1991). BEAST confirmed population growth of *scirpaceus* from c. 35,000 years with a faster diffusion through the last 10,000 years. During this time, global temperatures increased, the glaciers retreated, more suitable habitats were available and species expanded their geographic ranges out of their limited regions during the Last Glacial Maximum (Dubey *et al.* 2006; Hewitt 2000; Murray Gates 1993; Wright *et al.* 1993). Dry climate conditions in areas not covered by ice may have represented serious limiting factor for wetland species during glacial periods.

The historical demography of *fuscus* presents a slighter increase in population growth in comparison with *scirpaceus*. The reason can be the presence of less suitable marshland areas for *fuscus* in Asia than *scirpaceus* in Europe. Warblers depend on wetlands for breeding, nesting, brood rearing or shelter; and changes in population size are dependent on availability and quality of wetlands. Recent population expansions among birds in Northern hemisphere have also been reported for other avian species: MacGillivray's warbler and wood duck in North America (Milá *et al.* 2000; Peters *et al.* 2005) as well as Savi's warbler and eider duck across Europe (Neto *et al.* 2012; Tiedemann *et al.* 2004).

Origin of wintering birds in Africa

In spite of the small sample size of wintering *A. scirpaceus* (N = 2), our results indicate that the bird in Kenya corresponds to *fuscus*, whereas the sample of Nigeria corresponds to *scirpaceus.* This agrees with migration studies on Eurasian reed warblers (Cramp & Perrins 1994; Pearson *et al.* 2002).

3.3 Mitochondrial Evidence for Genetic Diversity and Low Phylogeographic Differentiation in the Marsh Warbler *Acrocephalus palustris* (Aves: Acrocephalidae)

3.3.1 Abstract

We analyzed levels of genetic variability in a long-distance migratory reed warbler, the marsh warbler (*Acrocephalus palustris*), by using 611 nt sequences of the mitochondrial cytochrome oxidase *c* subunit I gene (*COI*). We obtained sequences from 229 individuals from 10 sampling sites that include breeding, wintering and migrating birds. Overall, 44 haplotypes were detected, which reflect high levels of genetic variation in this species, but most of this variation corresponds to individual differences within populations. We also analyzed 829 nt of *cyt b* from 49 selected individuals of different populations to evaluate the reliability of the *COI* results. Our analyses based on of both mtDNA loci could not detect any population subdivision or phylogeographic structure, indicating high levels of gene flow between breeding populations (*Nm* = 13.69). The split between the marsh warbler and its sister species, the Eurasian reed warbler (*A. scirpaceus*), could be dated for the Lower Pliocene (about 3.8 million years ago). The time to the most recent common ancestor (TMRCA) among marsh warbler haplotypes was estimated as 0.45 My, indicating their recent origin during the last glacial periods. Low nucleotide diversity, a shallow phylogenetic tree, a star-like haplotype network, and a unimodal mismatch distribution point to a sudden increase of the effective population size (probably after the last glaciation period) and a recent range expansion likely from a single refuge.

3.3.2 Introduction

Organisms live in an environment that is changing over time. The current patterns of diversity are affected by both past evolutionary history like climatic and environmental fluctuations, and current population processes such as population size and dispersal ability. Molecular techniques have provided objective means to measure the current genetic variation within and among populations and describe the historical evolutionary processes that have created present diversity (Avise 2004).

Marsh warblers (*Acrocephalus palustris*) are Old World warblers of the family Acrocephalidae. They are small warblers with a plain olive-brown plumage. Because of dull colours and lack of distinctive characters, the individuals of this species can be easily misidentified with close relatives. Marsh warblers occupy a large distribution area; breed

throughout Western Europe (excluding the Iberian Peninsula) to Southern Scandinavia, North-western Russia and Western Asia (Fig. 3.3.1). They are long distance migrants and winter in south-east Africa (del Hoyo *et al.* 2006; Leisler & Schulze-Hagen 2011).

Assessments of population genetic structure are currently lacking for the marsh warblers. This study is the first to use molecular genetics techniques to measure the degree of genetic differentiation between the populations in order to understand the factors that have shaped present day genetic patterns and current distributions of this species. Mitochondrial genes are widely used in phylogeographic studies due to the maternal inheritance, conserved structure, fast mutation rate and non-recombining nature of the haplotypes which reflects patterns of historical fragmentation and changes in population size and distribution (Avise 1989, 2000).

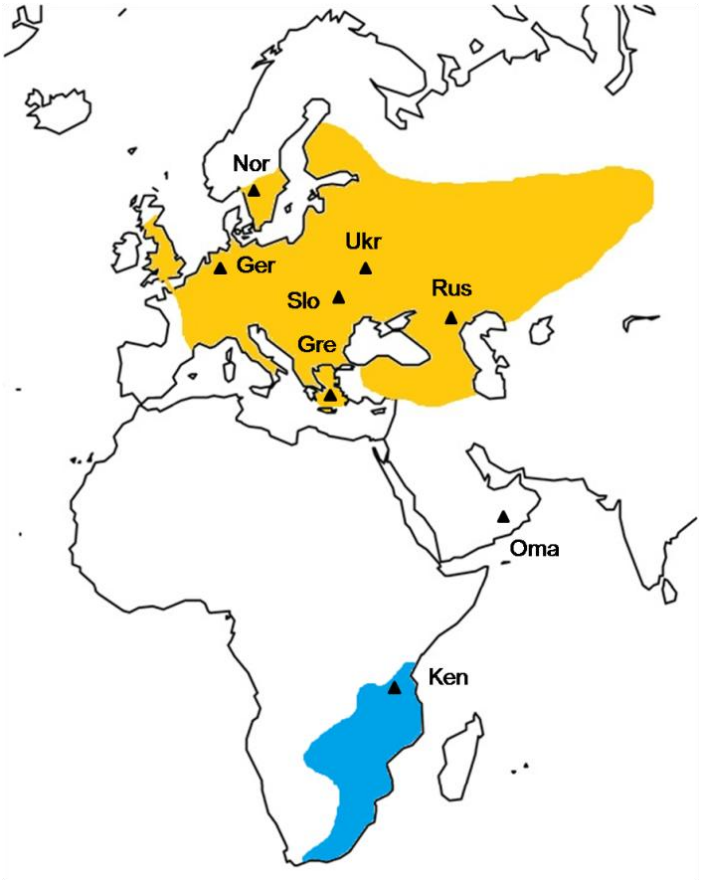

Fig. 3.3.1 Approximate locations of the sampling sites of *A. palustris*. Breeding (yellow) and wintering (light blue) distribution (del Hoyo *et al.* 2006). The names of localities are abbreviated as in Table 3.3.1. Germany includes Franconia, Treysa and Saar

In the present study, first we analyzed 611 nt of the mitochondrial cytochrome oxidase c subunit I gene (*COI*) for a total of 229 marsh warblers, and furthermore we added 829 nt of mitochondrial cytochrome *b* (*cyt b*) of 49 individuals from different populations to increase the accuracy of the analyses. The reasons were 1: recent concerns over studies which are based on a single locus (Edwards *et al.* 2005) and 2: the publication of (Boonseub *et al.* 2009) which compared three most commonly used mitochondrial genes for avian studies (*cyt b*, *COI* and *ND2*) and indicated that *cyt b* is the best one for evolutionary studies. We aimed to (a) evaluate the genetic structure and levels of gene flow, (b) estimate the time since the marsh warbler diverged from its sister species, and (c) draw the demographic history of the population including possible past expansion.

3.3.3 Methods

Sampling, amplification and sequencing

Blood samples of 220 marsh warblers were collected during the breeding, migration and winter time from 10 populations (Table 3.3.1) ranging from Western Asia to Western Europe and Eastern Africa (Fig. 3.3.1). We amplified and sequenced 611 nucleotides (nt) of cytochrome *c* oxidase subunit I gene (*COI*) from all individuals and 829 nt of the cytochrome *b* (*cyt b*) from 49 selected individuals from the same sample collection. Nine *COI* sequences of Norway and Russia and four *cyt b* sequences from Oman were obtained from other studies (Johnsen *et al.* 2010; Kerr *et al.* 2009; Leisler *et al.* 1997).

Table 3.3.1 Localities, number of samples and collectors

Code	Sample site	*N*	Status	Collector
Fra	Franconia, Germany	77	Breeding	B. Leisler
Tre	Treysa, Germany	44	Migrant	H.-H. Witt
Saa	Saar, Germany	25	Migrant	R. Klein, F. Feß, L. Hayo
Slo	Slovakia	37	Breeding	H. Hoi
Ken	Kenya	24	Wintering	G. Nikolaus
Oma	Oman	7	Migrant	G. Nikolaus
Rus	Russia	6	Breeding	Kerr *et al*. (2009)
Ukr	Ukraine	4	Migrant	G. Nikolaus
Nor	Norway	3	Migrant	Johnsen *et al*. (2010)
Gre	Greece	2	Migrant	D. Ristow

Genomic DNA was extracted from 100 µL blood using standard proteinase K (Merck, Darmstadt, Germany) and phenol-chloroform extraction protocols (Sambrook *et al.* 1989). The DNA was adjusted to a concentration of 100 ng/µl and stored at -20 °C until use. The *COI* gene was amplified using PasserF1/PasserR1 and ExtF/BirdR2 (Johnsen *et al.* 2010; Sheldon *et al.* 2009) and L14990/H16065 (Hackett 1996; Kocher *et al.* 1989) for *cyt b*. PCR amplifications were carried out in the following reaction mixture (total volume of 50 µL): 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris (pH 8.5), 50 mmol/L KCl, 100 µmol/L dNTPs, 0.2 units of *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng DNA, and 5 pmol of primers. Optimal annealing temperatures were established by gradient PCR in a Tgradient thermocycler (Biometra, Gottingen, Germany). The samples were denatured at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 51.5 °C for 1 min, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 10 min. PCR products were precipitated with 4 M NH_4Ac and ethanol (1:1:6) and centrifuged; for 15 min (13,000 rpm). Sequencing was performed on a ABI 3730 automated capillary sequencer (Applied Biosystems, CA, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (carried out by STARSEQ GmbH, Mainz, Germany). Sequencing primers correspond to the primers used for PCR amplifications. The sequences were deposited at GenBank under the accession numbers listed in Table 3.3.2.

Phylogenetic and population genetic analyses

Sequences were aligned using Clustal W (Thompson *et al.* 1994) and confirmed by visual inspection in BioEdit version 7.0.9.0 (Hall 1999). For phylogenetic reconstruction, we made use of Maximum Likelihood implemented in MEGA version 5.1 (Tamura *et al.* 2011). The phylogenetic reconstruction was rooted with closely related taxa (Leisler *et al.* 1997) such as *A. scirpaceus*, *A. baeticatus*, *A. dumetorum*, *A. agricola*, *A. concinens* and *A. tangorum*, while node support was evaluated by 1000 bootstrap replicates. The phylogeographic structure was assessed by constructing a median-joining network with Network version 4.6.1.1 (Bandelt *et al.* 1999).

Within populations the genetic diversity was measured by the number of haplotypes, number of segregating sites, haplotype diversity, average number of pairwise differences and nucleotide diversity using Arlequin version 3.5.1.2 (Excoffier & Lischer 2010). We performed two neutrality tests of Tajima's *D* (Tajima 1989) and Fu's Fs (Fu 1997) which are based on allele frequency. Mismatch distributions (Rogers & Harpending 1992) and the time of population expansion ($\tau = 2\ \mu T$) were also estimated in Arlequin with 1000 bootstrap

replicates and compared statistically against models of sudden population expansion (100 replicates) using the sum of squared deviations test. We calculated overall and pairwise F_{ST} with AMOVA (Excoffier *et al.* 1992) in order to obtain population differentiation. The significance of the variance components was determined using 1000 permutations. The R2 test (Ramos-Onsins & Rozas 2002) and gene flow (*Nm* based on F_{ST}) among populations (Hudson *et al.* 1992) were conducted with DnaSP version 5.1 (Librado & Rozas 2009). None of the diversity indices were calculated for small population sizes ($N < 6$).

Divergence time and the age of the most recent common ancestor (TMRCA) were estimated in BEAST version 1.7.5 (Drummond & Rambaut 2007a) assuming a population expansion model, uncorrelated log-normal relax clock, GTR substitution model and a mutation rate uniform distribution ranging from 0.01105 to 0.02500 substitutions/site/lineage/million years (Neto *et al.* 2012; Weir & Schluter 2008). We conducted an analysis consisting of 20 million generations sampled at intervals of 1000. BEAST was also used to model the demographic changes of populations using the Bayesian skyline plot analysis (Drummond *et al.* 2005) with a chain length of 100 million generations sampled every 2000. Effective sample size (ESS) values of at least 200 were evaluated with TRACER version 1.5 (Rambaut & Drummond 2007). 10% of the initial samples were removed as burn-in and maximum clade credibility trees were reconstructed with TreeAnnotator 1.7.5 (http://beast.bio.ed.ac.uk/TreeAnnotator) and visualized using FigTree 1.4.1 (http://beast.bio.ed.ac.uk/FigTree).

3.3.4 Results

Diversity indices

Among the 611 nucleotides of *COI* from a total of 229 individuals 47 polymorphic sites were detected leading to the definition of 44 haplotypes (Table 3.3.3). The most frequent haplotype H1 was found in all populations ($N = 152$), 17 haplotypes were shared between 7 and 2 individuals and 25 haplotypes were unique for single birds. For each population, the genetic diversity was relatively high ($Hd = 0.43–0.80$) while the nucleotide diversity was low ($\pi = 0.001–0.002$). Genetic diversity and nucleotide diversity were both lowest for the origin Saar, while the origin Russia showed the highest diversity for these parameters (Table 3.3.4). Sequence analyses of 49 individuals based on cytochrome *b* gene showed 31 polymorphic sites among 24 haplotypes. The first and second most frequent haplotypes were found in all populations ($N = 22$) and 15 haplotypes were unique for different individuals (Table 3.3.5).

Table 3.3.2 IPMB (Institute of Pharmacy and Molecular Biotechnology) collection numbers, haplotypes (H), origins, collectors and GenBank accession numbers. Data are sorted according to their haplotype (H) number. The number of individuals sharing both the haplotype and the locality are shown between parentheses (*N*)

IPMB	H	Country	Locality (*N*)	Collector	GenBank No. (*COI*)	GenBank No. (*cyt b*)
31707	1	Germany	Franconia (38)	B. Leisler	KF843766	KJ408239
31708	1	Germany	Franconia	B. Leisler	-	KJ408240
31728	1	Germany	Franconia	B. Leisler	-	KJ408241
31747	1	Germany	Franconia	B. Leisler	-	KJ408242
31758	1	Germany	Franconia	B. Leisler	-	KJ408243
31761	1	Germany	Franconia	B. Leisler	-	KJ408244
31771	1	Germany	Franconia	B. Leisler	-	KJ408245
31773	1	Germany	Franconia	B. Leisler	-	KJ408246
31779	1	Germany	Franconia	B. Leisler	-	KJ408247
31784	1	Germany	Franconia	B. Leisler	-	KJ408248
31815	1	Germany	Franconia	B. Leisler	-	KJ408249
6554	1	Oman (4)	-	G. Nikolaus	-	-
6563	1	Ukraine (4)	-	G. Nikolaus	-	KJ408221
19601	1	Greece (2)	-	D. Ristow	-	KJ408222
23402	1	Slovakia (13)	Bratislava	H. Hoi	-	KJ408223
23415	1	Slovakia	Bratislava	H. Hoi	-	KJ408225
23427	1	Slovakia	Bratislava	H. Hoi	-	KJ408226
23448	1	Slovakia	Bratislava	H. Hoi	-	KJ408227
23449	1	Slovakia	Bratislava	H. Hoi	-	KJ408228
23477	1	Slovakia	Bratislava	H. Hoi	-	KJ408229
23483	1	Slovakia	Bratislava	H. Hoi	-	KJ408230
23519	1	Slovakia	Bratislava	H. Hoi	-	KJ408232
23452	1	Slovakia	Bratislava	H. Hoi	-	KJ408233
23550	1	Slovakia	Bratislava	H. Hoi	-	KJ408234
46392	1	Kenya (12)	Ngulia	D. J. Pearson	-	KJ408250
46393	1	Kenya	Ngulia	D. J. Pearson	-	KJ408251
46394	1	Kenya	Ngulia	D. J. Pearson	-	KJ408252
46398	1	Kenya	Ngulia	D. J. Pearson	-	KJ408253
46400	1	Kenya	Ngulia	D. J. Pearson	-	KJ408254
46403	1	Kenya	Ngulia	D. J. Pearson	-	KJ408257
52342	1	Germany	Treysa (26)	H.-H. Witt	-	KJ408258
52393	1	Germany	Treysa	H.-H. Witt	-	KJ408259
52642	1	Germany	Treysa	H.-H. Witt	-	KJ408261
54197	1	Germany	Treysa	H.-H. Witt	-	KJ408262
54210	1	Germany	Treysa	H.-H. Witt	-	KJ408263
56873	1	Germany	Saar (19)	R. Klein, F. Feß, L. Hayo	-	KJ408264
-	1	Norway	Telemark	Johnsen *et al*. (2010)	GU571216	-
-	1	Norway	Ostfold	Johnsen *et al*. (2010)	GU571217	-

IPMB	H	Country	Locality (*N*)	Collector	GenBank No. (*COI*)	GenBank No. (*cyt b*)
-	1	Norway	Ostfold	Johnsen *et al*. (2010)	GU571215	-
-	1	Russia	Astrakhan	Kerr *et al*. (2009)	GQ481278	-
-	1	Russia	Kirovskaya Oblast	Kerr *et al*. (2009)	GQ481276	-
-	1	Russia	Kirovskaya Oblast	Kerr *et al*. (2009)	GQ481273	-
31727	2	Germany	Franconia (2)	B. Leisler	KF843767	-
52361	2	Germany	Treysa	H.-H. Witt	-	-
56912	2	Germany	Saar (2)	R. Klein, F. Feß, L. Hayo	-	-
23596	2	Slovakia	Bratislava	H. Hoi	-	KJ408237
23615	2	Slovakia	Bratislava	H. Hoi	-	KJ408238
31737	3	Germany	Franconia	B. Leisler	KF843768	-
46401	3	Kenya	Ngulia	G. Nikolaus	-	KJ408255
46402	3	Kenya	Ngulia	G. Nikolaus	-	KJ408256
23409	3	Slovakia	Bratislava (2)	H. Hoi	-	KJ408224
31789	4	Germany	Franconia (2)	B. Leisler	KF843769	-
60759	4	Germany	Treysa	H.-H. Witt	-	-
6560	4	Oman	-	G. Nikolaus	-	-
31883	5	Germany	Franconia (3)	B. Leisler	KF843770	-
54353	5	Germany	Treysa	H.-H. Witt	-	-
31729	6	Germany	Franconia (2)	B. Leisler	KF843771	-
60755	6	Germany	Treysa	H.-H. Witt	-	-
31701	7	Germany	Franconia	B. Leisler	KF843772	-
60760	7	Germany	Treysa (2)	H.-H. Witt	-	-
54432	8	Germany	Treysa	H.-H. Witt	KF843773	-
6556	8	Oman	-	G. Nikolaus	-	-
56877	8	Germany	Saar	R. Klein, F. Feß, L. Hayo	-	KJ408265
54449	9	Germany	Treysa	H.-H. Witt	KF843774	-
-	9	Russia	Krasnodarskiy Kray	Kerr *et al*. (2009)	GQ481274	-
23551	9	Slovakia	Bratislava	H. Hoi	-	KJ408235
60687	10	Germany	Treysa	H.-H. Witt	KF843775	-
6507	10	Germany	Franconia	B. Leisler	-	-
23491	10	Slovakia	Bratislava	H. Hoi	-	KJ408231
31844	11	Germany	Franconia (2)	B. Leisler	KF843776	-
31817	12	Germany	Franconia (2)	B. Leisler	KF843777	-
31704	13	Germany	Franconia (2)	B. Leisler	KF843778	-
31797	14	Germany	Franconia (2)	B. Leisler	KF843779	-
54515	15	Germany	Treysa	H.-H. Witt	KF843780	-
23570	15	Slovakia	Bratislava	H. Hoi	-	-
23524	16	Slovakia	Bratislava (2)	H. Hoi	KF843781	-
23529	17	Slovakia	Bratislava (2)	H. Hoi	KF843782	-
64468	18	Kenya	Ngulia	G. Nikolaus	KF843783	-
23569	18	Slovakia	Bratislava	H. Hoi	-	-
64469	19	Kenya	Ngulia	G. Nikolaus	KF843784	-
31801	20	Germany	Franconia	B. Leisler	KF843785	-

IPMB	H	Country	Locality (*N*)	Collector	GenBank No. (*COI*)	GenBank No. (*cyt b*)
31900	21	Germany	Franconia	B. Leisler	KF843786	-
-	22	Russia	Krasnodarskiy Kray	Kerr *et al*. (2009)	GQ481277	-
31821	23	Germany	Franconia	B. Leisler	KF843787	-
31767	24	Germany	Franconia	B. Leisler	KF843788	-
-	25	Russia	Kursk Oblast	Kerr *et al*. (2009)	GQ481275	-
31895	26	Germany	Franconia	B. Leisler	KF843789	-
31733	27	Germany	Franconia	B. Leisler	KF843790	-
31867	28	Germany	Franconia	B. Leisler	KF843791	-
56878	29	Germany	Saar	R. Klein, F. Feß, L. Hayo	KF843792	-
31858	30	Germany	Franconia	B. Leisler	KF843793	-
31827	31	Germany	Franconia	B. Leisler	KF843794	-
56976	32	Germany	Saar	R. Klein, F. Feß, L. Hayo	KF843795	-
60741	33	Germany	Treysa	H.-H. Witt	KF843796	-
46395	34	Kenya	Ngulia	G. Nikolaus	KF843797	-
64452	35	Kenya	Ngulia	G. Nikolaus	KF843798	-
64456	36	Kenya	Ngulia	G. Nikolaus	KF843799	-
52633	37	Germany	Treysa	H.-H. Witt	KF843800	KJ408260
60757	38	Germany	Treysa	H.-H. Witt	KF843801	-
6553	39	Oman	-	G. Nikolaus	KF843802	-
54202	40	Germany	Treysa	H.-H. Witt	KF843803	-
56945	41	Germany	Saar	R. Klein, F. Feß, L. Hayo	KF843804	-
23571	42	Slovakia	Bratislava	H. Hoi	KF843805	KJ408236
23442	43	Slovakia	Bratislava	H. Hoi	KF843806	-
23620	44	Slovakia	Bratislava	H. Hoi	KF843807	-
-	-	Oman	-	Leisler *et al*. (1997)	-	AJ004293
-	-	Oman	-	Leisler *et al*. (1997)	-	AJ004294
-	-	Oman	-	Leisler *et al*. (1997)	-	AJ004344
-	-	Oman	-	Leisler *et al*. (1997)	-	AJ004345

Population genetic structure

Considering breeding populations, AMOVA indicates that 98.21% of the total genetic variability was distributed within populations (Φ_{ST} = 0.02, P = 0.05); considering all populations the total amount of genetic variability (100.66%) exists within populations (Φ_{ST} = -0.01, $P > 0.05$; Table 3.3.6). Pairwise F_{ST} values were low between breeding populations: Franconia/Slovakia (0.01), Franconia/Russia (0.03) and Slovakia/Russia (0.05), and was significant only between Franconia and Slovakia ($P < 0.05$).

AMOVA shows a lack of geographic and population genetic structure in marsh warbler which agrees with high levels of gene flow, for instance, between breeding populations (Franconia-Slovakia-Russia; Nm = 13.69). Moreover, the star-like haplotype-

spanning network (Fig. 3.3.2) shows low levels of sequence divergence (differences of 1–2 nucleotides between individuals), a high frequency of single haplotypes radiating from the most common haplotype (H1) and no indication of haplotype clusters. Maximum Likelihood analysis recognized *A. palustris* as a well-supported monophyletic lineage with little structure among haplotypes (not shown).

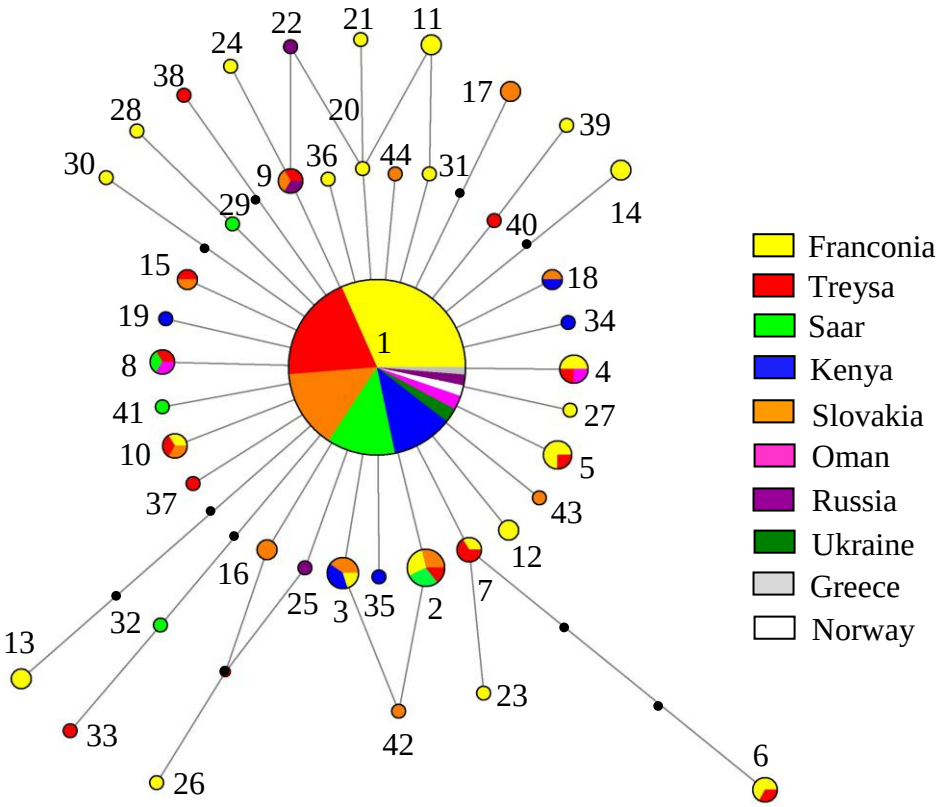

Fig. 3.3.2 Minimum-spanning network for 44 marsh warbler haplotypes (see Table 3.3.3). Circles are drawn proportional to sample size

Demographic analysis

Tajima's *D* values and Fu's *Fs* estimates were negative for each population and highly significant for the complete samples rejecting the null hypothesis of constant size (Table 3.3.4). These results were also supported by the agreement between observed and expected mismatch distributions assuming a model of sudden expansion (Fig. 3.3.3). Additionally, the Bayesian Skyline Plot (Fig. 3.3.3) indicates a gradual population growth of this species with a faster expansion in the last 5,000 years (the time after the last glaciation when most parts of

Europe became suitable for marsh warblers). The phylogenetic analysis showed that *A. palustris* diverged from its sister species, *A. scirpaceus*, approximately 3.8 million years ago (95% HPD: 1.6–6.2 MYA) and we estimated 0.45 MYA for the time to the most recent common ancestor (TMRCA) of *A. palustris* populations (Fig. 3.3.4).

Table 3.3.3 Variable sites among *A. palustris* haplotypes (*COI*). Numbers between parentheses indicate the number of individuals sharing the same haplotype in each site

H	*N*	Alignment position 11 1111222222 2222222233 3333444444 5555555 244556901 4689133345 5666789902 3578124778 0012289 9125173051 7595014732 8147382467 0754197174 1762584	Sample sites
1	152	TGGATACGAG GTATCGGAGC AGAGCTTAAC CAGTGCACAT ATCAACA	Fra(48),Tre(30),Slo(22),Saa(19),Ken(17), Ukr(4),Oma(4),Nor(3),Rus(3),Gre(2)
2	7	....A.	Slo(2),Fra(2),Saa(2),Tre(1)
3	5	....G.	Slo(2),Ken(2),Fra(1)
4	4	..... .C.....	Fra(2),Tre(1),Oma(1)
5	4	....C....	Fra(3),Tre(1)
6	3	.....G. ....A..... .....T..	Fra(2),Tre(1)
7	3	....T..	Tre(2),Fra(1)
8	3	....C.....	Tre(1),Saa(1),Oma(1)
9	3	.....A	Tre(1),Rus(1),Slo(1)
10	3	....T.....	Tre(1),Fra(1),Slo(1)
11	2	.A..C.....	Fra(2)
12	2	....C...	Fra(2)
13	2	....TG... .C.....	Fra(2)
14	2	...G..... ....C	Fra(2)
15	2	.....G....	Tre(1),Slo(1)
16	2	....A.....	Slo(2)
17	2	C..... ....G..	Slo(2)
18	2	......T...	Ken(1),Slo(1)
19	1	..A.....	Ken(1)
20	1	.A.....	Fra(1)
21	1	.A..... ..... G.....	Fra(1)
22	1	.A.....A	Rus(1)
23	1	..... A..... ....T..	Fra(1)
24	1	.....A A.....	Fra(1)
25	1	....A....	Rus(1)
26	1	....A.... ...A..... ....G	Fra(1)
27	1	....A...	Fra(1)
28	1	.... .A..... ....A.....	Fra(1)
29	1	.... .A.....	Saa(1)
30	1	....G.. ....T....	Fra(1)
31	1	....C.....	Fra(1)
32	1	....T ....T.	Saa(1)
33	1	....T ....G.T.	Tre(1)
34	1	.... .G.....	Ken(1)
35	1	....G.....	Ken(1)
36	1	....C.....	Ken(1)
37	1	....G.	Tre(1)
38	1	....T ..... G.....	Tre(1)
39	1	....G.. T.....	Oma(1)
40	1	.... T.....	Tre(1)
41	1	....A.....	Saa(1)
42	1	....A. .....G.	Slo(1)
43	1	.....A..	Slo(1)
44	1	....T.....	Slo(1)
Total	229	47 variable sites	10 populations

Field identification errors

Genetic analyses indicate that a total of 4.1% of the marsh warblers were wrongly identified by the ringers. Most of the misidentifications occurred between *A. palustris* and *A. scirpaceus* (3.2%); even lower error rates concerned *A. schoenobaenus*. An alternative and plausible explanation is mitochondrial introgression due to hybridization, which seems to occur quite regularly in these birds, for instance, *A. palustris* x *A. scirpaceus* (Lemaire 1977; Otterbeck *et al.* 2013), *A. palustris* x *A. schoenobaenus* (Lifjeld *et al.* 2010), and *A. palustris* x *A.*

dumetorum (Koskimies 1980; Lindholm *et al.* 2007; Loon & Keijl 2001; Trnka 2004). Mitochondrial introgression among closely related species is well documented in birds (Taylor *et al.* 2012).

Table 3.3.4 Diversity indices (±SD) across different populations of *A. palustris*. Summary statistics include *N* = sample size; *H* = number of haplotypes; *S* = number of segregating sites; *Hd* = haplotype diversity; *K* = average number of pairwise differences; π = nucleotide diversity; R2 = Ramos-Onsins and Rozas test; τ = time of population expansion ($\tau = 2\ \mu T$)

Locality	*N*	*H*	*S*	*Hd*	*K*	π	Fu's Fs	Tajima's *D*	R2	τ
Franconia (breeding)	77	21	27	0.61±0.07	1.23± 0.79	0.002±0.001	–20.37***	–2.41***	0.02***	0.11
Treysa (breeding, migrant)	44	14	17	0.54±0.09	0.86±0.62	0.001±0.001	–14.003***	–2.49***	0.04***	0.79
Saar (migrant)	25	6	6	0.43±0.12	0.55±0.47	0.001±0.001	–3.85***	–1.95**	0.08^{ns}	0.50
Slovakia (breeding)	37	12	11	0.65±0.09	0.95±0.66	0.001±0.001	–9.99***	–1.97**	0.04***	0.99
Kenya (wintering)	24	7	6	0.50±0.12	0.58±0.48	0.001±0.001	–5.40***	–1.94**	0.07***	0.70
Oman (migrant)	7	4	4	0.71±0.18	1.14±0.83	0.002±0.002	–1.22^{ns}	–1.43^{ns}	0.18^{ns}	1.25
Russia (breeding)	6	4	3	0.80±0.17	1.20±0.88	0.002±0.002	–1.45*	–0.45^{ns}	0.18*	1.37
All breeding populations	120	31	33	0.63±0.05	1.15±0.75	0.002±0.001	–29.20***	–2.45***	0.02***	1.56
Population	229	44	47	0.56±0.04	0.92±0.64	0.001±0.001	–29.92***	–2.58***	0.01**	0.76

Note—Diversity indices were not calculated for populations with small sample sizes, i.e. Ukraine, Norway and Greece.
ns = not significant; *** = P < 0.001; ** = P < 0.01; * = P < 0.05

Table 3.3.5 Variable sites among *A. palustris* haplotypes (*cyt b*). Numbers between parentheses indicate the number of individuals sharing the same haplotype in each site

H	*N*	Alignment position 111112233 3334444445 5566666678 8 1157891203 4591234581 7800126770 1 4794994033 8461337133 3834576519 4	Sample sites
1	17	TTGGGATATA AGCATAGTAA CAACAACCCG C	Slo(7),Oma(4),Tre(3),Fra(1)
2	5	....A...	Slo(2),Tre(2),Ken(1)
3	2	.......G.. .AT.....	Fra(2)
4	2	....T... .	Fra(1),Slo(1)
5	2	...A...... ....C..	Slo(2)
6	2	....TG..... .	Fra(2)
7	2	.... A	Fra(1),Slo(1)
8	2	....T. .	Fra(1),Slo(1)
9	2	....G.....G	Ken(2)
10	1	.......G.. ..T.....	Ken(1)
11	1	.......G.. ..T..... ....C .	Ken(1)
12	1	....A..... ..T.....	Slo(1)
13	1	....T....C..	Gre(1)
14	1	.......G.. ..T..G....	Ukr(1)
15	1	.C........	Saa(1)
16	1	.... .G........ .	Saa(1)
17	1	..A.......	Ken(1)
18	1	..A...C...	Fra(1)
19	1	........C.	Slo(1)
20	1	.... T......... .	Ken(1)
21	1	.... C.......G.	Tre(1)
22	1	C......... ....C..... .....G.... .	Fra(1)
23	1	....G....T.. A	Ken(1)
24	1	.....GC..G	Fra(1)
Total	49	31 variable sites	8 populations

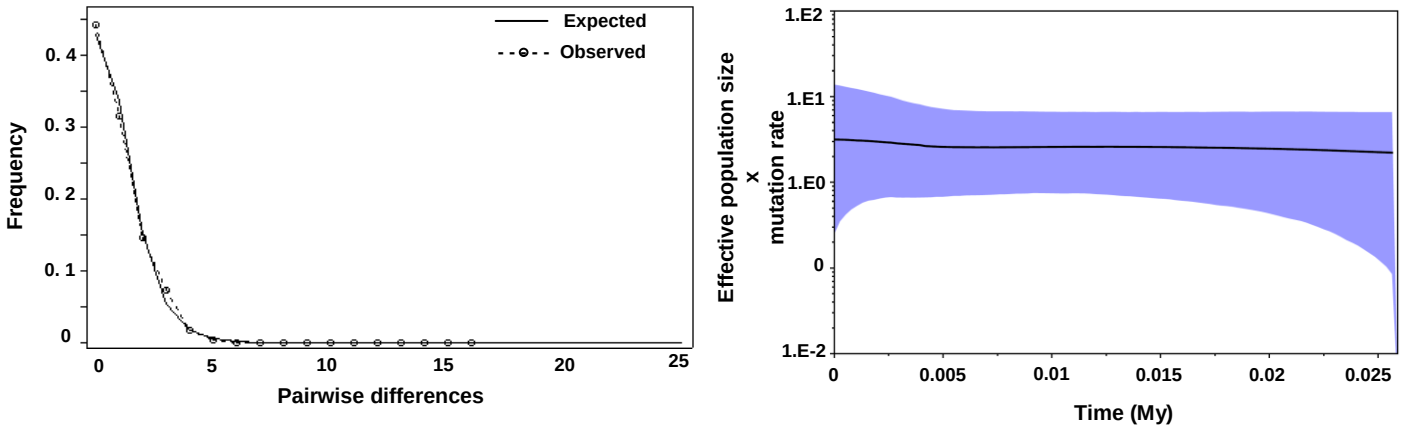

Fig. 3.3.3 Left: Mismatch distribution. Expected (solid line) and observed (broken line) mismatch distribution under population expansion model. Right: Bayesian skyline plot representing the historical demographic trend of mitochondrial lineages of *A. palustris* populations. The mean estimates are shown as thick solid lines, and the light blue area corresponds to the 95% highest posterior density (HPD).

Table 3.3.6 Analysis of molecular variance (AMOVA) based on pairwise differences

Among all populations						
Source of variation	d.f.	SS	Variance	% var.	Φ-stat.	P
Among populations	9	3.62	–0.003	–0.66	–0.01	0.74
Within populations	219	101.78	0.46	100.66		
Total	228	105.40	0.46			
Between breeding populations (Franconia, Slovakia and Russia)						
Source of variation	d.f.	SS	Variance	% var.	Φ-stat.	P
Among populations	2	1.75	0.01	1.79	0.02	0.05
Within populations	117	66.65	0.57	98.21		
Total	119	68.40	0.58			

3.3.5 Discussion

Our genetic analyses revealed high levels of variation within marsh warbler populations and low levels of differentiation among populations. The lack of genetic structure observed in two loci of the mitochondrial genome may be due to high levels of gene flow among breeding populations (Edwards 1993; Zink 1994), and/or historical demographic events such as recent origin and insufficient time to differentiate genetically (Rogers 1995).

In our study, different analyses collectively revealed a recent population expansion, most likely from a single glacial refuge, as a consequence of homogeneity across populations

(Ruegg & Smith 2002). Such a range expansion from a population of small effective size can be visualized by shallow phylogenetic trees and star-shaped haplotype networks with single high-frequency haplotypes and numerous low-frequency variants that differed from each other by a few mutations (Slatkin & Hudson 1991). Poisson distribution of pairwise nucleotide differences among haplotypes (Rogers & Harpending 1992) and a smooth and unimodal shape of a mismatch distribution also support the idea of a recent range expansion. Finally, negative and significant values of neutrality tests in *A. palustris* together with high haplotype diversity and low levels of nucleotide diversity point to the same hypothesis (Avise 2000; Grant & Bowen 1998; Rogers & Harpending 1992).

The genetic makeup of marsh warbler populations has probably been changed within a young coalescent scenario, as revealed by short divergence times to the most recent common ancestor (TMRCA = 0.43 MYA). This shallow time frame might have been not long enough for the establishment of distinct phylogeographic lineages considering the vagrant behaviour of the species.

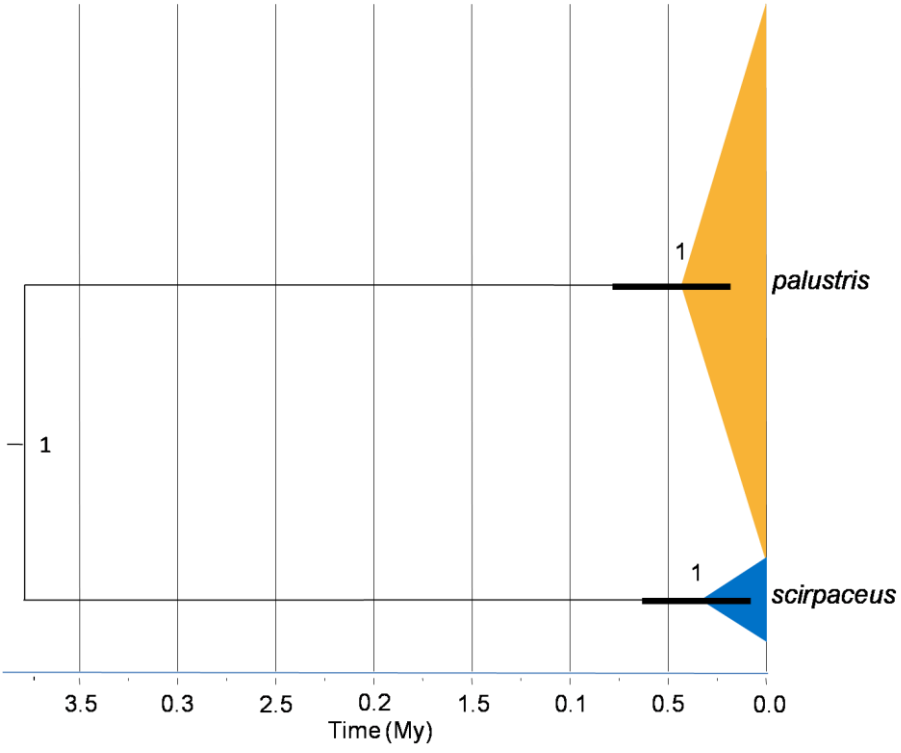

Fig. 3.3.4 Phylogenetic chronogram, a coalescent analysis implemented in BEAST with lognormal relax clock (uniform distribution and 0.01105–0.02500 substitutions per million years) and population expansion model as priors. Thick horizontal bars represent the 95% HDP of the age of major nodes and numbers indicate node posterior probability values

High levels of gene flow among breeding populations (Birky *et al.* 1983) and low F_{ST} values indicate high degree of admixture, enough to avoid allele fixation and strong divergence (Hedrick 2000; Wang 2004). Gene flow may increase the genetic variability within a population by introducing alleles from the other populations, but it results in high genetic similarity between localities and shallow population subdivision by decreasing the influence of genetic drift (Hutchison & Templeton 1999; Ramakrishnan *et al.* 2010). The pattern of low genetic structure is expected for species with high dispersal capacity and of recent range expansion. *Acrocephalus palustris* is a long-distance migrant, for which long geographical distances or landscape obstructions do not represent significant barriers to gene flow, leading to a lack of a phylogeographic pattern over our study area. Moreover, many breeding populations are connected seasonally by overlapping migration routes and shared wintering areas, enhancing the exchange of individuals among populations (Grinnell 1922; Helbig 2003).

Lack of phylogeographical structure due to recent expansion during the Pleistocene and high levels of gene flow due to long distance dispersal, has been reported in close relatives. Studies of the genetic structure in breeding populations of paddyfield warbler (*Acrocephalus agricola*) from NE Bulgaria, SE Russia and S Kazakhstan, based on mitochondrial control region sequences and five microsatellite loci (Zehtindjiev *et al.* 2011) and in reed warbler (*Acrocephalus scirpaceus*) populations across Europe by comparing ten polymorphic microsatellite loci (Procházka *et al.* 2011), show relatively low levels of genetic differentiation among populations in these species. Also, limited availability of refugia and repeated mixing of populations during the climate fluctuations explains low levels of diversification in species of the Northern Hemisphere (Webb & Bartlein 1992). Moreover, extensive regions of Europe were either covered by ice or had steppe tundra vegetation until 12000 years ago that was not suitable as breeding areas for marsh warblers. Thus, only after climate warming, new habitats appeared that could have been recently colonized by marsh warblers giving place to a scenario of low genetic differentiation.

In conclusion, our investigation of the population structure of marsh warblers revealed limited differentiation among populations due to both historical and current-day processes. Population bottlenecks caused by Pleistocene glaciations, and the concomitant loss of genetic diversity, may be followed by postglacial rapid expansion, large population sizes, high dispersal capabilities and thus high levels of gene flow, all leading to a genetically homogeneous population without phylogeographically defined lineages.

4 Conclusions

In this thesis, the phylogenetic relationships in the family Acrocephalidae were investigated using three mitochondrial (*cyt b*, *COI* and *ND2*), five nuclear (myoglobin, *ODC*, *LDH*, b-fibrinogen and *RAG-1*) as well as ISSR markers. Furthermore, two mitochondrial loci (*cyt b* and *COI*) were employed to study the phylogeographic patterns and evolutionary history of populations of Eurasian reed warbler (*Acrocephalus scirpaceus*) and marsh warbler (*Acrocephalus palustris*). Based on the new results presented here, the following points can be concluded:

1. Phylogenetic study

The purpose of this study is evaluation the phylogenetic relationships among 35 species of Acrocephalidae, the most monomorphic passerines (Leisler & Schulze-Hagen 2011), by using the three most common mitochondrial genes in avian species identification and five unlinked nuclear loci as well as ISSR genomic fingerprinting. Findings from the current study result in improved support values and an overall better corroborated of relationships between the genera and species than the previous molecular studies (Fregin *et al.* 2009; Helbig & Seibold 1999; Leisler *et al.* 1997). However, despite the use of 6280 nt, some relationships and some internal nodes in the tree are still poorly resolved. Lack of resolution is the result of rapid divergence, as suggested by Helbig and Seibold (1999) and Fregin *et al*. (2009). Estimated divergence times, based on Bayesian inference, confirmed their suggestion and revealed an evolutionary time framework within the last 12.5 million years. Resolving evolutionary relationships in groups that underwent rapid radiation is a major challenge for molecular phylogeny (Pisani *et al.* 2012). When divergence occurred rapidly and stem lineages are of short duration, accurate phylogenetic reconstruction is difficult and addition of further loci may fail to resolve the phylogenic relationships (Belfiore *et al.* 2008; Wiens *et al.* 2008).

The results obtained in the present study are largely in accordance with the molecular study of Fregin *et al*. (2009). Based on the analyses, the major clades of *Acrocephalus*, *Iduna* (except *I. aedon*), *Hippolais*, *Nesillas* and *Calamonastides* were recovered. The current taxonomic position of *Calamonastides gracilirostris* as a monotypic genus in the tree was supported. The analyses could neither support nor reject the inclusion of *I. natalensis* and *I. similis* in genus *Iduna* but strongly reject the inclusion of *I. aedon* in this group. The inclusion of *aedon* in *Iduna* clade proposed by Fregin *et al*. (2009) was based on comparatively little

genetic evidence, and hence not very well corroborated. The results from eight molecular loci and ISSR could not confirm the inclusion of *aedon* in *Iduna* clade; therefore the resurrection of genus *Phragamaticola* for this different taxon is proposed.

2. Phylogeographic studies

Pleistocene biogeographic events and changes in habitat distribution of the temperate Northern Hemisphere, specifically restriction of vegetation patches, caused by cyclical glaciations over the last 2 million years (Frenzel 1973; Hewitt 2004; Wright *et al.* 1993) appear to have played a major role on the present-day diversity and phylogeographical pattern of species (Avise 2000; Hewitt 2000). The study of mtDNA genetic distances in birds revealed that the main phylogeographic subdivisions within avian species and most sister-species separations dated to the Pleistocene (Klicka & Zink 1997; Zink *et al.* 2004). Avise *et al*. 1998 studied the evolutionary history of a total of 63 avian species. Their studies showed two basic patterns: First, 37 of the species (59%) showed deeply divergent in mtDNA lineages within a species and 28 of these separations (76%) dated to the Pleistocene glaciations (Avise & Walker 1998; Hewitt 2001). Second, the other taxa exhibited shallow genetic differentiation in populations expanded from glacial refugia. They concluded that multiple distinct phylogroups within a species or species complex often indicate the occupancy of multiple glacial refugia. Isolation in refugia leads to genetic divergence between populations from different refugia and over long time frames leads to development of distinct lineages while shallow mtDNA differences that contain no distinct phylogroups may indicate the occupancy of a single glacial refugium (Avise *et al.* 1998). The present study on phylogeography of two widespread and long distance migratory birds, using the sequences of two mitochondrial loci obtained across their vast breeding range, provided sufficient data to recover the Pleistocene and recent history of them. The phylogeographic analyses on Eurasian reed warbler (*Acrocephalus scirpaceus*) and marsh warbler (*Acrocephalus palustris*) present both explained patterns, as further described below.

Molecular analyses on *Acrocephalus scirpaceus* revealed three main lineages within the species that diversified during the Pleistocene, expanding to colonize Asia and Europe from a probable Eastern African origin. These distinct phylogroups (corresponding to the three subspecies: *A. s. scirpaceus, A. s. fuscus* and *A. s. avicenniae*) show a long-term divergence during the Quaternary climatic fluctuations. They are well differentiated in mtDNA; none of the haplotypes are shared between the clades and form monophyletic groups that strongly support their independent history. European subspecies (*A. s. scirpaceus*)

probably expanded from a glacial refugium located in the western part of the species breeding area, somewhere in Southern Europe, while Asian subspecies (*A. s. fuscus*) derived from a refugium in the eastern part of the breeding range. The primitive subspecies (*A. s. avicenniae*) may have survived in one of the African refugia, likely low forest refugium of the Ethiopian Highlands during the Last Glacial Maximum (Levinsky *et al.* 2013). This pattern is quite similar to the origin of the Old World Sparrows (Allende *et al.* 2001). Gene flow analysis on two subspecies of *A. s. scirpaceus* and *A. s. fuscus* across Europe by Procházka *et al.* (2011) confirmed barriers of nuclear gene flow that corresponds to a pattern of Pleistocene differentiation in an Eastern European/Middle East refuge. Two mtDNA data in the present study strongly corroborate an Eastern European/Middle East Pleistocene biogeographical division in *A. s. scirpaceus*/*A. s. fuscus*. This pattern is commonly found in the other passerine birds e.g. great reed warbler (Hansson *et al.* 2008), chiffchaffs (Helbig *et al.* 1996), the subalpine warbler (Brambilla *et al.* 2006), and flycatchers (Hogner *et al.* 2012; Lehtonen *et al.* 2009) as well as many different avian species (Avise & Walker 1998).

In contrast to the results of *Acrocephalus scirpaceus*, *A. palustris* exhibits low levels of differentiation among populations which indicate occupation of a single glacial refugium during the Pleistocene epoch. High levels of gene flow among breeding populations (Franconia-Slovakia-Russia; *Nm* = 13.69) is another factor which affects the lack of geographic and population genetic structure in *A. palustris*. High current gene flow leads to high degree of admixture, enough to avoid allele fixation and strong divergence (Hedrick 2000; Wang 2004) and results in high genetic similarity between localities and shallow population subdivision by decreasing the influence of genetic drift (Hutchison & Templeton 1999; Ramakrishnan *et al.* 2010). The pattern has also been observed in breeding populations of paddyfield warbler (*Acrocephalus agricola*) across a vast distribution area of Bulgaria, Russia and Kazakhstan (Zehtindjiev *et al.* 2011).

Moreover, The Bayesian Skyline Plot indicates the population growth of both species after cold and dry climate of glaciation, when more suitable habitats were available and species could expand their geographic ranges out of their limited regions during the Last Glacial Maximum (Dubey *et al.* 2006; Hewitt 2000; Murray Gates 1993; Wright *et al.* 1993). Recent population expansions among birds in Northern hemisphere have been reported for other avian species: MacGillivray's warbler and wood duck in North America (Milá *et al.* 2000; Peters *et al.* 2005) as well as Savi's warbler and eider duck across Europe (Neto *et al.* 2012; Tiedemann *et al.* 2004).

3. *Additional findings of the research*

DNA analyses revealed high number of identification errors between reed warblers in the field. The plumages of Eurasian reed warbler subspecies and closely related species especially marsh warbler (*Acrocephalus palustris*) are very similar and present a significant identification problem for many birders. In order to distinguish among these taxa, close attention to the combinations of morphological, biometrical and vocal characters are needed (Leisler & Schulze-Hagen 2011; Pearson *et al.* 2002). DNA sequence data can discover and reduce these kinds of mistakes. The other important finding of this research is presenting the first genetic evidence of Asian subspecies (*Acrocephalus scirpaceus fuscus*) in Central Europe (Treysa, Germany). In recent years, there have been several reports of *A. s. fuscus* from Britain, but they remained equivocal due to suspected identification mistakes (Pearson *et al.* 2002).

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Affidavit for Dissertation

I hereby declare that this thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

_____26 June 2014_____

Tayebeh Arbabi