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Mitochondrial phylogeny of the Eurasian/African reed warbler complex (*Acrocephalus*, Aves). Disagreement between morphological and molecular evidence and cryptic divergence: A case for resurrecting *Calamoherpe ambigua* Brehm 1857

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ABSTRACT

A tree based on the mitochondrial cyt *b* gene for 278 samples from throughout the range of the Eurasian Reed Warbler *Acrocephalus scirpaceus* – African Reed Warbler *A. baeticatus* complex shows well-supported geographically structured divergence for eight distinct lineages. The phylogenetic structuring together with the clarification of priority, provided by sequence data from seven type specimens, suggests that both taxonomy and distribution boundaries are in need of revision. The Iberian and Moroccan populations form a well-supported clade, and we propose that these are treated as taxonomically distinct, under the name *ambiguus* (Brehm, 1857). We propose that the names *scirpaceus*, *fuscus*, *avicenniae*, *ambiguus*, *minor*, *cinnamomeus*, *hallae* and *baeticatus* are used for the well supported clades in the complex, which we recommend to treat as one polytypic species, *A. scirpaceus*, pending studies of gene flow and assortative mating in the contact zones.

1. Introduction

The reed warblers (*Acrocephalus*, Acrocephalidae) comprise 37 species as currently recognized, most of them occurring in wet habitats, such as reedbeds and other marsh vegetation, but also in bush and scrubland (Cramp, 1992; Pearson, 2006; Kennerley and Pearson, 2010; Leisler and Schulze-Hagen, 2011; Clements et al., 2012; Dickinson and Christidis, 2014; Gill and Donsker, 2015). A large proportion of the species have rather plain, nondescript plumage, with brownish or greyish upperparts in various shades, and whitish underparts with more or less distinct buff hues (Pearson, 2006; Kennerley and Pearson, 2010).

One group of smaller reed warblers with unclear taxonomy is distributed throughout Europe, western Asia and Africa, differing mainly in hues of coloration, sometimes also average differences in measurements of bill, wing or tail-lengths. The Eurasian Reed Warbler *Acrocephalus scirpaceus* (Hermann, 1804), is a polytypic species breeding from Europe to central Asia and North Africa. Nominate *scirpaceus* breeds in the western part of the range, from North Africa and Spain to Greece, north to Scandinavia and east to Western Russia, Ukraine and Crimea (Kennerley and Pearson, 2010), although Iberian populations south of Catalonia differ genetically from the rest of Europe (Procházka et al., 2011). The subspecies *fuscus* (Hemprich and Ehrenberg, 1833) occupies the eastern part of the range, breeding in the Middle East, Cyprus and Levant, and from the northern Caspian area east to Kazakhstan and extreme north-west China (western Xinjiang), south to Iran and north-west Afghanistan (Kennerley and Pearson, 2010). Both

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are summer visitors to the northern hemisphere and winter in sub-Saharan Africa. Kralj et al. (2010) reported differences in wing shape and indications of restricted gene flow between coastal and inland breeding populations in Croatia, and proposed that these differences may imply that the two populations may use different migratory routes and/or winter in different areas. They did not evaluate the phylogenetic position of these two populations in relation to a larger data set. To the south, distinct populations breeding in coastal mangroves of the Red Sea (Sudan, Eritrea, western Arabia and north Somalia) are recognized as the subspecies *avicenniae* (Ash et al., 1989). These were previously suspected to be closely related to the African Reed Warbler *A. baeticatus* (Vieillot, 1817) (Cramp, 1992), but were suggested to be closer to *A. scirpaceus* by Leisler et al. (1997). Morgan (1998) remarked that reed warblers breeding in Israel showed characters recalling *A. s. avicenniae*, rather than *A. s. fuscus*, and Hering et al. (2009) reported *A. s. avicenniae* from a desert oasis in north-western Egypt.

South of the Sahara, the African Reed Warbler *A. baeticatus* (Vieillot, 1817) is patchily distributed across much of the continent. The relationship between Eurasian *A. scirpaceus* and the sub-Saharan *A. baeticatus* is unclear. There are consistent differences in biometrics, but these could be adaptations to different migration strategies of the various taxa – migratory populations being generally larger with longer and more pointed wings than sedentary populations. Most recent authors have considered *A. scirpaceus* and *A. baeticatus* as two distinct species (e.g. Kennerley and Pearson, 2010; Pearson, 2006; Gill and Donsker, 2015), whereas other authors have lumped them in a single species, because of similar vocalizations (Dowsett-Lemaire and Dowsett, 1987; Dickinson and Christidis, 2014). Clancey (1975) splits the African taxa in two species, *A. cinnamomeus* to the north, and *A. baeticatus* to the south. Early molecular studies of Palearctic and African *Acrocephalus* warblers (including the taxa *scirpaceus*, *fuscus*, *avicenniae* and *guiersi*) using mitochondrial cytochrome *b* (cyt *b*) gene sequences (Helbig and Seibold, 1999; Leisler et al., 1997) could not confidently resolve the phylogenetic relationships within the *A. scirpaceus–baeticatus* complex. None of these included any *A. baeticatus* populations other than *guiersi* from Senegal. *A. baeticatus* is currently separated into five subspecies (Pearson, 2006; Kennerley and Pearson, 2010; Gill and Donsker, 2015): (1) *baeticatus* (Vieillot, 1817) – north Botswana and Zimbabwe to south and south-east South Africa; (2) *hallae* (White, 1960) – west South Africa, Namibia, west Botswana, to Angola and South Zambia; (3) *suahelicus* (Grote, 1926) – coastal Tanzania (mainly in mangroves) to east Mozambique and north-east South Africa (KwaZulu-Natal); (4) *guiersi* (Colston and Morel, 1984) – northern Senegal (along Senegal river); (5) *cinnamomeus* (Reichenow, 1908) – patchily across the Sahel from southern Senegal, south Mali, east Niger, Nigeria, Cameroon, Gabon, Chad and from Sudan to Ethiopia and Somalia, also in Congo, Zambia, Malawi and Mozambique. No phylogenetic study to date has included all these taxa.

Eurasian Reed Warblers breeding in North Africa – from Morocco to Libya – have long been considered to belong to *A. s. scirpaceus* (Clements et al., 2012; Cramp, 1992; Pearson, 2006; Leisler and Schulze-Hagen, 2011; Dickinson and Christidis, 2014; Gill and Donsker, 2015), and individuals identified as this taxon by molecular markers were recently reported to breed close to Benghazi in Libya (Hering et al., 2009; Hering et al., 2010). However, at the same site breeding individuals reported to be genetically close to *A. baeticatus guiersi* were present in apparently greater numbers than *A. s. scirpaceus* (Hering et al., 2009, 2010). Also in Morocco the breeding populations consist of individuals phenotypically recalling *A. baeticatus* (Jiguet et al., 2010; Amezian et al., 2010), but in contrast to the situation in Libya evidence of breeding *scirpaceus* are lacking. Based on phylogenetic analysis of mitochondrial control region 2 markers, Winkler et al. (2013) showed that reed warblers from Libya are part of the same clade as individuals from Portugal and Spain. This clade, in turn, was indicated to be most closely related to individuals sampled in Senegal, and Winkler et al. (2013) suggested that these two clades are more closely related to European Reed Warblers than to *A. baeticatus* from South Africa and could deserve subspecific or perhaps even specific status. Winkler et al. (2013) also pointed out that Brehm (1857) gave the name *ambigua* to the Reed Warblers inhabiting Valencia and Murcia, but considered this name unavailable as they deemed the original description inadequate and were unable to locate a type specimen.

Several previous studies contributed to the understanding of the phylogeny of the *A. scirpaceus–baeticatus* complex by molecular methods (Arbabi et al., 2014; Fregin et al., 2009; Helbig and Seibold, 1999; Hering et al., 2009; Hering et al., 2010; Leisler et al., 1997; Leisler and Schulze-Hagen, 2011; Winkler et al., 2013), but most of these focused on only part of the range of the complex and none included all taxa. We gathered DNA data from throughout the range of the complex, and here present the results of a molecular study that includes representatives of all of the currently recognized taxa in the complex, including sequence data from a majority of the type specimens, combined with a comprehensive morphometric analysis of the breeding populations of the Iberian Peninsula.

2. Materials and methods

2.1. Molecular analyses

2.1.1. DNA sampling and sequencing

The analyses were based on DNA sequences from a total of 278 Eurasian and African Reed Warblers, sampled from throughout the range (Supplementary Table 1, Fig. 1). Most samples were from fresh blood or feathers, but samples from the type specimens and a few others were from toepads. 17 of the samples were kindly provided by the Muséum National d'Histoire Naturelle, Paris, France (MNHN), the Natural History Museum, Tring, UK (NHM), and the American Museum of Natural History, New York, USA (AMNH). Among these were the lectotype and two paratypes of *Calamoherpe ambigua* (Brehm, 1857) (AMNH SKIN 455328, and 455326 and 455327; Hartert, 1918), collected on 19 June, in August and on 19 July at Jativa, province of Valencia, Spain, respectively, and the holotypes of *guiersi* (Colston and Morel, 1984), *hopsoni* (Fry et al., 1974), *minor* (Lynes, 1923), *avicenniae* (Ash et al., 1989), *hallae* (White, 1960) and *nyong* (Bannerman, 1936). Additionally, 54 GenBank sequences of a selection of *Acrocephalus scirpaceus* and *A. baeticatus* were added to the data set. Two *Acrocephalus palustris* were used as outgroup for rooting the phylogeny, based on Fregin et al. (2009).

DNA was extracted using QIA Quick DNEasy Kit (Qiagen, Inc.), according to the manufacturer's instructions, but with 30 µl DTT added to the initial incubation step for the extraction from feathers and toe-pads. We sequenced the mitochondrial cytochrome *b* (cyt *b*) gene following the protocols described in Olsson et al. (2005) for the amplification and sequencing of the fresh samples. For the toe-pad samples, cyt *b* was sequenced in small fragments, using a range of specific primer combinations (Supplementary Table 2). All sequences have been submitted to GenBank (Supplementary Table 1).

2.1.2. Phylogenetic analyses

Sequences were aligned using MegAlign 4.03 in the DNAstar package (DNAstar Inc.). The choice of substitution model was determined based on the Bayesian Information Criterion (Schwarz, 1978) calculated in jModeltest (Darriba et al., 2012;

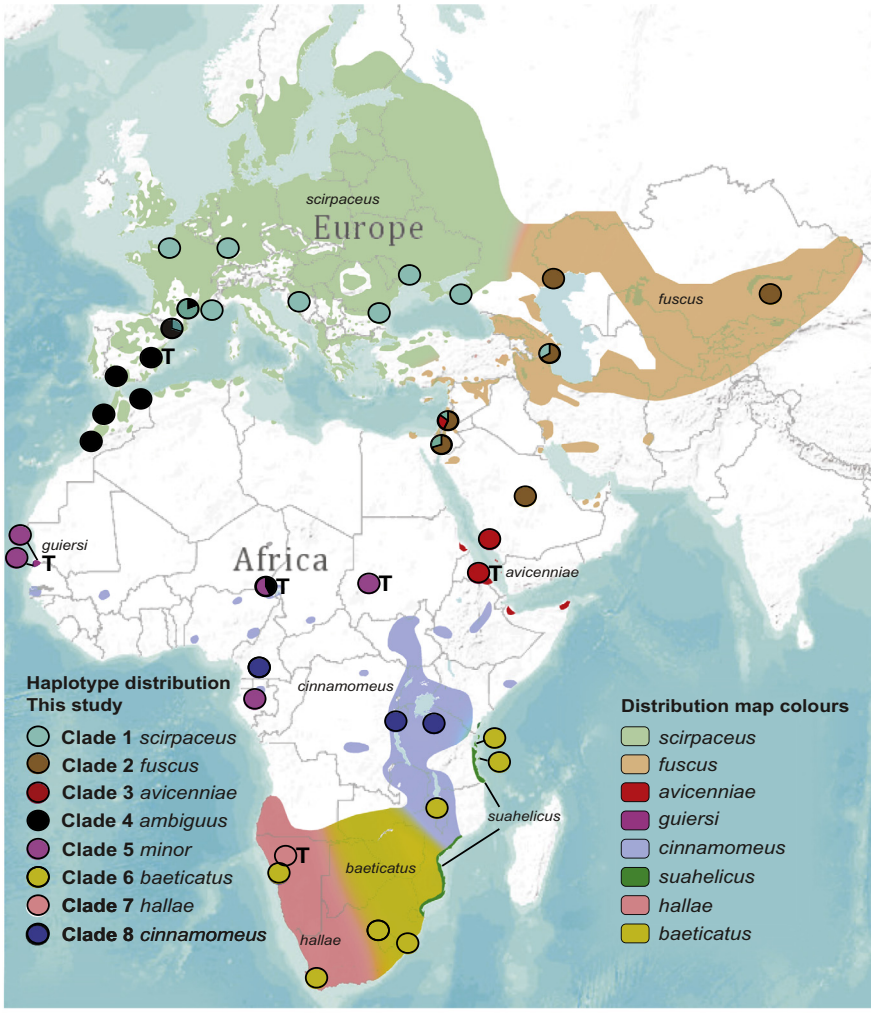

Fig. 1. Sampling localities and haplotype distribution superimposed on distribution ranges based on BirdLife International and NatureServe (2014). Bird species distribution maps of the world, with approximate ranges of the different taxa based on Pearson (2006). Localities where more than one haplotype has been recorded are given as pie charts, with the size of the sections proportional to the number of samples. If a holotype was sequenced from a locality, this is indicated by a T to the right of a circle.

Guindon and Gascuel, 2003). The preferred model was the Tamura-Nei (TrN = TN93) model (Tamura and Nei, 1993) assuming variable base frequencies, equal transversion rates and variable transition rates. We also estimated the phylogeny under the GTR + G model, as this was the model used by Weir and Schluter (2008).

The cyt *b* dataset was analyzed using BEAST version 2.2.1 (Bouckaert et al., 2014). Xml files for the BEAST analyses were generated in BEAUti 2 (Bouckaert et al., 2014). We ran several analyses with and without outgroup included, and under different models. The MCMC output was analyzed in Tracer version 1.6.0 (Rambaut and Drummond, 2013) to determine the effective sample size of each parameter and evaluate whether valid estimates of the posterior distribution of the parameters had been obtained. Comparisons of clocklike and non-clocklike analyses determined that the substitution rates were not clocklike, and hence a lognormal prior was used for the final analyses. Final phylogenetic analysis and molecular dating were obtained from the same runs using a Yule process tree prior and a clock rate of 2.1%/million years (my) (cf. Weir and Schluter, 2008). Other priors were used with default values. 2×10^8 generations were run, sampled every 10,000 generations. Every analysis was run twice. The first 25% of the generations were discarded as "burn-in", well after stationarity of chain likelihood values had been established. Trees were summarized using TreeAnnotator version 2.2.1 (Rambaut and Drummond, 2015), choosing "Maximum clade credibility tree" and "Mean heights", and displayed in FigTree version 1.4.2 (Rambaut, 2014). Tracer version 1.6.0 was used to evaluate the performance of the MCMC.

2.1.3. Demographic analyses

Descriptive statistics of nucleotide variation and population differentiation were calculated in DNAsp 5.0 (Librado and Rozas, 2009) for the six main clades that contained more than three samples. We performed three statistical tests to evaluate evidence for or against the populations being in demographic equilibrium, using different sources of information. Coalescent simulations were performed to test for statistical significance. We chose one method using information from the distribution of mutation frequencies, R_2 (Ramos-Onsins and Rozas, 2002). In a growing population, an excess of recent mutations would be expected to produce an excess of singletons (substitutions present in only one sampled sequence) (Slatkin and Hudson, 1991; Tajima, 1989). R_2 is based on the difference between the number of singleton mutations and the average number of nucleotide differences. The null hypothesis is a

population at demographic equilibrium, and after a recent strong population growth, values of R_2 are expected to become lower. Fu's F_S test statistic (Fu, 1997) and Tajima's D (Tajima, 1989), on the other hand, are based on haplotype distribution. Also in this case, the null hypothesis is a population at demographic equilibrium, and an excess of singleton mutations caused by an expansion would result in low values. Simulations by Fu (1997) and Ramos-Onsins and Rozas (2002) suggest that Tajima's D is the least powerful of these methods, but we include it for comparison, as it is widely used (e.g. by Arbabi et al., 2014). According to Ramos-Onsins and Rozas (2002), the most powerful tests for detecting population growth are Fu's F_S test and their own R_2 test. They found the behavior of the R_2 test to be superior for small sample sizes, whereas F_S performed better for large sample sizes (Ramos-Onsins and Rozas, 2002). For the four clades for which population expansion was inferred, we calculated the time since expansion from the equation $t = \tau/2u$, using the mismatch calculator by Schenekar and Weiss (2011; tool provided by Weiss) where τ is a unit of mutational time and u is the cumulative substitution rate per generation across the DNA fragment.

2.2. Morphometric analyses

158 different individuals were examined and measured, including live birds captured by mist-netting in Spain and southern France, and specimens held at MNHN and AMNH. 30 *A. s. scirpaceus* were studied in the MNHN (28 from France and 2 from Morocco, the two latter with reference numbers CG1970-566 and CG1970-568), and 11 in the AMNH, originating from France (1), the United Kingdom (3), Germany (1), Romania (2), Congo (1), Algeria (1, AMNH594589, male collected in Algerian Sahara, 15 May 1912, wing length 68 mm) and Spain (2; AMNH455324, collected in Galicia, June 1854, wing length 70 mm; AMNH455334, collected in Catalonia, 2 May 1856, wing length 67 mm).

Museum specimens from North African breeding populations included a single specimen at the AMNH (from Oued Souss near Taroudant, Morocco) and 3 at the MNHN (1 from Algeria and 2 from Morocco, Saïdia and Agadir).

We gathered biometric data from live birds: 5 from Etang de Canet, Pyrénées Orientales, south France; 69 from Spain (Doñana National Park, Andalucía, June 2011, $n = 22$; Valencia province, 9 at Torreblanca and 12 at Castellon, June 2011, $n = 21$; and Catalonia, at Embassament d'Utxesa, Torres de Segre, Lleida, 21 June 2011, $n = 26$). This North African/Iberian group was complemented with the three specimens preserved at AMNH as the lectotype and paralectotypes of *Calamoherpe ambigua* (Brehm, 1857) (AMNH455328, AMNH455326 and AMNH455327, respectively; Hartert, 1918). We did not obtain comparable measurements for live birds of Moroccan breeding populations.

From the sub-Saharan population, we gathered biometric data from 10 live '*guiersi*' captured in southern Mauritania close to the Senegal River, and 8 specimens of the same subspecies in the MNHN.

In addition to these, we measured a number of museum specimens (names according to labels) 1 *hallae* (Zimbabwe); 2 *cinnamomeus* (also sampled for the mtDNA analysis: one from Gabon, haplotype of clade 5, and one from Burundi, haplotype of clade 8) held in the MNHN; 4 *baeticatus* (Transvaal, Cape in South Africa; 7 *cinnamomeus* (Congo); 2 *hallae* (Angola); and 1 *suahelicus* (Zanzibar) held in the AMNH; and the holotype of *guiersi* held in the collection of The Natural History Museum, Tring, UK (BMNH 1983.4.1).

We obtained 13 biometric measures on these birds: length of flattened wing, tarsus, bill (tip to skull), head plus bill, and hind claw (with a precision of 0.1 mm). We further measured (to the nearest half mm) the distance of primaries (P) 2 to 7 (numbered descendently P2 to P7) to the wing tip, the length of the notch on the inner web of P2, and the length of P1 compared to the tip of the primary coverts. The measurements were taken by Frédéric Jiguet for all museum specimens (at AMNHN and MNHN; except AMNH 455327 and 455328 measured by Peter Capainolo, and BMNH 1983.4.1 measured by Jose Luis Copete). Live birds were measured by Julien Fouchet (*guiersi* captured in Mauritania); José Luis Arroyo (Andalusia); German Lopez (El Hondo NP, Valencia), Sergi Sales (Catalonia) and Lionel Courmont (Etang de Canet, France).

2.3. Principal Component Analysis on biometrics

We performed a Principal Component Analysis using the 13 biometric variables from 158 individuals. The biometric variables were first normalized to account for individual variation in body size. To do so, we first multiplied tarsus length, wing length, head plus bill length and bill length for each individual then we calculated the quadratic root of this product of 4 linear dimensions, and further divided each of the 13 biometric measures by this estimate of individual relative body size.

3. Results

3.1. Molecular phylogenetics

3.1.1. Molecular analyses

We obtained sequences of the cyt *b* gene from 281 individuals in the *Acrocephalus scirpaceus–baeticatus* complex, including the type specimens, with the exception of one of the paralectotypes of *Calamoherpe ambigua*, from which no useful sequence data was obtained. From most of the samples a contiguous 886 base pair portion was obtained, but for some of the samples from GenBank or museum specimens sequences were incomplete (Supplementary Table 1). In a preliminary phylogenetic analysis three GenBank sequences (AJ004297, AJ004299, AJ004300) appeared to be spurious and were excluded from the final analysis. The final phylogenetic analyses of the light strand of the cyt *b* gene were based on 278 sequences and contained 886 characters, of which147 (16.5%) were parsimony informative, 681 (77%) constant, and 58 (6.5%) variable but parsimony-uninformative. No unexpected stop codons, indels or distinct double peaks in the chromatograms that would indicate the presence of nuclear pseudogenes (e.g. Sorenson and Quinn, 1998) were found. Details of origin and GenBank accession numbers are given in Supplementary Table 1.

3.1.2. Phylogenetic and dating analyses

Eight main clades or lineages were identified (Fig. 2, analyzed without outgroup). The main clades are well supported, but internal nodes are insufficiently supported, and the branching pattern among these clades is uncertain. Estimated mean ages for the main nodes in the *A. scirpaceus–baeticatus* complex are given in Supplementary Fig. S1.

The basal divergence is estimated at 0.77 million years ago (mya) (95% highest posterior distribution [HPD]: 0.57–1.02 mya) (Supplementary Fig. S1), and separates an insufficiently supported northern group (clades 1–3, Figs. 2 and 3) from a likewise insufficiently supported clade, containing four entirely African clades, and one which extends to the Iberian peninsula (clades 4–8) (Figs. 2 and 4). The basal divergence in the northern group (clades 1–3) is inferred to 0.58 mya (HPD: 0.41–0.79 mya) (Supplementary Fig. S1). Clades 1–3 contains samples from the range of *scirpaceus*, *fuscus* and *avicenniae* (Figs. 1 and 3). Clades 2 and 3 are inferred to be sisters, but the support is insufficient.

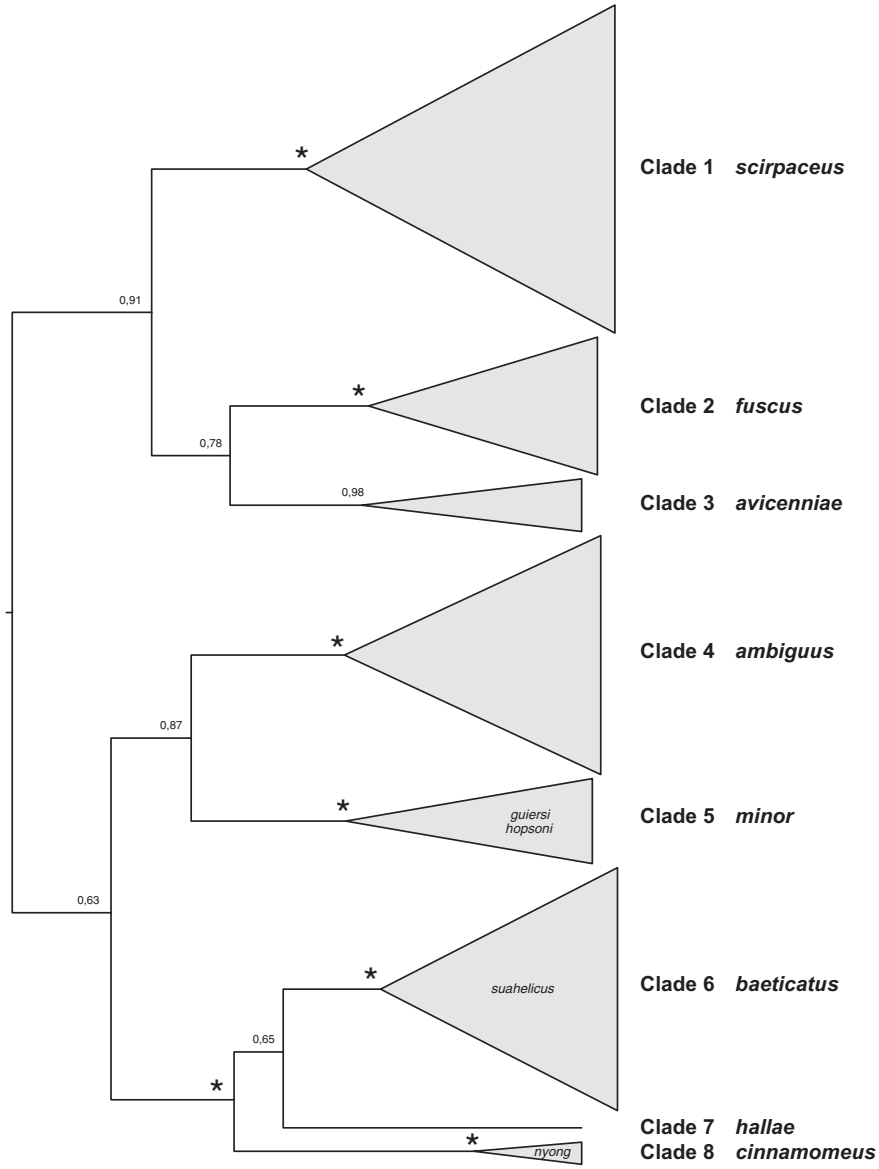

Fig. 2. Cytochrome *b* tree for 278 samples of the *Acrocephalus scirpaceus–A. baeticatus* complex. Numbers above the nodes denote posterior probability (PP), a * representing a PP value of 1.00.

The African clades (4–8) are inferred to have diverged into a northern (clades 4–5) and a southern group (clades 6–8) approximately 0.64 mya (HPD: 0.45–0.85 mya) (Supplementary Fig. S1). Clade 4 contains Iberian and Moroccan samples and is inferred to have diverged from clade 5, containing samples primarily from Senegal, Lake Chad and Sudan (Figs. 1, 2 and 4), 0.53 MYA (HPD: 0.36–0.72 mya) (Supplementary Fig. S1). The Iberian/Moroccan clade includes the lectotype of *Calamoherpe ambigua* (Brehm, 1857), the majority of the samples from Morocco and Spain, and also three samples from southern France and three from Lake Chad. Clade 5 contains sequences from the holotypes of *guiersi*, *hopsoni* and *minor*.

The inclusivity of the sub-Saharan African clades (clades 5–8; Figs. 1, 2, 4 and 5) is at odds with current taxonomy (e.g. Kennerley and Pearson, 2010; Pearson, 2006), as populations from Lake Chad are not included in the same clade as samples from further south in Africa, clades 6–8. The distribution boundaries of the central and southern African taxa also appear to be different from those generally recognized (e.g. Kennerley and Pearson, 2010; Pearson, 2006) in that topotypical *suahelicus* are subsumed in clade 6, which also includes all our samples from south of the Sahel region with only five exceptions: one sample from Gabon nested in clade 5; one sample from inland Tanzania, one from Burundi, near the type locality of *cinnamomeus*, and the holotype of *nyong*, the three latter together making up clade 8. Unexpectedly, the holotype of *hallae* is distinct from all other samples from south-western Africa, including those from the immediate vicinity of the type locality (Figs. 2 and 5). The divergence between clades 6–8 is inferred to 0.47 mya (HPD: 0.31–0.66 mya) (Supplementary Fig. S1).

3.1.3. Demographic analyses

The population genetics analyses of the sequence data, summarized in Table 1, suggest significant departures from the null hypotheses for clades 1, 2, 4 and 6, both for Fu's F_S, R_2 and Tajima's D. For clade 1, all estimators indicate population expansion. The onset of an expansion is estimated to 205 thousand years ago (kya), a period of cooling, during the transition from the marine

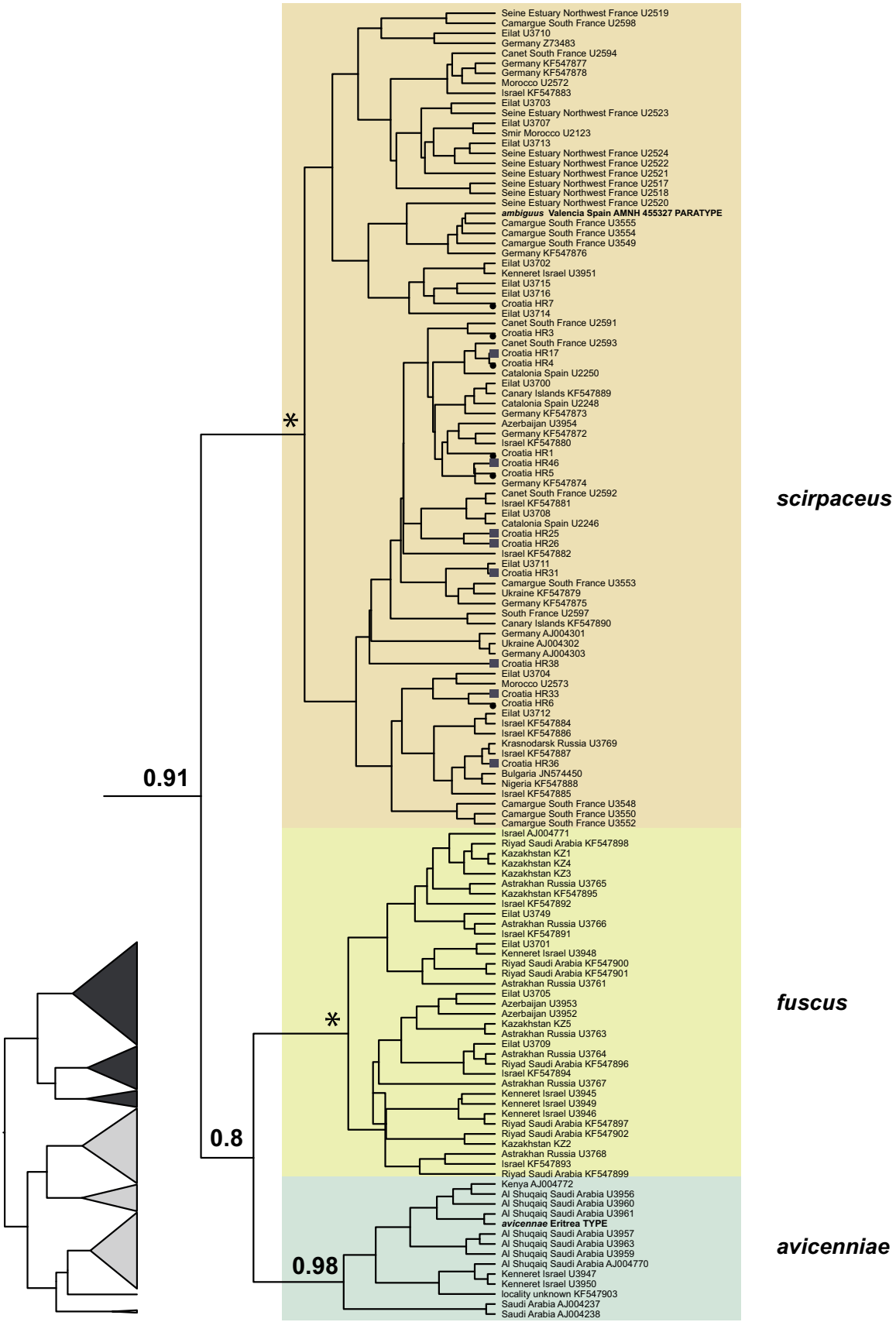

Fig. 3. Details of clades 1–3 from Fig. 2. Entries in bold represent sequences from type specimens. The paralectotype of *Calamoherpe ambigua* (Brehm, 1857) is found in clade 1, and the holotype of *avicenniae* (Ash et al., 1989), is found in clade 3. Filled circles indicate samples from an inland locality in Croatia (Draganić); shaded squares are samples from coastal localities in Croatia (Neretva and Vransko Lake).

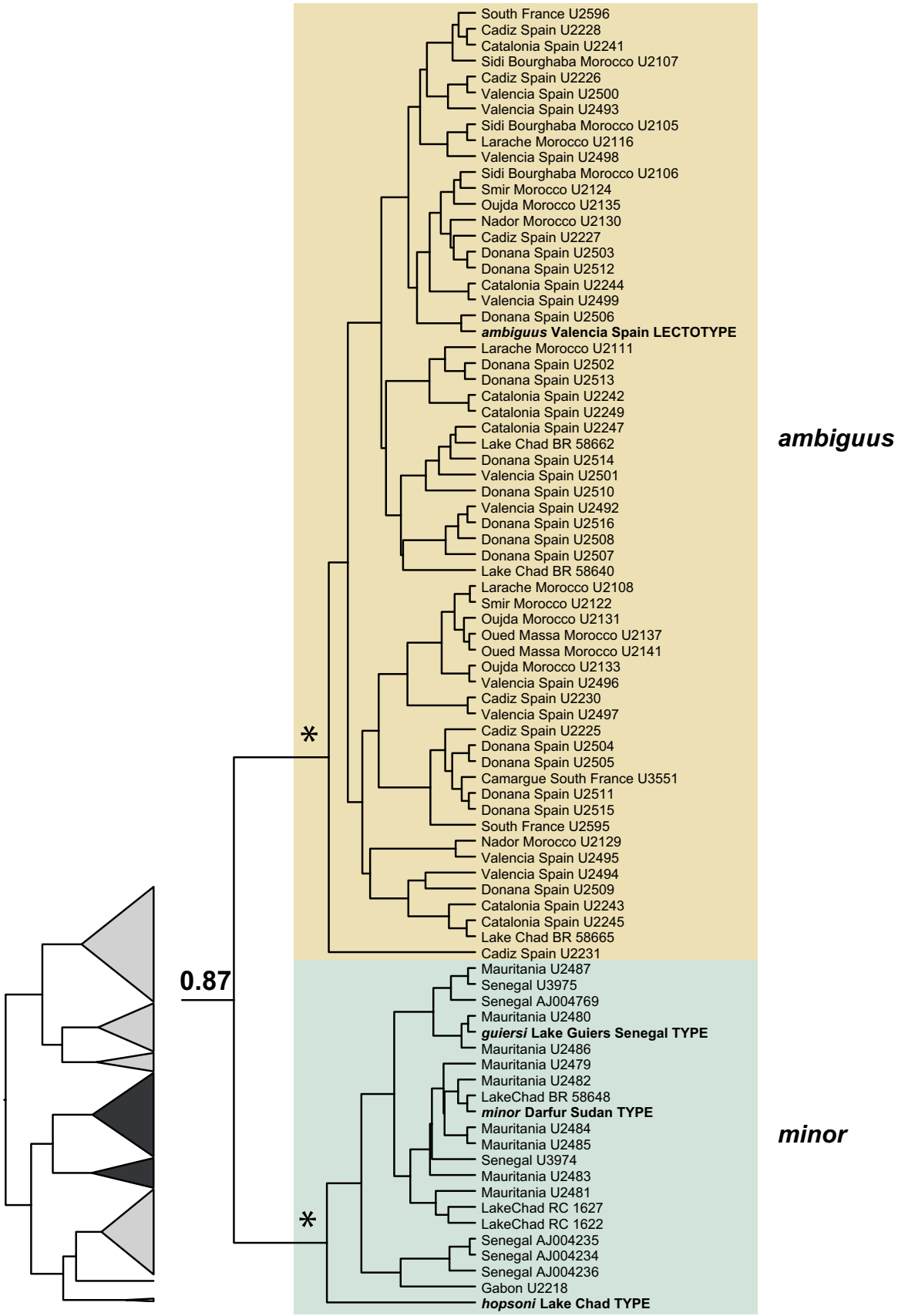

Fig. 4. Details of clades 4–5 from Fig. 2. Entries in bold represent sequences from type specimens. The lectotype of *Calamoherpe ambigua* (Brehm, 1857) is found in clade 4, and the holotypes of *guiersi* (Colston and Morel, 1984), *hopsoni* (Fry et al., 1974) and *minor* (Lynes, 1923), are found in clade 5.

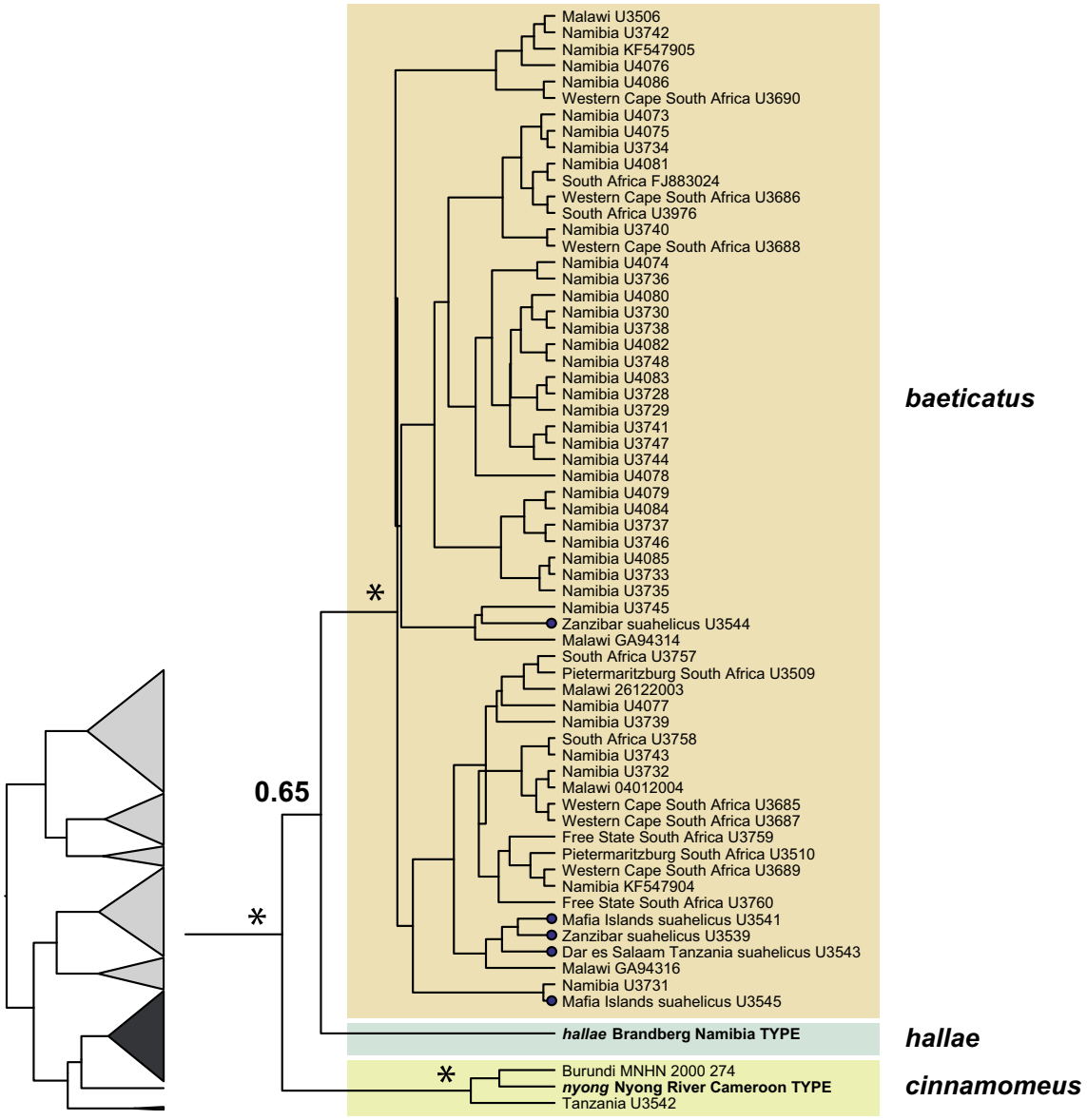

Fig. 5. Details of clades 6–8 from Fig. 2. Entries in bold represent sequences from type specimens. The holotype of hallae White, 1960 is the only representative of lineage 7, and *nyong* (Bannerman, 1936) is found in clade 5. All specimens from the range of suahelicus are found in clade 6 (filled circles).

isotope stage 7 (MIS7) interstadial (*sensu* Lisiecki and Raymo, 2005) to the stadial MIS6. Also for clade 2, all estimators indicate population expansion, with the onset of an expansion estimated to 30 kya, which coincides with the Last Glacial Maximum (LGM). For clade 4, Fu's F_S, R_2 and Tajima's D indicate departure from the null hypothesis of a population at demographic equilibrium, although the level of significance is lower for R_2 and Tajima's D. The onset of an expansion is estimated to 133 kya, which would coincide with MIS5, a warmer period during the climatically instable last interglacial. We also analyzed the Iberian and Moroccan samples separately, which indicated a stable population in Morocco, but deviation from demographic equilibrium for the Iberian population in which a population expansion was indicated to have begun 100 kya. For clade 6, all estimators indicate population expansion, with the onset of an expansion estimated to 125 kya, which would coincide with MIS5e (Eemian), also a warmer period during last interglacial.

3.2. Morphometrics: principal component analysis

The first two principal components (PC) explained 75.1% of the total variance, with 59.5% for PC1 and 15.6% for PC2. Table 2 gives the significant contributions of the variables to the first two PCs. The first axis is clearly related to the wing length and shape, higher value on this axis means a short and rounded wing (with short second primary and short intervals between wing tip and other inner primaries). The second axis is related to wing length, and notch on P2. A high value on this axis means a long wing, a short head and bill length but a long bill, and a short notch on P2. The different individuals, according to their taxon and origin, are plotted along the first two principal components of the PCA (PC1 and PC2) (Fig. 6). Museum skins are dry and might produce shorter measurements than live birds (Svensson, 1992), but live and museum specimens of a same group (here for Mauritanian samples '*guiersi*', but also for Iberian birds with the lectotypes of

Table 1
Descriptive statistics on genetic variation in the *Acrocephalus scirpaceus-baeticatus* complex, with significance determined using coalescent simulations; all calculated in DNASP 5.10. (Librado and Rozas, 2009). Ramos-Onsins and Rozas (2002) found the behavior of Fu's F_S better for sample sizes above 20–30, whereas the R_2 test was superior for smaller sample sizes. In these results, R_2 would be most reliable for *avicenniae* and *minor*, and Fu's F_S for the remainder, except for *cinnamomeus* and *hallae* for which the sample size is too small for any demographic calculations.

	N	Hd	π	θ_w	*S*	*Π*	F_S	R_2	Tajima's D	τ	t_{exp}
Clade 1 – *scirpaceus*	83	0.972	0.00502	0.01420	54	3.828	-54.405^{***}	0.0307^{***}	-2.10604^{***}	3.828	205
Clade 2 – *fuscus*	38	0.936	0.00590	0.01248	39	4.504	-12.575^{***}	0.0486^{**}	-1.86718^{**}	0.564	30
Clade 3 – *avicenniae*	16	0.858	0.00449	0.00513	13	3.425	-0.151^{ns}	0.1247^{ns}	-0.48506^{ns}	1.359	Stable
Clade 4 – *ambiguus*	59	0.901	0.00280	0.00729	30	2.477	-12.439^{***}	0.0409^{**}	-2.00356^{**}	2.477	133
ambiguus Morocco	15	0.819	0.00254	0.00278	8	2.248	-1.529^{ns}	0.1442^{ns}	-0.31841^{ns}	2.248	Stable
ambiguus Iberian	38	0.889	0.00268	0.00618	23	2.378	-7.089^{***}	0.0524^{**}	-1.91552^{**}	1.864	100
Clade 5 – *minor*	22	0.797	0.00307	0.00529	15	2.541	-2.067^{ns}	0.0877^{ns}	-1.52942^{*}	1.110	Stable
Clade 6 – *baeticatus*	61	0.928	0.00358	0.00916	38	3.174	-14.963^{***}	0.0400^{**}	-2.01089^{**}	2.331	125
Clade 7 – *hallae*	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Clade 8 – *cinnamomeus*	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

1. N – Number of sequences analyzed.
2. Hd – Haplotype diversity (Nei, 1987).
3. π – Nucleotide diversity.
4. θ_w – The population mutation parameter theta, estimated from No. of segregating sites.
5. *S* – No. of segregating sites.
6. *Π* – Average number of nucleotide differences between sequence pairs (Nei, 1987).
7. F_S – Fu's statistic (Fu, 1997).
8. R_2 – Ramos-Onsin and Roza's statistic (Ramos-Onsins and Rozas, 2002).
9. τ – Age of expansion in units of mutational time
10. t_{exp} – Time since onset of population expansion (kilo years ago), in populations inferred to deviate from demographic equilibrium.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
Coordinates of biometric variables significantly contributing to the first two axes of the Principal Component Analysis.

Variables	PC1	PC2
Wing	−0.542	0.556
Head and bill	0.117	−0.303
Bill to skull		0.424
Tarsus		−0.168
Claw		−0.115
P1	0.146	0.207
P2	0.272	
P4	−0.144	0.166
P5	−0.301	0.165
P6	−0.435	0.182
P7	−0.517	0.180
Notch P2	0.134	−0.444

Calamoherpe ambigua) are globally grouped and do not mix with other groups, so we can confidently consider that the biometrics of live birds and dry skins are comparable. In this figure, two groups segregate along the first component PC1, while the second component (PC2) shows a weak separation between *scirpaceus* and *baeticatus*. According to the contributions of the morphometric variables to the axes, an increasing coordinate along PC1 corresponds to birds with short and more rounded, less pointed wings. In these respects, Iberian/North African birds appear quite similar to *A. s. scirpaceus*. PC2 seems to better segregate birds breeding in Europe (north of Spain) from those breeding in Spain and Africa. Along this axis, the Iberian/North African birds have lower values than *scirpaceus* and are more similar to the *baeticatus* group, so they share a short wing, long notch on second outermost primary, short bill but long head with the other African Reed Warblers. As all biometric values have been standardized for body size, these comparisons are relative to the size of an individual. The three lectotypes of *Calamoherpe ambigua* are deliberately separated in Fig. 6 and appear within the Iberian/North African group owing to their morphometry (though at least one has a *scirpaceus* haplotype). The *guiersi* holotype appears clearly within the cloud of sub-Saharan taxa.

4. Discussion

4.1. Phylogeny and demography

There is well-supported geographically structured divergence for eight distinct lineages in the *Acrocephalus scirpaceus–baeticatus* complex. The earliest divergence among the reed warblers is inferred to have occurred between an Afrotropical lineage (clades 6–8) and a mainly Palaearctic one (also including populations from the Sahel region) (clades 1–5). The structure largely corroborates the distributions outlined by Kennerley and Pearson (2010), but there are some notable instances where our data are at odds with these. Our results concerning the northern taxa are also congruent with two other studies (Arbabi et al., 2014; Winkler et al., 2013), except regarding demographic inferences in the former, and in that the latter does not recover a separate *avicenniae* clade.

The dating analysis suggests that the basal split took place more or less at the start of the Ionian subdivision of the Pleistocene and that subsequent divergence in the clade primarily occurred during this stage, but the uncertainty is substantial due to large confidence intervals. All clades are clearly indicated to have diverged earlier than the most recent glacial maximum, corroborated by the maternal demographic history. For clades indicated to deviate from population equilibrium, population expansions are suggested to have taken place during at least three different periods in the middle and late Pleistocene. The earliest one concerns clade 1, and is inferred to have started approximately 205 kya. The next period of population expansions involves clades 4 and 6, and is inferred to have occurred around 125–133 kya. As the latter two clades have widely disjunct ranges, the drivers of population changes affecting these may be different, even though they occurred relatively close in time. The most recent population expansion concerns clade 2, and is inferred to have commenced approximately 30 kya. All inferred population expansions, except for clade 2, appear to roughly coincide with episodes of warmer climate. For clade 2, the estimated onset of a population expansion is inferred to coincide with the Last Glacial Maximum (LGM; MIS2). This may suggest that different factors promoting population

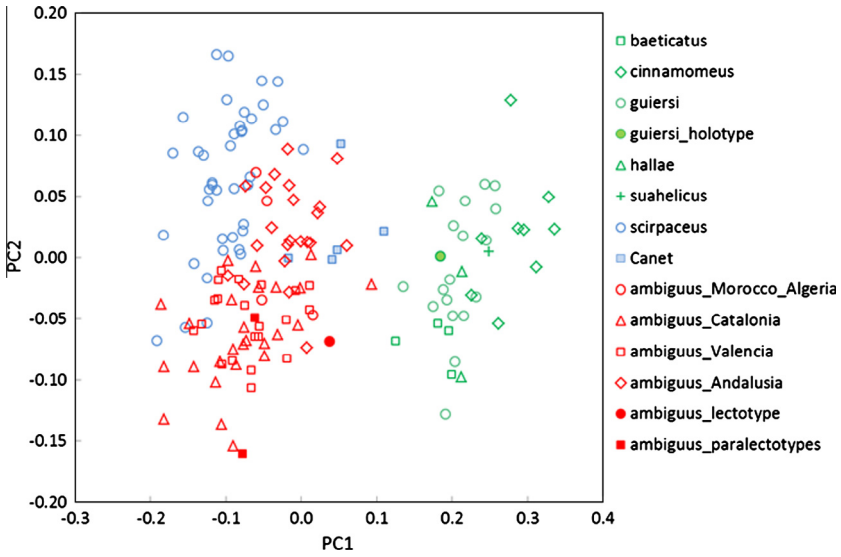

Fig. 6. Representation of the 158 specimens included in the morphometric multivariate analysis along the first two principal component axes. The different groups are represented with symbols of different colours: green for subspecies of the *baeticatus* group (symbols vary with taxon; plain dot for the *guiersi* holotype), blue for *scirpaceus* (plain blue square for samples of Etang de Canet, southern France), and red for *ambiguus* (symbols vary with location; plain dot for the lectotype and plain squares for the paralectotypes hosted at the AMNH; the paralectotype with a *scirpaceus* haplotype is the upper one).

expansions may have influenced populations in different regions and at different times, but there is a considerable amount of uncertainty in these estimates. Transitions between stadials and interstadials during the fluctuating Pleistocene climate seem to have been rather abrupt. Consequently hypotheses about climate related drivers of population expansions are highly vulnerable to slight adjustments of priors, e.g. the substitution rate, by which a slight shift of the estimated time since the start of expansion could make it coincide with an entirely different climatic scenario. Although this would not change the overall pattern of largely pre-LGM expansions in the present study, hypotheses about specific climate related drivers of population expansions remain speculative. One conclusion that may be drawn is that climate fluctuations during the Holocene may have affected distributions within the *Acrocephalus scirpaceus* complex, but both the divergence behind the phylogenetic patterns and the onset of population expansions, with the exception of clade 2, appear much older for all clades (see Table 3).

Our interpretation of the shaping of present day ranges differ from Arbabi et al. (2014) in that our data mainly indicate pre-LGM population expansions. Their cyt *b* sequences deposited in GenBank are included in our data set, but for the demographic analyses our results are very different from theirs. We used their τ (Table 1 in Arbabi et al., 2014) to re-calculate the time since the onset of a population expansion based on their data, and arrived at estimates of a similar magnitude as those derived from our own data (Supplementary Table 3), but for cyt *b* is 5 and 11 times older than the estimates of Arbabi et al. (2014) from their Bayesian Skyline Plot (BSP) for *scirpaceus* and *fuscus*, respectively. For their CO1 data, the recalculated time estimate for *scirpaceus* is the same as from their BSP. For *fuscus*, the recalculated time estimate based on τ is 8 times older than that of their BSP, although it should be noted that in their data the signal of deviation from demographic equilibrium is not significant, except weakly for Fu's F_S ($P < 0.05$) (Arbabi et al., 2014, their Table 1). This places the timing of the onset of the expansions based on the demographic data of Arbabi et al. (2014) before the LGM (*contra*, Arbabi et al., 2014), except for the time estimate for *scirpaceus* based on CO1, which has an exceptionally low value of τ. The reason for this is not clear, but may be an artifact of a sampling bias, as over 91% of their CO1 sequences come from samples collected in a rather restricted area in Germany, and may only represent the demography of the local population, whereas the cyt *b* data from both this study and Arbabi et al. (2014) come from locations representing a larger part of the entire range.

Table 3
Summary of some biometric measurements, as average ± s.d. (min.–max.), for all specimens studied in the morphometric analyses.

Taxon (sample size)	*Scirpaceus* (*n* = 41)	*Ambiguus* (*n* = 76)	*Guiersi* (*n* = 18)	*Cinnamomeus* (*n* = 9)	*Baeticatus* (*n* = 4)	*Hallae* (*n* = 3)	*Suahelicus* (*n* = 1)
Wing length	66.1 ± 1.9 (63–71)	63.7 ± 1.9 (59–67.5)	56.7 ± 1.8 (54–60.5)	54.7 ± 2.1 (51–57.5)	62.4 ± 0.8 (62–63.5)	59.0 ± 1.3 (57.5–60)	60
Tarsus length	22.7 ± 0.9 (21.0–24.1)	22.3 ± 0.8 (19.1–23.8)	21.5 ± 0.9 (20.1–23.0)	20.9 ± 0.7 (19.9–21.9)	22.7 ± 0.9 (21.7–23.9)	21.5 ± 0.7 (20.8–22.1)	22.7
Head & bill length	32.6 ± 1.3 (29.0–34.6)	32.4 ± 1.1 (29.2–35.9)	31.7 ± 0.9 (29.5–33.5)	31.8 ± 1.3 (29.3–33.9)	32.7 ± 0.6 (32–33.2)	30.8 ± 1.5 (29.1–31.8)	34.3
Bill to skull	17.9 ± 1.0 (15.7–20.1)	15.8 ± 1.3 (15.0–18.0)	16.0 ± 0.8 (14.7–17.1)	16.2 ± 1.1 (14.8–18.3)	16.5 ± 0.5 (16.0–17.2)	16.8 ± 1.3 (15.4–17.9)	16.9
Distance P2 to wing tip	2.2 ± 0.7 (0–3)	2.4 ± 0.8 (1.5–4.5)	4.0 ± 0.8 (2.5–5)	5.4 ± 0.9 (4–6.5)	5.1 ± 0.6 (4.5–6)	5.8 ± 0.3 (5.5–6)	6
Distance P7 to wing tip	9.5 ± 0.7 (8–11)	7.9 ± 1.1 (6–11)	3.9 ± 0.7 (2.5–5)	3.2 ± 0.7 (2.5–4.5)	4.4 ± 0.9 (3.5–5.5)	3.8 ± 1.0 (3–5)	4
Notch on P2	11.7 ± 1.1 (10–15)	12.5 ± 0.9 (11–14.5)	13.1 ± 2.0 (11–19.5)	12.4 ± 0.8 (11.5–13.5)	14.4 ± 0.3 (14–14.5)	13.2 ± 0.8 (12.5–14)	13

4.2. Northern clade (clades 1–3)

There is some support that clades 1–3 constitute a monophyletic group, possibly with clades 2–3 forming a sister clade to clade 1. The sequence of lineage divergence, if representing the true pattern, suggests that the ancestral population may have first become divided in a western and an eastern population during the lower Pleistocene, the latter then becoming further divided into a northern and a southern population. The western population (*scirpaceus*, clade 1) is, according to our data, mainly distributed from south-western France to the shores of the Black Sea. Possible sympatry with other clades, based on more than one clade being represented from the same locality, was found in Catalonia and southern France, in Azerbaijan and in Israel. In Catalonia the majority (70%, $n = 10$) of haplotypes belonged in clade 4, with the rest belonging in clade 1. In southern France the frequency of haplotypes were more or less reversed; 81% belonging in clade 1 and 19% in clade 4 ($n = 16$). In Azerbaijan, a single individual with the haplotype of clade 1 was collected from the same locality as two samples belonging in clade 2. All three Azerbaijan individuals were presumed by the collector to have been breeding locally. A sample of seven birds from Lake Kenneret, Israel, collected some time later than 21 May in 1995, contains representatives of clades 1, 2, and 3, but their status as breeders or migrants is unclear, and needs further study. There are four main alternative scenarios that could explain these cases: (1) one of the haplotypes may represent non-breeding stragglers; (2) the area may be a zone of secondary intergradation, as indicated by Procházka et al. (2011); (3) two forms may occur in sympatry without intergradation; or (4) the pattern may be the result of incomplete sorting of ancestral polymorphisms. Our data do not allow us to choose among these alternatives, and this should be studied further.

The morphological differences between coastal and inland populations in Croatia, reported by Kralj et al. (2010) are not accompanied by any discernible pattern among the haplotypes from these localities. The restricted gene flow and different wing shapes between these populations are thus more likely to have originated *in situ* due to differences in selection regimes of very recent origin, rather than secondary contact of populations that diverged in glacial refugee. The fact that there are morphological differences between geographically close populations indicates that there may be a high degree of site fidelity, which may in part explain the relatively high haplotype diversity in *scirpaceus*.

4.3. Iberian-northwest African clade (clade 4)

The Iberian and northwest African populations of reed warblers are shown to be genetically and morphologically distinct. Plumage differences from other populations are slight, explaining why the distinctness of this population has long gone unnoticed. The population reported from Libya as *A. baeticatus* by Hering et al. (2009) and Hering et al. (2010) most likely belongs to this clade, as one of their samples from Libya is placed in the same clade as their samples from the western and southern Iberian Peninsula in Winkler et al. (2013). The sister relationship between the Ibero-African clades (4–5) and the remainder of the African populations, to the exclusion of the northern (clades 1–3), is weakly supported and uncertain.

4.4. Sahel clade (clade 5)

The populations inhabiting the southern edge of the Sahara desert, from the lower reaches of the Senegal River to the mangroves of the Red Sea, have been subject to a number of taxonomic reappraisals (Lynes, 1923; Fry et al., 1974; Colston and Morel, 1984; Ash et al., 1989), leading to the description of four taxa from the area. Of these, *avicenniae* Ash et al. (1989) has since been found to be more closely related to *A. s. scirpaceus* than to *A. baeticatus*. The Lake Chad population was described as *hopsoni* Fry et al., 1974, and was subsequently considered a junior synonym of *cinnamomeus* Reichenow, 1908. The isolated *guiersi* Colston and Morel, 1984 has remained a subspecies of *A. baeticatus*, following the original designation. The Darfur taxon *minor* Lynes, 1923 disappeared into obscurity and was not considered by either of Ash et al. (1989), Colston and Morel (1984) or Fry et al. (1974).

Our results for this clade disagree with current taxonomy (Clements et al., 2012; Pearson, 2006; Kennerley and Pearson, 2010; Gill and Donsker, 2015), regarding all sampling sites. All samples from Lake Chad and Darfur come from within the alleged range of *cinnamomeus*, sensu del Hoyo et al. (2006) and Kennerley and Pearson (2010), and would have been expected to represent clade 8. Instead, most of the samples from this area, including all three holotypes of *guiersi* (Colston and Morel, 1984), *hopsoni* (Fry et al., 1974) and *minor* (Lynes, 1923), respectively, are part of clade 5. At Lake Chad haplotypes representing both clades 4 and 5 were collected in the first week of May 2000. Whether birds representing clades 4 and 5 breed there, or whether our samples represented migrating or straggling individuals remains to be clarified. No samples representing clade 8 have been obtained at Lake Chad or other places within the range of clade 5.

4.5. Tropical and southern African clade (clades 6–8)

All our samples from the respective ranges of *suahelicus*, *baeticatus* and *hallae* (sensu Pearson, 2006; Kennerley and Pearson, 2010) are part of clade 6, without any discernible geographic pattern. The only exception to this is the holotype of *hallae* (White, 1960), the haplotype of which forms a unique lineage. This pattern is unexpected, as we have several samples of breeding birds from the vicinity of the type locality of *hallae*, all of which belong in clade 6. One possible explanation is that intra-African migratory patterns obscure the geographic structure of these populations. Future investigations need to take the possibility of annual population turnover at specific localities into consideration, and the genetic composition at different times of year needs to be investigated in order to separate breeding ranges of the African taxa from non-breeding season distributions. Anyway, in our analyses we have taken great care to make sure that the sequence is not chimaeric or a numt (see Sorenson and Quinn, 1998).

4.6. Taxonomic considerations

The seven well-supported clades plus the single sample of clade 7 correspond to named taxa in current use (Fig. 2), with two exceptions (clades 4 and 5).

4.6.1. Clade 1 – scirpaceus

Most samples from Europe except from the Iberian Peninsula are part of this clade, here represented by samples from Bulgaria, Croatia, France, Germany, Russia and Ukraine. The eastern-most sampling localities in the presumed breeding range are in Krasnodarsk, Russia. One record from Azerbaijan in August was reported as a local breeder, but may represent an early migrant. In our material, there are several records of presumably migrating birds from Spain, Morocco and Israel, and one winter record from Nigeria. The museum specimen from Morocco has a long wing (66 mm) and appears clearly within *scirpaceus* in the biometric multivariate analysis. Judging from the dates, we consider the most likely explanation to be that all three Moroccan individuals (April, May and October) were on migration, but the possibility that they could be evidence of secondary intergradation, or that the two forms may occur in sympatry without intergradation can't be ruled out.

The taxon *scirpaceus* was found breeding in Libya (Hering et al., 2010), and could thus conceivably breed also in Morocco. One sample from Lake Kenneret, Israel, collected some time later than 21 May in 1995, may have been a local breeder, but this needs confirmation.

4.6.2. Clade 2 – *fuscus*

This well-supported clade contains all samples from Kazakhstan and several from Saudi Arabia and Israel, where four samples from Lake Kenneret, collected some time later than 21 May in 1995, may have been local breeders. The western-most sampling locality in the presumed breeding range are Astrachan and Azerbaijan, suggesting that the ranges of *fuscus* and *scirpaceus* abut somewhere between the Black and the Caspian Seas, consistent with Kennerley and Pearson (2010). The possible occurrence of both *fuscus* and *scirpaceus* in Azerbaijan during the breeding season needs further study.

4.6.3. Clade 3 – *avicenniae*

This clade contains the holotype of *avicenniae* Ash et al. (1989) from Eritrea and samples from breeding populations of *avicenniae* in Saudi Arabia, but also a sample from Kenya originally attributed to *fuscus*. As the latter was obtained away from the breeding grounds, misidentification is a possibility. Most probably this sample represents a migrant from the Red Sea mangrove population. The range of *avicenniae* is generally believed to be confined to mangroves bordering the Red Sea, recently extended to reach Egypt (Hering et al., 2011a), but our records from Lake Kenneret in inland Israel, and the observations by Morgan (1998) and Hering et al. (2009, 2011b), imply that this taxon may have a larger distribution and wider choice of habitats than previously known. Both *fuscus* and *scirpaceus* were also sampled at Lake Kenneret during the same period, warranting further study.

4.6.4. Clade 4 – *ambiguus*

Our cyt *b* gene tree, combined with differences in morphometrics, suggests that the *Acrocephalus* warblers breeding in south-western Europe and north-western Africa represent a distinct population, consisting of samples from Morocco, Spain, southern France and Lake Chad, Nigeria. This clade does not correspond to any taxon name in current use, but includes the lectotype of *Calamoherpe ambigua* (Brehm, 1857) (from Valencia, Spain). Both the morphometrics and haplotype of the lectotype (AMNH 455328, adult male, collected at 39.00 N, 00.32 W, Valencia, Spain, on 19 June 1856) are consistent with its position in clade 4. The morphometrics of the two paralectotypes fit those of other Spanish breeding birds sampled in this study, although the paralectotype AMNH 455327 has a *scirpaceus* haplotype. Our sequence of the second paralectotype AMNH 455326 is too short to be conclusive. As AMNH 455327 (clade 1 haplotype) was collected on 19 August (LeCroy, 2008), the possibility that it was an early migrant *scirpaceus* can't be rejected. Apart from this individual, all breeding birds sampled in Spain south of Catalonia are part of clade 4. We propose that the name *ambigua* is resurrected for this clade in the form *ambiguus*, which is in grammatical agreement with the gender of *Acrocephalus*.

The taxon *ambiguus* is a small reed warbler, similar to *scirpaceus* in plumage, but intermediate between *scirpaceus* and *baeticatus* in morphology. For a detailed description, see Jiguet et al. (2010). Present evidence suggests that the moult strategy differs from that of *scirpaceus* (Svensson, 1992). At least Moroccan birds undergo complete moult (wing and tail) in autumn (Amezian et al., 2010; Jiguet et al., 2010; Taillandier et al., 2006). In the Ebro delta, some adults have been observed to moult flight-feathers in August/September (David Bigas, in Amezian et al., 2010).

The taxon *ambiguus* breeds discontinuously in wetlands of Morocco and Spain (Andalusia to at least Valencia), and probably most of the Iberian Peninsula, with Catalonia and southern France possibly being a zone of secondary intergradation (cf. Procházka et al., 2011). In Morocco the taxon has been recorded to winter locally (Amezian et al., 2010). This taxon may also breed in Algeria (one specimen included in our morphometric analysis) and Tunisia. In the phylogenetic analysis by Winkler et al. (2013, their Fig. 4), the '*baeticatus*' clade contains samples from Spain, Portugal and Libya, indicating that *ambiguus* is one of the forms occurring in Libya. Sympatry with *A. s. scirpaceus* in the Benghazi area, Libya, was supported by both adults and eggs of both *scirpaceus* and "*baeticatus*" haplotype groups being recorded (Hering et al., 2010), but no data on degree of assortative mating was reported.

4.6.5. Clade 5 – *minor*

This clade consists of samples from Mauritania and Lake Chad, and includes the holotypes of three taxa from the southern edge of Sahara. The western-most population is currently recognized as *guiersi*, whereas *hopsoni* from Lake Chad and *minor* from Darfur are considered junior synonyms of *cinnamomeus*. Our samples from Malamfatori, Lake Chad, Nigeria, are part of Clades 4 and 5. This is the type locality of *hopsoni* (Fry et al., 1974), and the holotype of this taxon is part of this clade. As both the holotypes of *guiersi* (Colston and Morel, 1984) and *minor* (Lynes, 1923) are also part of clade 5, the taxonomy of the Sahel populations seems to be in need of revision. There is no apparent geographical structure within this clade, so based on our data the three taxa are synonymous, with *minor* (from Zalingei, Darfur, Sudan) having priority over both *hopsoni* and *guiersi*, and we propose this name to be used for the clade. However, an aberrant individual, described as *Acrocephalus albotorquatus* from Lado (vicinity of Juba, South Sudan) by Hartlaub (1880), was, with the exception of its albino nuchal collar, considered identical to the Zalingei population by Lynes (1923). Should this specimen prove to belong to this clade, the name *A. albotorquatus* (Hartlaub, 1880) has priority for the clade. A single sample from Gabon, collected in April, also belongs to Clade 5. This individual may have been a migrant or straggler from the Sahel area, but the possibility that clade 5 occupies larger parts of the range currently attributed to *cinnamomeus* needs to be further investigated.

4.6.6. Clade 6 – *baeticatus*

This clade contains a wide selection of samples from Malawi, Namibia, South Africa and Tanzania. All our samples from both Namibia and western South Africa belong in this clade, clearly separate from the holotype of *hallae*. In addition to this, all samples obtained from Zanzibar, the Mafia Islands and the coast of Tanzania, representing *suahelicus*, are nested in this clade. The latter samples were selected based on morphology and sampling locality, with collection dates including spring, summer and late autumn, and we are confident that they represent the local population. According to our results *suahelicus* should be considered a junior synonym of *baeticatus*, as proposed by White (1960). Five of the samples were collected in Malawi, i.e. within the presumed range of *cinnamomeus* (cf. Kennerley and Pearson, 2010).

The reed warbler populations of southern Africa are known to be migratory, but the migration patterns are insufficiently known. Wintering quarters of southern African birds have been suggested as Malawi or elsewhere in central Africa (Maclean, 1993), but Hanmer (1988), who found that African Reed Warblers were present in southern Malawi mainly between April and November, considered most to be *cinnamomeus*, with a few identified as *suahelicus*.

Our samples from Namiba include 21 breeding birds from January and 13 birds in post-breeding condition sampled in April, all

belonging in this clade and thus representing *baeticatus*. It is possible that some of our samples from both Malawi and the Western Cape, South Africa, represent migrants from a more restricted breeding range of *baeticatus*, and that we missed local breeding populations of *cinnamomeus* in Malawi. Our Malawi samples were collected in December and January, during the austral summer when South African breeders are presumed to be back at the breeding grounds, suggesting that the pattern of occurrence of African Reed Warblers in Malawi may be more complex than previously believed. Alternatively, the geographic circumscription of both *baeticatus* and *cinnamomeus* may be in need of revision.

4.6.7. Clade 7 – hallae

This clade contains a single sample from the holotype of *hallae*, collected in April at Brandberg, Namibia. This sequence is clearly divergent from samples collected from the breeding population in Swakopmund, near the type locality of *hallae*. We have no other samples from this area, and it is possible that there is a population turnover due to intra-African migration in this region. The type of *hallae* may thus represent a migratory population from an as yet undiscovered area, spending the austral winter in Namibia.

4.6.8. Clade 8 – cinnamomeus

This clade contains one sequence from Burundi and one from the Mt Meru area, Tanzania (the latter labeled *suahelicus* BMNH 1947.71.342), reasonably close to the type locality of *cinnamomeus*, Lake Edward in Uganda, to presume that they represent this taxon (Clements et al., 2012; Pearson, 2006; Kennerley and Pearson, 2010; Watson et al., 1986). The clade also includes the type of *nyong* (Bannerman, 1936), from Nyong River, Cameroon, which may thus be regarded as a junior synonym of *cinnamomeus* based on molecular evidence. Apart from the coastal regions, most of Africa between roughly 15° north and 15° south of the Equator is generally considered to be occupied by the taxon *cinnamomeus* (cf. Pearson, 2006; Kennerley and Pearson, 2010; Gill and Donsker, 2015). However, samples from the Sahel region and Malawi do not belong in this clade, challenging the circumscription and distribution of *cinnamomeus*, while samples from the type locality are needed to confirm that this clade is correctly named as *cinnamomeus*.

4.7. Taxonomic implications

This complex of populations is a classic showcase of the evolutionary stage where divergence appears to be in the neighborhood of the diffuse demarcation line between the categories of subspecies and species. The prevailing norm in decisions on species status under the "Biological Species Concept" (BSC; Mayr, 1942, 1963) is to demand persuasive evidence for reproductive isolation, whereas combining lineages into a polytypic species is the standard procedure when in doubt. Gill (2014) suggested shifting this burden of proof, such that manifest evolutionary lineages by default are treated as species until evidence for an absence of reproductive isolation is put forward. This seems to be in agreement with the suggestions by de Queiroz (2007), who considered "a separately evolving metapopulation lineage" to be the primary defining property of the species category, and argued that all properties that provide evidence of lineage separation should be relevant criteria in species delimitation. Regarding the major clades demonstrated in this study, the mere existence of geographic structure among mitochondrial haplotypes speaks against panmixis and in favor of essential reproductive isolation. However, we have very few samples from geographical areas where different taxa are supposed to meet, and very little evidence is available pertaining to the degree of actual assortative mating in these contact zones. The basal divergence, estimated to have occurred between 0.57 and 1.02 mya, between clades 1–3 and 4–8, is relatively deep and suggest long standing isolation, but is still considerably younger than the approximately 2 million years that has been suggested to be an approximate time required for reproductive isolation to be completed in birds (Price and Bouvier, 2002; Weir and Price, 2011). This places the *A. scirpaceus–A. baeticatus* complex in the company of some other taxonomically controversial warbler taxa, such as the *Phylloscopus collybita* (Helbig et al., 1996) and *Phylloscopus trochiloides* (Alcaide et al., 2014) complexes.

In the case of the *A. scirpaceus* s.l. complex, morphological differentiation, divergence and geographical structure in mitochondrial loci, and apparently disjunct ranges, support that the different major clades have been evolving as separate lineages for a considerable time and could thus be considered different species. Generally, the morphological differences that historically led researchers to appoint names to these populations are in agreement with the phylogenetic patterns, but there are also a number of cases of conflict between clades and arrangements based on phenotype. For example, the taxon *guiersi* was explicitly compared to other Sahel populations, and determined to differ sufficiently in morphology from these to deserve taxonomic recognition (Colston and Morel, 1984). Yet it is here demonstrated to belong to the same haplotype group as birds from Lake Chad and further east, raising the question of which should be regarded as more important if they are in conflict, genetics or morphology? To provide an answer, one would need more information about the reason for the conflict, which could be e.g. secondary introgression masking an ancient divergence or rapid phenotypical adaption to local conditions incorrectly giving the appearance of long standing isolation.

Regardless of their status as subspecies or species, we propose that the names *scirpaceus*, *fuscus*, *avicenniae*, *ambiguus*, *minor*, *cinnamomeus*, *hallae* and *baeticatus* are used for these lineages, as outlined above, and recommend further studies in the contact zones to aid decisions on species limits. Localities known to harbor representatives of more than one haplotype group, like Catalonia/southern France, Libya, Israel and Azerbaijan in the northern hemisphere, and Malawi and Namibia in the south, would be prime locations to study interactions and gene flow between different taxa. In the southern half of Africa, studies are recommended to pay particular attention to possible seasonal population turnover and the identity of the local breeding population, preferably based on molecular evidence.

We see four main taxonomic alternatives based on the present knowledge. The most conservative approach, consistent with Gill's (2014) H01 based on the BSC, would be to consider all taxa as subspecies of *A. scirpaceus*. Arguments in favor would be relatively short time since the most recent common ancestor and lack of proof of reproductive isolation between taxa. Three alternative classifications would probably be consistent with Gill's (2014) H02 and de Queiroz's (2007) unified concept of species. One of these would be to treat *scirpaceus*, *fuscus* and *avicenniae* as subspecies of *A. scirpaceus*, and *cinnamomeus*, *hallae* and *baeticatus* together with *ambiguus* and *minor* as subspecies of *A. baeticatus*, based on the basal split in the phylogeny. Another one would be to treat also *ambiguus* and *minor* as a species *sui generis*, *A. ambiguus*, in this case recognizing the presence of the three major clades as the species level justification. Under this alternative, the criteria for subspecies proposed by Patten (2015) are met by *guiersi*, which is phenotypically different from the other populations here suggested to be included in *minor*, in spite of no differentiation in the cyt *b* locus, adding an interesting twist to this discussion. The, under current norms, most radical alternative would be to treat all eight lineages as full species, pending the rejection of the null hypothesis of lack of free interbreeding.

Based on the fact that our data represent a single line of evidence of lineage divergence, and awaiting corroboration by

independent data, we tentatively recommend the most conservative approach, considering all taxa as subspecies of *A. scirpaceus*. We acknowledge that considering that a single migratory warbler species has a latitudinal distribution covering more than 100° is not satisfactory. Due to its ambiguous stage in the evolutionary process, further studies of this complex would make an ideal case study in the debate both about the subspecies category (e.g. Gill, 2014; Sackett et al., 2014; Patten, 2015) and species delimitation (e.g. de Queiroz, 2007; Tobias et al., 2010).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.05.026.

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