Evolutionary relationships of stonechats and related species inferred from mitochondrial-DNA sequences and genomic fingerprinting

Michael Wink, Hedi Sauer-Gürth and Eberhard Gwinner

ABSTRACT Sequence data of the mitochondrial *cytochrome b* gene and genomic fingerprinting provide good evidence that the geographically separated taxa of the Common Stonechat *Saxicola torquata* complex represent distinct genetic lineages, which became separated more than one million years ago. The distinct genetic pattern implies that hybridisation and gene flow between these lineages no longer takes place to a significant degree. Since these lineages also differ in morphology, breeding behaviour, vocalisations and physiological control of their annual cycles, we suggest treating European Stonechat *S. torquata*, African Stonechat *S. [t.] axillaris*, Reunion Stonechat *S. [t.] tectes*, Canary Islands Stonechat *S. dacotiae* and Siberian Stonechat *S. [t.] maura* as 'good' and distinct species.

Footnote: In this paper, the vernacular and scientific names used for the key taxa of interest are those preferred by the author, although this does not necessarily reflect editorial policy.

Introduction

Systematic classifications are usually based on similarity, with taxa that show the greatest similarity being considered to be closely related. Since taxa which are only distantly related may, however, come to show morphological similarities owing to adaptation to similar ecological constraints – 'convergent evolution', one example being penguins (Spheniscidae) and auks (Alcidae) – evolutionary trees based on the study of adaptive characters can lead to erroneous taxonomic conclusions.

The analysis of nuclear or mitochondrial marker genes has, during the last 15 years, become widely used to reconstruct phylogenetic relationships between taxa in all fields of zoology, including ornithology (see Avise 1994; Mindell 1999). Molecular data have the great advantage that convergent evolution does not impair the analysis to the same degree as it does for morphological data. If two or more taxa belong to the same species, their marker genes are identical, or almost identical, and intraspecific genetic distances are generally significantly smaller than those between established species. Molecular data may also provide an estimate for the timescale over which a particular evolutionary step has taken place (the 'molecular clock') and, therefore, allow both a phylogenetic and a geographical analysis of the unknown past of a group of organisms. Molecular data have consequently become an important tool for taxonomic and evolutionary studies.

Few molecular studies have addressed phylogenetic relationships in stonechats and other members of the family Turdidae. Based on the analysis of 300 base pairs of the mitochondrial *cytochrome b* gene, preliminary data have suggested that European Stonechat *Saxicola torquata*, Siberian Stonechat *S. [t.] maura* and

Robin Chittenden

220. Male European Stonechat *Saxicola torquata*, Suffolk, May 2001.

African Stonechat *S. [t.] axillaris* have already diverged to a high degree (Wittmann 1994; Wittmann *et al.* 1995).

For the analysis presented here, we sequenced most of the *cytochrome b* gene of 23 turdid taxa in seven genera, and also enlarged the dataset by including more stonechat taxa than in our previous study (Wittmann *et al.* 1995).

The fertilised egg contains only its mother's mitochondria but receives nuclear DNA from both parents. Since mitochondrial DNA (mtDNA) is inherited only through the female line, it does not fully reflect the evolution of all members of the species, and can give a distorted view of phylogenetic relationships. We have, therefore, additionally analysed the structure of the nuclear genome using the polymerase chain reaction (PCR) to produce 'Inter-Simple-Sequence Repeat' ('ISSR')-profiles, as described in an appendix at the end of this paper. ISSR-PCR is a very effective method by which to understand interspecific variation and genetic structure of populations (Damodar Reddy *et al.* 1999; Ge & Sun 1999), to sex individuals (Wink *et al.* 1998), to generate species-specific genomic fingerprints (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994) and to detect hybridisation between taxa (Wink *et al.* 2000).

Materials and methods

Blood and tissues were either preserved in an EDTA buffer (0.1 M Tris [pH 7.4], 10% EDTA, 1% NaF, 0.1% thymol) or in ethanol (Wink *et al.* 1998) and stored at -20°C until processing. Total DNA was extracted from the blood samples by an overnight incubation at 37°C in lysis buffer (10 mM Tris [pH 7.5], 25 mM EDTA, 75 mM NaCl, 1% SDS) including 1 mg of Proteinase K (Merck, Darmstadt), followed by a standard phenol/chloroform protein extraction. DNA was precipitated from the supernatant with 0.8 volume of cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer.

Full details of the sequencing technology, the ISSR-PCRs, and parameters and methodologies used to draw up the phylogenetic trees can be obtained from the authors.

Results and Discussion

The analysis was restricted mainly to species of the Western Palaearctic, and comprised the following genera: stonechats *Saxicola*, rock thrushes *Monticola*, redstarts *Phoenicurus*, wheatears *Oenanthe*, robins *Erithacus*, nightingales *Luscinia*, and thrushes *Turdus*. The dippers *Cinclus* were included as a possibly closely related comparison (ingroup), and the wrens *Troglodytes* as a distantly related outgroup. Although we sequenced several individuals of each taxon (between six and 20 in the case of stonechat subspecies), within any given subspecies all individuals clustered as a single genealogically related group derived from a single common ancestor (a monophyletic clade). One to three representatives of each taxon were chosen for an analysis which aimed to outline the patterns of phylogeny and speciation within stonechats and related Turdidae.

In fig. 1, the result of maximum parsimony and maximum likelihood analyses of mtDNA

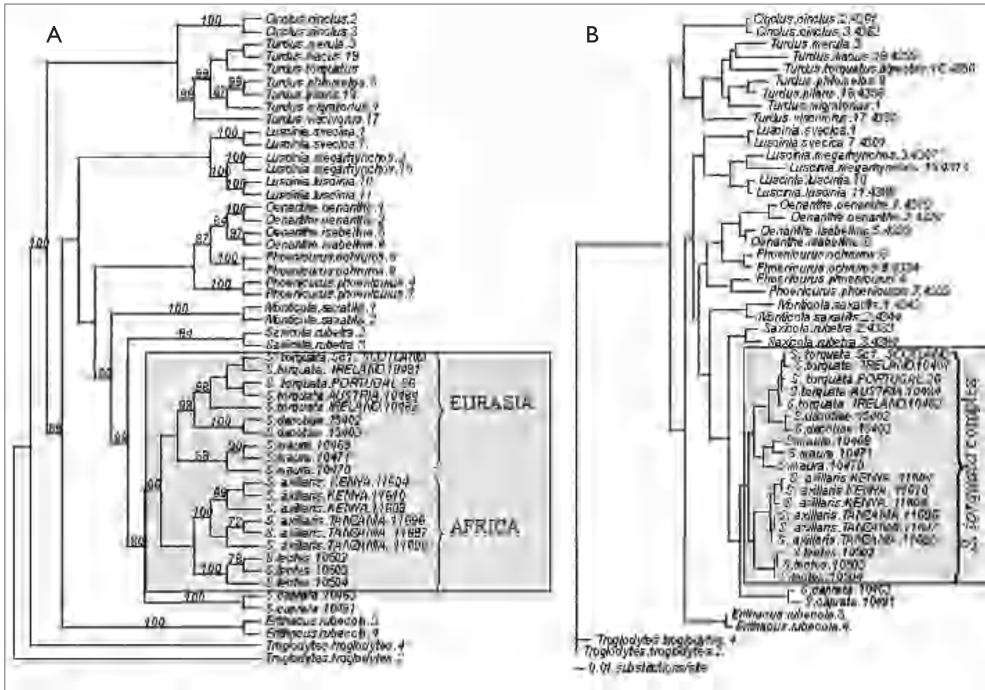

Fig. 1. A molecular phylogeny of stonechats and related turdids based on sequence data of the mitochondrial *cytochrome b* gene (see text for more details).
A. Maximum parsimony analysis; (bootstrap values above 60% are given at the corresponding bifurcations).
B. Maximum likelihood analysis; branch lengths correspond to genetic distances.
The maximum parsimony method takes the DNA sequences of the extant species and creates a phylogenetic tree linking them all, one which requires the least number of mutational changes during the evolution of all the species. It assumes that no more evolution will have taken place than was absolutely necessary. In contrast, the maximum likelihood method does not make this assumption. It takes specific mathematical models based on current knowledge of how DNA (or proteins) mutates during evolution, accepting that mutation is a random process. Using this knowledge, it is possible to work out the statistical likelihood of any phylogenetic tree based on the observed DNA sequences being the right ones. Of all possible trees, the one calculated as being 'most likely' is accepted.

are given. Both methods arrange the taxa into identical monophyletic groups and are also congruent in branching order. Members of the genus *Saxicola* form a clade (supported by a bootstrap statistical value of 88%), which derives from a common ancestor that is shared with Rock Thrush *Monticola saxatilis*. The *Monticola/Saxicola* clade shares ancestry with the genera *Luscinia, Oenanthe, Phoenicurus* and *Erithacus*. These genera are grouped in the apparently monophyletic tribe Saxicolini (supported by a bootstrap value of 89%).

Members of the genus *Turdus*, which form a monophyletic clade (88% bootstrap support), always cluster as a sister group to the tribe Saxicolini. The Dipper *Cinclus cinclus* shows no close affinity to members of the genus *Turdus*, (pairwise genetic distances 12-15%), or to wrens with which they have also been associated. We need a more complete dataset before considering the real affinities of dippers and wrens.

Relationships within the stonechat complex

Mitochondrial-DNA sequences show that, within the genus *Saxicola*, the Whinchat *S. rubetra* takes a basal position, followed by Pied Stonechat *S. caprata*. Both these species are, therefore, assumed to have split from the ancestors of European and Siberian Stonechats early in the evolution of the genus. The *S. torquata* complex, which includes *S. torquata, S. [t.] tectes, S. [t.] maura, S. [t.] axillaris* and *S. dacotiae*, clusters as a monophyletic group (with bootstrap support 86%). Within the complex the following relationships are apparent: the Canary Islands Stonechat *S. dacotiae* and the European Stonechat *S. torquata rubicola/hiber-*

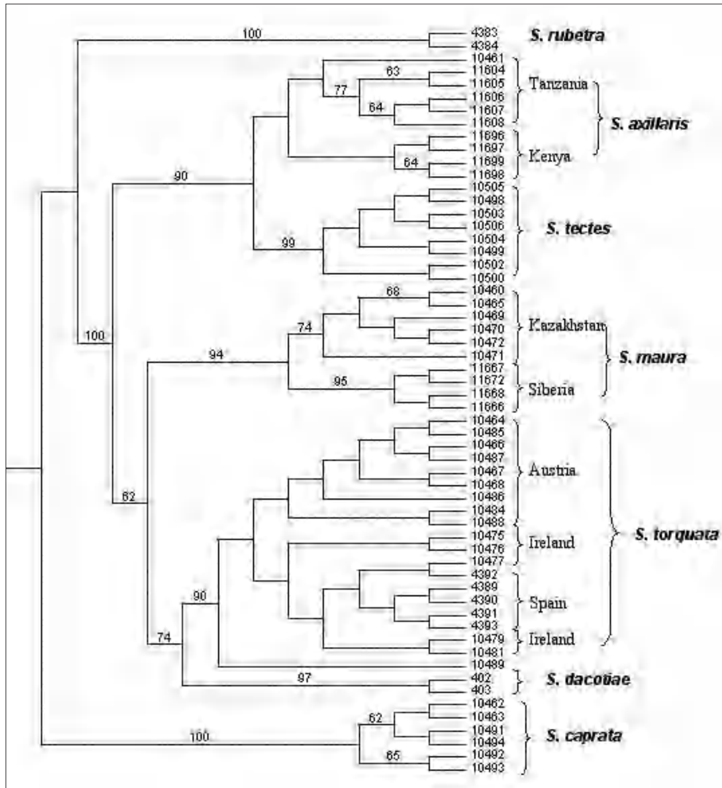

Fig. 2. UPGMA cluster analysis of ISSR-profiles. The pattern of polymorphic-ISSR fragments was recorded in a 0/1 matrix and the results were analysed by UPGMA bootstrap analyses (1,000 replications; bootstrap values above 60% are given at the corresponding bifurcations). Numbers following taxon names refer to internal accession numbers.

nans appear as sister taxa (supported by a bootstrap value of 98%). The Reunion Stonechat *S. [t.] tectes* and the African Stonechat *S. [t.] axillaris* form another sister pair. The Siberian Stonechat *S. [t.] maura* shows affinities to the *S. dacotiae/S. torquata* clade. Within the African Stonechat, two populations were studied, from Kenya and Tanzania, which show different and consistent mtDNA sequences (haplotypes), the genetic distances being 0.4-0.6%. Within *S. torquata*, a clear attribution of mtDNA haplotypes to subspecies (*S. t. hibernans* from Ireland, *S. t. rubicola* from central and western Europe) and/or geographic ranges was not apparent. It is not, therefore, possible to define subspecies of the European Stonechat on the basis of their mtDNA alone, although intraspecific variation is obvious (intraspecific distances 0.1-1.3%).

The Canary Islands Stonechat *S. dacotiae* (which occurs on Fuerteventura) is closely related to the European Stonechat *S. torquata*. The genetic distance of 2.7-3.5% implies that these taxa diverged about 1.3-1.7 million years ago, if the '2% per million years' rule (Wilson *et al.* 1987) is taken for calibration of mtDNA. Most likely, the Canary Islands Stonechat derived from a founder population of European Stonechats, which became resident on the Canary Islands, or from a population of Euro-

Robin Chittenden

221. Female European Stonechat *Saxicola torquata*, Suffolk, May 2001.

Robin Chittenden

222. First-winter European Stonechat *Saxicola torquata*, Norfolk, September 1996.

pean Stonechats living in northern Africa. Both taxa share common ancestry with the Siberian Stonechat, from which they differ respectively by 2.7% and 5.1% base-pair substitutions.

The African Stonechat *S. [t.] axillaris,* differs from the European Stonechat by 4.6-5.7% substitutions. It clusters as a sister taxon to the Reunion Stonechat *S. [t.] tectes*, which has sometimes been recognised as a distinct species (Hall & Moreau 1970). Pairwise distances amount to 4.4-5.1%.

Using genomic fingerprinting with ISSR-PCR, it is apparent that *S. t. rubicola/hibernans, S. [t.] axillaris, S. dacotiae, S. [t.] tectes* and S. *[t.] maura* form distinct genetic entities (fig. 2). Once again, *S. caprata* and *S. rubetra* cluster at the base of the tree, as in the analyses derived from the mitochondrial *cytochrome b* gene. The stonechats are again recognised as a monophyletic assemblage (bootstrap support 86%); the relationships within this complex are almost identical to those found for *cytochrome b*: *S. dacotiae* clusters as a sister group to *S. torquata rubicola/hibernans; S. [t.] axillaris* is a sister group to *S. [t.] tectes,* and *S. [t.] maura* is intermediate. Also, the phylogeographic divisions within *S. [t.] axillaris* and *S. [t.] maura* can be recovered by the ISSR-data. The ISSR-profiles, based on nuclear DNA, clearly indicate that the results obtained from mitochondrial *cytochrome b* sequences correctly reflect the phylogeny of this group of birds.

ISSR and *cytochrome b* sequences both show a certain degree of geographic variation in the DNA of stonechats (figs. 1-2). Because we have sampled a limited number of populations so far, our analysis of intraspecific variation must be regarded as preliminary. It would certainly be rewarding for further investigations to sample genetic variation across the complete geographic distribution range of stonechats.

The forms *S. [t.] tectes, S. [t.] maura, S. [t.] axillaris* and *S. dacotiae* have often been treated as subspecies of Common Stonechat *S. torquata* (see Sibley & Monroe 1990). On the basis of diagnostic differences in morphology, breeding biology and distribution, however, these taxa have already been recognised as distinct species in several bird guides and handbooks. If the members of the *S. torquata* complex were closely related subspecies, we should expect a cluster showing no (or just extremely small) genetic differences between each of the stonechat taxa. Instead, both the *cytochrome b* sequence data and ISSR-profiles exhibit a well-developed phylogenetic pattern with relatively long branch lengths separating them (figs. 1-2). Genetic distances between the taxa of the *S. torquata* complex range between 6.1% and 2.7%, whereas distances within a given subspecies do not exceed 1.5%. The genetic distances within the *S. torquata* complex imply divergence times of one to three million years. In our dataset, genetic distances for other sibling species are, for example, 6.6% for Rufous *Luscinia megarhynchos* and Thrush Nightingales *L. luscinia*; 5.0% for Northern *Oenanthe oenanthe* and Isabelline Wheatears *O.*

Arnoud B. van den Berg

223. Siberian Stonechat *Saxicola [torquata] maura*, Khao Yai, Thailand, November 1991.

isabellina; and 3.4% for Fieldfare *Turdus pilaris* and Song Thrush *T. philomelos*. These comparisons indicate that the genetic distance found between members of the *S. torquata* complex are in a similar range to other closely related, but distinct species. It is, therefore, highly likely that the taxa within the stonechat complex represent distinct genetic lineages. Not only do they differ in genetic terms, but they also differ by their allopatric distribution, ecology, physiology (Gwinner & Scheuerlein 1999; Helm & Gwinner 1999) and morphology (Starck *et al.* 1995; Helm & Gwinner 2001). As a consequence, European Stonechat, African Stonechat, Canary Islands Stonechat, Reunion Stonechat and Siberian Stonechat can be regarded as distinct species, not only within the Phylogenetic Species Concept but also within the Biological Species Concept.

Acknowledgments

We thank U. Wittmann and P. Heidrich for preliminary studies and D. Ristow, W. Bednarek, H. H. Witt and Heidrun Albrecht for providing samples for the present study.

References

Avise, J. C. 1994 *Molecular markers, natural history and evolution*. London.

Damodar Reddy, K., Nagaraju, J., & Abraham, E. G. 1999. Genetic characterization of the silkworm *Bombyx mori* by simple sequence repeat (SSR)-anchored PCR. *Heredity* 83: 681-687.

Ge, X. J., & Sun, M. 1999. Reproductive biology and genetic diversity of a cryptoviviparous mangrove *Aegiceras corniculatum* (Myrsinaceae) using allozyme and intersimple sequence repeat (ISSR) analysis. *Molecular Ecology* 8: 2061-2069.

Gupta, M., Chyi, Y-S., Romero-Severson, J., & Owen J. L. 1994. Amplification of DNA markers from evolutionary diverse genomes using single primers of simple-sequence repeats. *Theoretical Applied Genetics* 89: 998-1006.

Gwinner, E., & Scheuerlein, A. 1999. Photoperiodic responsiveness of equatorial and temperate-zone stonechats. *Condor* 101: 347-359.

Hall, B. P. & Moreau, R. E. 1970. *An atlas of speciation in African passerine birds*. Brit. Mus. (Nat. Hist.), London.

Helm, B., & Gwinner, E. 1999. Timing of postjuvenile molt in African (*Saxicola torquata axillaris*) and European (*S. t. rubicola*) Stonechats: Effects of genetic and environmental factors. *Auk* 116: 589-603.

— & — 2001. Nestling growth and post-juvenile moult under a tight seasonal schedule in Stonechats *Saxicola torquata maura* from Kazakhstan. *Avian Science* 1: 31-42.

Huelsenbeck, J. P., & Crandall, K. A. 1997. Phylogeny estimation and hypothesis using Maximum Likelihood. *Ann. Rev. Ecol. Syst.* 28: 437-466.

Mindell, D. P. 1999. *Avian molecular evolution and systematics*. Academic Press, San Diego.

Sibley, C. G., & Monroe, B. L. 1990. *Distribution and Taxonomy of Birds of the World*. New Haven.

Starck, J. M., König, S. M., & Gwinner, E. 1995. Growth of Stonechats *Saxicola torquata* from Africa and Europe: an analysis of genetic and environmental components. *Ibis* 137: 519-531.

Swofford, D. L. 2001. PAUP-Phylogenetic analysis using parsimony. Version PAUP*4.0b4a.

—, Olsen, J. G., Waddell, P. J., & Hillis, D. M. 1996. Phylogenetic interference. In: Hillis, D. M., Moritz, C., & Mable, B. K. (eds.), *Molecular Systematics*. 2nd edn. Sunderland, Mass.

Wilson, A. C., Ochman, H., & Prager, E. M. 1987. Molecular timescale for evolution. *Trends Genetics* 3: 241-247.

Wink, M., Sauer-Gürth, H., Martinez, F., Doval, G., Blanco, G., & Hatzofe, O. 1998. Use of GACA-PCR for molecular sexing of Old World vultures (Aves: Accipitridae). *Molecular Ecology* 7: 779-782.

—, Guicking, D., & Fritz, U. 2000. Molecular evidence for hybrid origin of *Mauremys iversoni* Pritchard & McCord, 1991, and *Mauremys pritchardi* McCord, 1997 (Reptilia: Testudines: Bataguridae). Zoologische Abhandlungen, Staatl. Mus. Tierkunde Dresden 51: 41-49.

—, Sauer-Gürth, H., Heidrich, P., Witt, H.-H., & Gwinner, E. In press. A molecular phylogeny of stonechats and related turdids. In: Urquhart, E. *Stonechats*. London.

Wittmann, U. 1994. Zur molekularen Phylogenie der Drosseln (Aves: Familie Turdidae). Thesis, University Heidelberg.

Wittmann, U., Heidrich, P., Wink, M., & Gwinner, E. 1995. Speciation in the Stonechat (*Saxicola torquata*) inferred from nucleotide sequences of the cytochrome b gene. *J. Zoo. Syst. Evol. Research* 33: 116-122.

Zietkiewicz, E., Rafalski, A., & Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (ISSR)-anchored polymerase chain reaction amplification. *Genomics* 20: 176-183.

Michael Wink and Hedi Sauer-Gürth, Institut für Pharmazeutische Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany

Eberhard Gwinner, Max-Planck-Research Centre for Ornithology, Von der Tannstr. 7, 82346 Andechs, Germany

Appendix

ISSR-PCR

The genomes of all animals and plants contain stretches of small repeated sequences of DNA such as .CACACACACACACACACA. that occur because of faults during the processes of replication and repair of DNA. These are called 'microsatellites'. In general, if there is gene flow between two different populations or subspecies, their patterns of microsatellites will be broadly identical (although mutations may still be found). Once gene flow is restricted, however, such as between closely related species or isolated subspecies that no longer breed with each other, the number, length and arrangement of these repeats starts to diverge randomly. 'ISSR-PCR' attempts to turn this divergence into a form that humans can see and score. It uses a small manufactured stretch of DNA (a primer) that binds specifically to some of these satellites in DNA isolated from the taxa of interest. An enzyme is used to create many thousands or millions of copies of the DNA that lies between these microsatellites. These copies can be separated and visualised according to their size by running them through a gel that acts like a sieve. Individuals with identical microsatellite patterns in their DNA will produce identical bands on the gel, but if mutations have occurred, different individuals will show slightly different banding patterns. The presence or absence of bands on the gel can be scored for each individual, and these scores fed into the algorithm that draws up phylogenetic trees. Taxa with the most similar banding patterns will tend to be the most closely related.