

A MOLECULAR PHYLOGENY OF STONECHATS AND RELATED TURDIDS

by

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INTRODUCTION

Systematic relationships are usually based on similarity, and taxa showing the least difference are considered to be closely related. Since morphological similarity in two lineages of organisms can be due to adaptation to similar ecological constraints, adaptive characters can lead to incorrect taxonomic classifications.

The analysis of nuclear or mitochondrial marker genes has become a widely applied tool in all fields of zoology, including ornithology, to reconstruct phylogenies and phylogeographic relationships (see overviews in Avise 1994 and Mindell 1999). Molecular data have the great advantage that convergence does not impair an analysis to the same degree as morphological data. If taxa belong to the same species, their marker genes are identical and do not show genetic distances that are typical for established species. Molecular data also give evidence for the timescale in which a particular evolutionary step has taken place ('molecular clock') and therefore allow both a phylogenetic and phylogeographic analysis of the unknown past of a group of organisms. Sequence data have therefore become an important tool for taxonomy and evolutionary studies. In our laboratory we have used sequences of mitochondrial DNA (mtDNA) to infer the phylogeny of diurnal and nocturnal raptors, shearwaters, gulls, bustards and warblers (Gaucher *et al.* 1996; Heidrich & Wink 1998; Heidrich *et al.* 1995, 1996, 1998; Helbig *et al.* 1995, 1996; Leisler *et al.* 1997; Seibold *et al.* 1996; Wink 1995, 1998; Wink & Heidrich 1999; Wink *et al.* 1996, 1998).

Few molecular studies have addressed phylogenetic relationships in stonechats and other turdids. Based on the analysis of a partial sequence (300 base pairs) of the mitochondrial cytochrome-*b* gene, preliminary data have suggested that the European Stonechat *Saxicola torquata*, Siberian Stonechat *Saxicola maura* and African Stonechat *Saxicola axillaris* have already diverged to a high degree (Wittmann 1994; Wittmann *et al.* 1995)¹.

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

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 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 (Boehringer Mannheim), 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. The mitochondrial cytochrome-*b* gene was amplified by PCR using primers MT-A3 GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G, MT-F2 CTA AGA AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC CAA TG OR MT-FS TAG TTG GCC AAT GAT GAT GAA TGG GTG TTC TAC TGG GTT. PCR products were sequenced directly using the dideoxy chain termination method with the cycle sequencing kit (Amersham Life Science, RPN 2438/RPN 2538) in combination with internal CY-5 labelled primers. For cycle sequencing a two-stage programme containing an initial denaturing step at 94°C for 4 min and 25 cycles at 60°C (40sec), and 94° (30sec) was used. The primers employed were MT-C2-CY XGA GGA CAA ATA TCA TTC TGA GG, MT-U2-CY XGG GGT GAA GTT TTC TGG GTC, MT-C4-CY XAG TGT TGG GTT GTC TAC TGA, MT-V-cy TGG AGG GGR AAR AAT CCG T, MT-b2-cy GCC CAG AAK CAT ATT TGT CCA CA, MT-le-cy TCA AAC CCG AAT GAT AYT TCC TAT T, and MT-c5-cy TCA GTA GAY AAC CCM ACA CT. CY-5 labelled fragments

¹Wink *et al.* (2002) use of binominals for European Stonechat (*S. torquata*) and African Stonechat (*S. axillaris*) differ from those used throughout this book. In the Common Stonechat Complex account and European, Siberian and African Stonechat accounts, European Stonechat is *S. rubicola* and African Stonechat *S. torquata* the reasoning for this is given on pages 133-134.

were analysed on an automated DNA sequencer (Amersham Pharmacia Biotech, ALF-Express II). Sequences of >1000 nt were obtained directly from ALF-Express and aligned. The phylogenetically informative characters of turdids are illustrated in Table 1.

Sequences of ca. 900 nt length were used to reconstruct phylogenetic trees, employing PAUP*4.0b8a (Swofford 1998) with maximum parsimony (MP). More details on the methodology can be found in Wink (1998) and Heidrich *et al.* (1998).

RESULTS AND DISCUSSION

The analysis was restricted mainly to species of the western Palaearctic and comprised the following genera: stonechats and bushchats *Saxicola*, rock thrushes *Monticola*, redstarts *Phoenicurus*, wheatears *Oenanthe*, robins *Erithacus*, nightingales and Bluethroat *Luscinia*, and thrushes *Turdus*. The dippers *Cinclus* were included as a possibly related ingroup and the wrens *Troglodytes* as a distantly related outgroup. Although we sequenced several individuals of each taxon (between 6 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 or two representatives of each taxon were chosen for an analysis which aimed to outline the patterns of phylogeny and speciation within stonechats and related Turdidae. A more complete analysis will be published elsewhere (see Wink *et al.* 2002).

In Figures 1a and 1b the results of maximum parsimony of mtDNA are given. Members of the genus *Saxicola* form a monophyletic clade (supported by a bootstrap value of 92%), which derives from a common ancestor that is shared with Rock Thrush *Monticola saxatilis*. Within the *Saxicola* clade either the Whinchat *Saxicola rubetra* or the Pied Bushchat *Saxicola caprata* take a basal position. The Canary Island Stonechat *Saxicola dacotiae*, the European Stonechat *Saxicola torquata*, the Siberian Stonechat *Saxicola maura*, the Réunion Stonechat *Saxicola tectes* and the African Stonechat *Saxicola axillaris* follow as a well-supported monophyletic clade (bootstrap 88%). The latter forms have often been treated as subspecies of a single species, Common Stonechat *S. torquata* (Sibley & Monroe 1993), but because of diagnostic differences in morphology, breeding biology and distribution these taxa have also been recognised as distinct species in recent literature.

If the members of the *S. torquata* complex were closely related subspecies, we should expect a cluster showing little or no genetic differences between each of the taxa. Instead, the cytochrome-*b* sequence data exhibit a well-developed phylogenetic pattern with relatively long branch lengths separating them (Figures 1a and 1b; Table 2). Genetic distances between the taxa of the *S. torquata* complex range between 4.3% and 6.8%, whereas distances within a given subspecies do not exceed 1% (data, based on larger samples of 10 to 20 individuals, will be documented elsewhere).

The Canary Island Stonechat *S. dacotiae* is closely related to the European Stonechat *S. torquata*; the genetic distance of 4.7% implies that both taxa have diverged about 2.3 million years ago (if the '2% per million years' rule [Wilson *et al.* 1987] is used for calibration of mtDNA). Most likely, the Canary Island Stonechat derived from a migratory population of European Stonechats which became resident on the Canary Islands. Both taxa share common ancestry with the Siberian Stonechat *S. maura*, from which they differ by 4.3% and 5.3% nucleotide substitutions respectively.

The African Stonechat *S. axillaris* differs from the European Stonechat *S. torquata* by 5.7% nucleotide substitutions. It clusters as a sister taxon to the Réunion Stonechat *S. tectes* which has recently been recognised as a distinct species. Pairwise distances amount to 5.3%. The *axillaris* group and the *rubicola* group form a sister group in all reconstructions, deriving from a common ancestor. These differences imply divergence times of 2–3 million years.

Genetic distances in our dataset for other closely related but distinct species such as Common Nightingale *Luscinia megarhynchos* and Thrush Nightingale *Luscinia luscinia*, Northern Wheatear *Oenanthe oenanthe* and Isabelline Wheatear *Oenanthe isabellina*, and Fieldfare *Turdus pilaris* and Song Thrush *Turdus philomelos* (Table 2) are similar in range to members of the *S. torquata* complex.

It is, therefore, highly likely that the taxa within the 'Common Stonechat' complex represent distinct genetic lineages which deserve species status. Not only do they differ in genetic terms, they also differ by their distinct allopatric distribution, ecology, physiology (Gwinner & Scheuerlein 1999; Helm & Gwinner 1999) and morphology. Using genomic fingerprinting with ISSR-PCR, we could confirm that the *S. t. rubicola*, *S. axillaris* and *S. maura* complex forms three distinct genetic entities (complete data will be published elsewhere), indicating that the results obtained from mitochondrial cytochrome-*b* sequences correctly reflect the phylogeny of this group of birds.

The *Monticola-Saxicola* clade is phylogenetically related to the genera *Luscinia*, *Oenanthe*, *Phoenicurus* and *Erithacus*. These genera form a well-supported monophyletic clade with a bootstrap value of 96%. Members of the genus *Turdus*, which also form a monophyletic clade (88% bootstrap support), always cluster as a sister group to the stonechat-wheatear assemblage. The White-throated Dipper *Cinclus cinclus*,

which superficially resembles a blackbird, shows no close affinity to members of the genus *Turdus*, (pairwise genetic distances of 12-15%), or to wrens, with which they have also been associated. We need a more complete dataset of all Passeriformes before considering the real affinities of dippers and wrens.

CONCLUSIONS

Sequence data of the mitochondrial cytochrome-*b* gene and genomic fingerprinting provide good evidence that the geographically separated taxa of the stonechats in this study represent distinct genetic lineages which have become 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, otherwise the differences would not be so clear-cut. Since these lineages also differ in morphology, breeding behaviour, vocalisations and annual rhythmicity, we believe that it is justified to treat them as good species (as many field guides already do).

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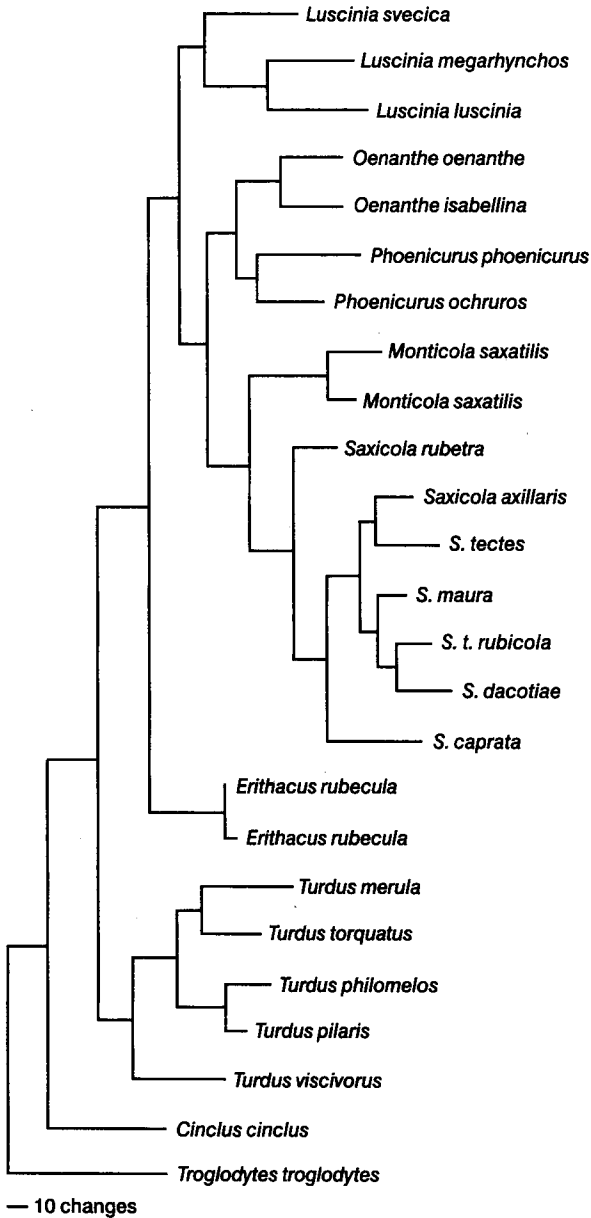


Figure 1a A molecular phylogeny of stonechats and related turdids based on sequence data of the mitochondrial cytochrome-*b* gene.

Maximum parsimony analysis (unweighted analysis); representation as a phylogram (branch lengths correspond to the number of character changes) of one of three equally parsimonious trees.

Tree length: 1197 steps; HI= 0.499; CI= 0.501; RI= 0.542; of 904 analysed characters, 425 characters are variable and 271 parsimony informative.

Bootstrap

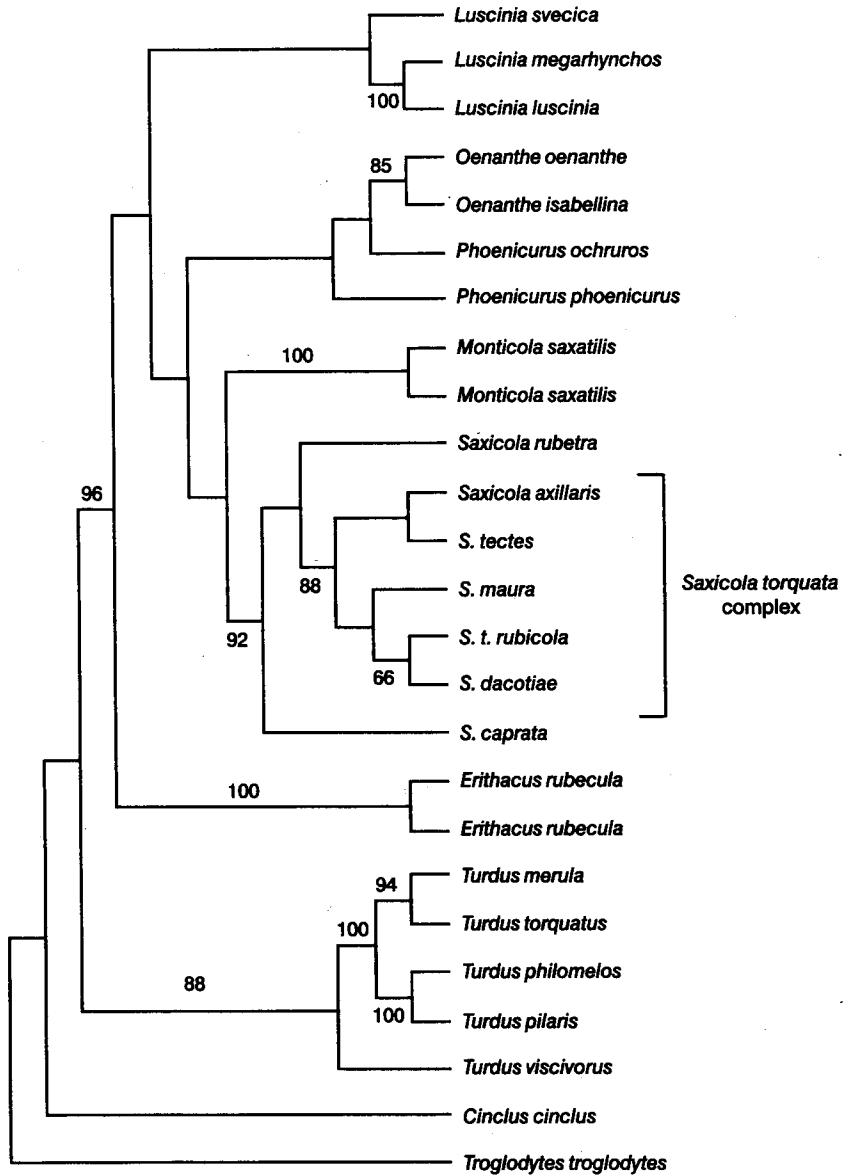


Figure 1b A molecular phylogeny of stonechats and related turdids based on sequence data of the mitochondrial cytochrome-*b* gene.

Maximum parsimony analysis; representation as a bootstrap cladogram (bootstrap values above 60% are given at the corresponding bifurcations).

Tree length: 1197 steps; HI= 0.499; CI= 0.501; RI= 0.542; of 904 analysed characters, 425 characters are variable and 271 parsimony informative.

Table 2 Genetic distances based on pairwise nucleotide substitutions (uncorrected p distance) in the cytochrome-*b* gene.

	2	4	7	14	18	21	25
2 <i>Luscinia svecica</i>	-						
4 <i>Luscinia megarhynchos</i>	0 12456	-					
7 <i>Luscinia luscinia</i>	0 13300	0 10060					
14 <i>Oenanthe oenanthe</i>	0 11202	0 12570	0 14199	-			
18 <i>Oenanthe isabellina</i>	0 11946	0 13742	0 14866	0 07010	-		
21 <i>Ph. phoenicurus</i>	0 12545	0 12709	0 14215	0 11338	0 11071	-	
25 <i>Ph. ochruros</i>	0 10761	0 13582	0 15098	0 09779	0 07793	0 09003	-
33 <i>Erithacus rubecula</i>	0 10527	0 12671	0 13088	0 11645	0 10677	0 10450	0 10214
34 <i>Erithacus rubecula</i>	0 10964	0 13109	0 13426	0 12091	0 11134	0 10999	0 10659
35 <i>Monticola saxatilis</i>	0 13145	0 15352	0 15794	0 12826	0 13583	0 11731	0 13071
36 <i>Monticola saxatilis</i>	0 11197	0 13350	0 13774	0 11435	0 12061	0 11107	0 11323
37 <i>Turdus merula</i>	0 14721	0 15563	0 16547	0 14279	0 16118	0 13616	0 13844
39 <i>Turdus philomelos</i>	0 15056	0 14886	0 16648	0 14073	0 16318	0 13425	0 14301
41 <i>Turdus torquatus</i>	0 14522	0 15231	0 16562	0 14758	0 16242	0 13337	0 14885
43 <i>Turdus viscivorus</i>	0 13660	0 14845	0 16185	0 13448	0 14453	0 13364	0 14692
44 <i>Turdus pilaris</i>	0 14759	0 14700	0 16687	0 14439	0 16356	0 12899	0 14333
46 <i>Cinclus cinclus</i>	0 15827	0 14453	0 16862	0 14845	0 15294	0 14421	0 15521
49 <i>T. troglodytes</i>	0 16836	0 16612	0 18779	0 16520	0 15969	0 15655	0 16186
63 <i>Saxicola rubetra</i>	0 11294	0 12005	0 14414	0 10865	0 10921	0 11539	0 11085
96 <i>S. axillaris</i>	0 12631	0 13586	0 15424	0 11872	0 12056	0 10994	0 11656
111 <i>S. maura</i>	0 12650	0 13626	0 15354	0 11221	0 11976	0 11367	0 11690
131 <i>S. t. rubicola</i>	0 12634	0 14039	0 16215	0 11988	0 12857	0 11105	0 11772
138 <i>S. caprata</i>	0 13298	0 14581	0 15108	0 11327	0 12186	0 12102	0 11885
141 <i>S. dacotiae</i>	0 13807	0 14904	0 16888	0 12359	0 13152	0 12489	0 12809
151 <i>S. tectes</i>	0 13002	0 14401	0 15805	0 11905	0 11178	0 11243	0 12133
	33	34	35	36	37	39	41
33 <i>Erithacus rubecula</i>	-						
34 <i>Erithacus rubecula</i>	0 00553	-					
35 <i>Monticola saxatilis</i>	0 12927	0 13484	-				
36 <i>Monticola saxatilis</i>	0 10650	0 11200	0 04172	-			
37 <i>Turdus merula</i>	0 13371	0 13931	0 15185	0 13728	-		
39 <i>Turdus philomelos</i>	0 12736	0 13287	0 15082	0 14071	0 10689	-	
41 <i>Turdus torquatus</i>	0 13089	0 13640	0 14974	0 14089	0 07985	0 08536	-
43 <i>Turdus viscivorus</i>	0 12326	0 12880	0 15774	0 13569	0 10929	0 10901	0 10473
44 <i>Turdus pilaris</i>	0 12536	0 13088	0 14783	0 13547	0 08903	0 03772	0 06885
46 <i>Cinclus cinclus</i>	0 14063	0 14615	0 16517	0 14732	0 14598	0 13275	0 13744
49 <i>T. troglodytes</i>	0 15404	0 15846	0 17730	0 16405	0 17093	0 16494	0 16190
63 <i>Saxicola rubetra</i>	0 10987	0 11515	0 11564	0 10192	0 13922	0 12611	0 14407
96 <i>S. axillaris</i>	0 11969	0 12417	0 11231	0 10976	0 14724	0 15057	0 14639
111 <i>S. maura</i>	0 11576	0 12121	0 10505	0 10902	0 14769	0 14330	0 14681
131 <i>S. t. rubicola</i>	0 12760	0 13302	0 11799	0 12201	0 15607	0 14506	0 15310
138 <i>S. caprata</i>	0 12316	0 12861	0 12242	0 12194	0 15281	0 14403	0 14539
141 <i>S. dacotiae</i>	0 12930	0 13487	0 12518	0 13262	0 16372	0 15816	0 15731
151 <i>S. tectes</i>	0 12676	0 13227	0 11712	0 12009	0 15545	0 14878	0 15354
	43	44	46	49	63	96	111
43 <i>Turdus viscivorus</i>	-						
44 <i>Turdus pilaris</i>	0 10256	-					
46 <i>Cinclus cinclus</i>	0 12089	0 13529	-				
49 <i>T. troglodytes</i>	0 15779	0 16090	0 15829	-			
63 <i>Saxicola rubetra</i>	0 13334	0 13086	0 15044	0 15385	-		
96 <i>S. axillaris</i>	0 14569	0 14543	0 15387	0 16506	0 06754	-	
111 <i>S. maura</i>	0 14393	0 14257	0 15302	0 16324	0 06781	0 04455	-
131 <i>S. t. rubicola</i>	0 15457	0 14542	0 15722	0 17726	0 08868	0 05655	0 04337
138 <i>S. caprata</i>	0 14693	0 14446	0 15285	0 17615	0 09081	0 08425	0 08449
141 <i>S. dacotiae</i>	0 16773	0 15290	0 17165	0 18633	0 08081	0 06397	0 05302
151 <i>S. tectes</i>	0 14827	0 15464	0 15211	0 17331	0 08663	0 05338	0 06248
	131	138	141	151			
131 <i>S. t. rubicola</i>	-						
138 <i>S. caprata</i>	0 08869	-					
141 <i>S. dacotiae</i>	0 04716	0 10213	-				
151 <i>S. tectes</i>	0 05105	0 09448	0 06860	-			