

Mitochondrial phylogeny of *Locustella* and related genera

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We used maximum likelihood analysis of complete mitochondrial ND2 sequences (1041 bp) to clarify the taxonomy and relationships of various species and genera of grass and bush warblers. The tree revealed two clades of grass and bush warblers. One clade was comprised of all four western Palearctic *Locustella* and two species of Asian *Bradypterus*. The other clade included five eastern Palearctic *Locustella* (including the distinctive Sakhalin warbler *Locustella amnicola*) and the marsh grassbird *Megalurus pryeri*. African *Bradypterus* and Australian little grassbird *Megalurus gramineus* were distantly related to their Asian congeners. Therefore, current taxonomy of these genera does not reflect their evolutionary history and needs revision. It is proposed that a phylogenetic analysis of morphology and ecological preferences would show that the current taxonomy of grass and bush warblers reflects species' habitat preferences and morphology related to locomotion and foraging in their habitats, rather than their shared ancestry. Distinct clades were found in grasshopper warbler *Locustella naevia* and Pallas's grasshopper warbler *L. certhiola*. Detailed phylogeographic studies are needed to elucidate the species status of the clades within these two species.

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Among the Old World warblers, several eco-morphological groups can be defined (Baker 1997). In this paper, we focus on two such groups, the “bush warblers” and “grass warblers.” “Bush warblers” include species in two genera, *Cettia* and *Bradypterus*. Differences between the genera are slight and some overlap exists. These differences involve tail shape, rectrix number and barring pattern, and size of rectal bristles. “Grass warblers” include two groups, one including the genera *Megalurus*, *Chaetornis*, *Grammicolia* and *Schoenicola*, and the other one consisting of species in *Locustella*. Differences between the two grass warbler groups include tail length and shape, bill robustness and size of rectal bristles.

However, this is some overlap, and members of both groups are skulking in nature, staying low in thick shrubbery, grass or reeds. Thus, members of these two groups provide challenges to field identification as well as to constructing stable classifications.

In this paper, we test aspects of the classification of grass and bush warblers, concentrating on the genus *Locustella*. *Locustella* includes nine species (Sibley and Monroe 1990, Stepanyan 1990, 2003, Monroe and Sibley 1993, Baker 1997), all of which inhabit middle latitudes in Eurasia. Three species (grasshopper warbler *Locustella naevia*, Eurasian river warbler *L. fluviatilis*, and Savi's warbler *L. luscinioides*) are widely distributed in

the western Palearctic, three species (lanceolated warbler *L. lanceolata*, Pallas's grasshopper warbler *L. certhiola*, and Gray's grasshopper warbler *L. fasciolata*) have large eastern Palearctic ranges, and the remaining three species (Sakhalin warbler *L. amnicola*, Middendorff's grasshopper warbler *L. ochotensis*, and Pleske's grasshopper warbler *L. pleskei*) are restricted to the Asian Pacific coast and islands. Only Savi's warbler is found outside Eurasia, in northwestern Africa. Despite the agreement on number of species included in the genus, different taxonomic arrangements have been proposed. For example, Sibley and Monroe (1990), Stepanyan (1990, 2003), and Monroe and Sibley (1993) recognize the Sakhalin warbler. However, Baker (1997) treats the Sakhalin warbler as conspecific with Gray's grasshopper warbler, but he assigns the marsh grassbird *Megalurus pryeri* to *Locustella*, resulting in a total of nine species. Although the taxonomy of *Locustella* is relatively stable, the relationships among species are virtually unknown.

We analyzed complete sequences of the mitochondrial ND2 gene (1041 bp) of all species of *Locustella* to test the monophyly of and relationships within the genus, and its relationship to other genera, specifically *Megalurus*, *Cettia*, *Bradypterus* and *Urosphena*. Because the general external morphology and habitat requirements of species in these genera are similar, the possibility exists that classifications will not reflect patterns of evolutionary history owing to convergence obscuring true relationships. Thus, a molecular phylogenetic perspective provides a test of current taxonomy.

Materials and methods

We used 38 samples representing all 9 species of *Locustella* (*sensu* Sibley and Monroe 1990) and 21 individuals from 10 species representing other warbler genera (Table 1). Total genomic DNA was extracted from frozen or 96% ethanol-preserved tissue samples using QIAamp Tissue Kit (QAIGEN Inc.). For each individual complete mitochondrial ND2 gene (1041 bp) was amplified with Perkin-Elmer PCR reagents and primers L5215 (Hackett 1996) and H1064 (CTTTGAAGGCCTTCGGTTTA, designed by SVD). The PCR profile included 2.5 minutes preheating at 94°C and 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C. PCR cycles were followed by 10 minutes of extension at 72°C. The PCR fragments were sequenced directly on ABI-377 or ABI-3700 sequencers using BigDye chemistry (Applied Biosystems). Amplification primers and primer L347 (CCATTCCACTTCTGATTCCC, designed by SVD) were used for sequencing.

Sequences were aligned automatically using Sequencher 3.0 (Gene Codes Corporation). The alignment did not require editing because there were no indels in

the ND2 sequences. A maximum likelihood (ML) phylogenetic analysis was performed using PAUP* (Swofford 1998). ML models and parameters were determined by Modeltest 3.06 (Posada and Crandall 1998). Taxa were added randomly for both ML and its bootstrap analysis (100 replicates). The tree was rooted with a composite outgroup consisting of Cetti's warbler *Cettia cetti*, Japanese bush-warbler *C. diphone*, and Asian stubtail warbler *Urosphena squameiceps*.

Results

There were 50 haplotypes among 59 warbler sequences (Fig. 1). The large number of haplotypes within species and the absence of unexpected stop codons suggest that our sequences are mitochondrial and not nuclear in origin. Both the Hierarchical Likelihood Ratio Test and the Akaike information criterion (minimum theoretical information criterion; Akaike 1974) implemented in Modeltest 3.06, selected the GTR+G+I model (Rodríguez et al. 1990) for complete ND2 sequences of all warblers used in our study. The G+I or "gamma+invariant" model (Gu et al. 1995) in the GTR+G+I model indicates that a portion of sites are invariable, and the discrete gamma approximation of substitution rates applies only to variable sites. Guanine was underrepresented, and cytosine was over represented in ND2 sequences (A = 34.75%, C = 39.32%, G = 7.29%, T = 18.64%; G-test: P = 0.001). All taxa shared this nucleotide bias and there was no evidence of heterogeneity of base composition among taxa. GenBank accession numbers for ND2 sequences are AY 382341–AY 382399.

The ML analysis of unique haplotypes resulted in a single tree (Fig. 1). The likelihood ratio test rejected the molecular clock assumption for our tree ($-\ln L$ without molecular clock enforced = 7708.16353, $-\ln L$ with molecular clock = 7745.22725; $-2\Delta\ln L = 74.12744$, $df = 48$, $P = 0.009$).

None of the four genera represented by multiple taxa in our study (*Bradypterus*, *Locustella*, *Megalurus*, and *Cettia*) were monophyletic (Fig. 1). Members of the genus *Locustella* were divided into two clades. One of these clades, which included Savi's warbler, Eurasian river warbler, lanceolated warbler, and grasshopper warbler, also included two Asian *Bradypterus*, Chinese bush-warbler *B. tacsanowskii* and chestnut-backed bush-warbler *B. castaneus*. Furthermore, the grasshopper warbler was more closely related to both of these Asian *Bradypterus* than to other *Locustella*. The other clade included five Asian *Locustella*: Sakhalin warbler, Gray's grasshopper warbler, Pallas's grasshopper warbler, Middendorff's grasshopper warbler, and Pleske's grasshopper warbler, as well as marsh grassbird. The little grassbird *Megalurus gramineus* was a distant sister

Table 1. Tissue samples and GeneBank accession numbers.

Species name	Institution	Sample ID	GenBank accession	Locality
Evergreen forest warbler	LSU	B21098*	AY382341	Tanzania: Iringa
<i>Bradypterus mariae</i>	LSU	B21099*	AY382342	Region
African bush-warbler	UWBM	lkb143	AY382343	South Africa:
<i>B. baboecola</i>	UWBM	lkb144	AY382344	QwaZulu/Natal
Chestnut-backed	AMNH	rdw24616	AY382345	Indonesia:
bush-warbler	AMNH	rdw24619	AY382346	Sulawesi
<i>B. castaneus</i>	AMNH	rwd24655	AY382347	
	AMNH	rwd24661	AY382348	
Cinnamon bracken-	LSU	B21093*	AY382349	Tanzania: Iringa
warbler <i>B. cinnamomeus</i>	LSU	B21094*	AY382350	Region
Chinese bush-warbler	UWBM	csw5963	AY382351	Mongolia: Töv Aymag
<i>B. tacsanowskii</i>	UWBM	svd495	AY382352	Russia: Irkutsk
Cetti's warbler	UWBM	bks1163	AY382353	Russia: Astrakhan'
<i>Cettia cetti</i>	UWBM	svd1083	AY382354	
Japanese bush-warbler	UWBM	sar6474	AY382355	Russia: Sakhalin
<i>C. diphone</i>	UWBM	sar6475	AY382356	
Sakhalin warbler	UWBM	ty180	AY382357	Russia: Sakhalin
<i>Locustella amnicola</i>	UWBM	vm245	AY382358	
Pallas's grasshopper	UWBM	cds4887	AY382359	Russia: Buryatiya
warbler <i>L. certhiola</i>	UWBM	csw4382	AY382360	Russia: Magadan
	UWBM	csw4831	AY382361	Russia: Khabarovsk
	UWBM	msuzm1999/n116	AY382362	Russia: Tyva
	UWBM	svd2495	AY382363	Russia: Primor'ye
	UWBM	svd2509	AY382364	
	UWBM	svd515	AY382365	Russia: Irkutsk
	UWBM	svd594	AY382366	Russia: Krasnoyarsk
	UWBM	svd2609	AY382367	Russia: Primor'ye
Gray's grasshopper warbler				
<i>L. fasciolata</i>				
Eurasian river	UWBM	bks1871	AY382368	Russia: Ekaterinburg
Warbler	UWBM	msuzm2000/n017	AY382369	Russia: Moscow
<i>L. fluviatilis</i>	BMUM	svd2747	AY382370	Russia: Arkhangel'sk
	BMUM	svd2774	AY382371	
Lanceolated warbler	UWBM	bks1863	AY382372	Russia: Ekaterinburg
<i>L. lanceolata</i>	UWBM	bks4106	AY382373	Mongolia: Töv Aymag
	UWBM	csw4783	AY382374	Russia: Magadan
	UWBM	csw5927	AY382375	Mongolia: Dornod Aymag
	UWBM	sar6205	AY382376	Russia: Kamchatka
	UWBM	msuzm1999/p089	AY382377	Russia: Tyva
	UWBM	svd503	AY382378	Russia: Irkutsk
	UWBM	vm515	AY382379	Russia: Khabarovsk
Savi's warbler <i>L. luscinioides</i>	UWBM	svd2399	AY382380	Russia: Krasnodar
Grasshopper warbler	UWBM	rcf1803	AY382381	Russia: Tyva
	UWBM	msuzm1999/n008	AY382382	
<i>L. naevia</i>	UWBM	svd1743	AY382383	Russia: Krasnodar
	UWBM	svd2054	AY382384	
	UWBM	svd2106	AY382385	
	UWBM	svd2113	AY382386	
	UWBM	svd2114	AY382387	
Middendorff's	UWBM	sar6214	AY382388	Russia: Kamchatka
Grasshopper warbler	UWBM	sar6237	AY382389	
<i>L. ochotensis</i>	UWBM	svd286	AY382390	Russia: Sakhalin
	UWBM	svd287	AY382391	
Pleske's grasshopper	NIES	L7*	AY382392	Japan: Fukuoka
warbler <i>L. pleskei</i>	NIES	L13*	AY382393	
	NIES	L14*	AY382394	
Marsh grassbird	BITU	jpn2L90219*	AY382395	Japan: Aomori
<i>Megalurus pryri</i>	BITU	jpn2L90208*	AY382396	Prefecture
Little grassbird <i>M. gramineus</i>	UWBM	sar7205	AY382397	Australia: New South Wales
Asian stubtail warbler	UWBM	csw4866	AY382398	Russia: Krasnodar
<i>Urosphena squameiceps</i>	UWBM	vm247	AY382399	Russia: Sakhalin

AMNH – American Museum of Natural History, BITU – Biological Institute, Tohoku University, BMUM – University of Minnesota Bell Museum, LSU – Louisiana State University Museum of Natural History, NIES – National Institute for Environmental Studies, Japan, MSUZM – Moscow State University Zoological Museum, UWBM – University of Washington Burke Museum. * Identifies samples with no voucher specimens (blood/DNA sample only).

to these two clades. The African bush-warbler *Bradypterus baboecola*, evergreen forest warbler *B. mariae*, and cinnamon bracken-warbler *B. cinnamomeus* formed a sister clade to the prior three clades. In the outgroup clade, the two *Cettia* were not sisters.

Although haplotypes for Pleske's grasshopper warbler and Middendorff's grasshopper warbler appeared reciprocally monophyletic, the difference between these two taxa (0.61% GTR + G + I corrected sequence divergence excluding intraspecific variation) was just slightly greater

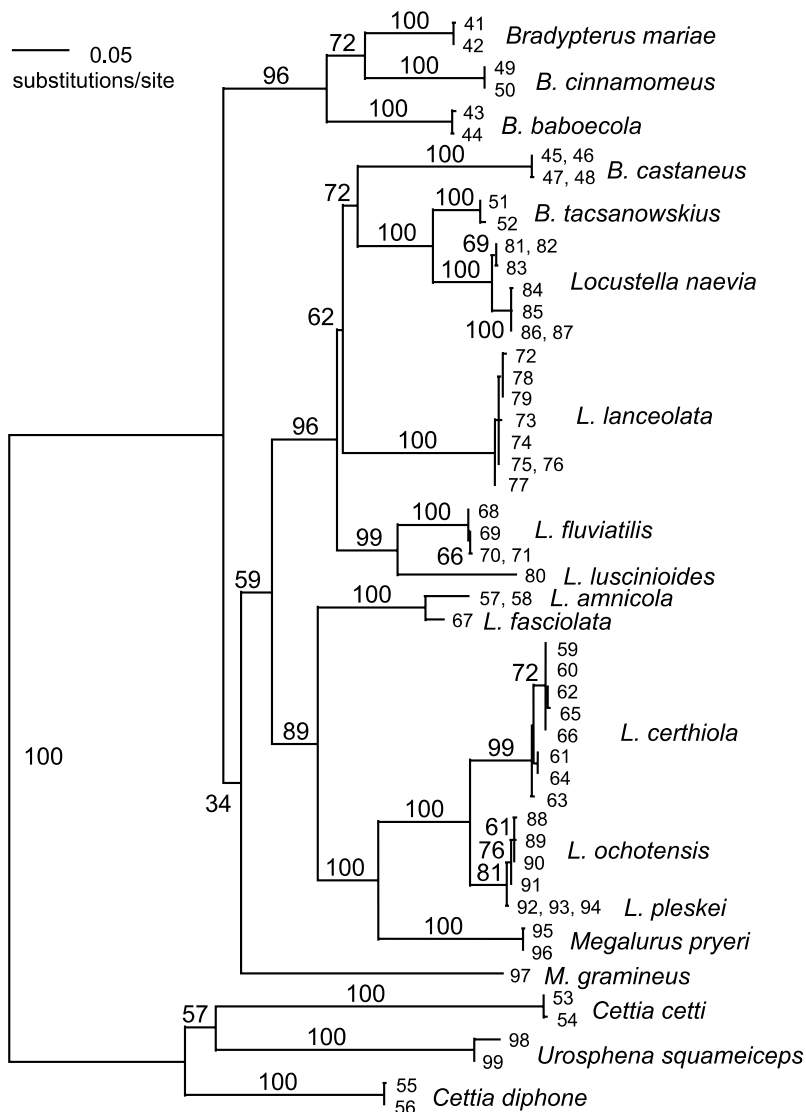


Fig. 1. Maximum likelihood tree based on GTR+G+I model. Numbers next to branches represent bootstrap values (100 replicates with random addition of taxa). Individual sequences identified by the last two digits of their GenBank accession number. Complete GenBank accession numbers are presented in Table 1.

than the largest difference within Middendorff's grasshopper warblers (0.45%; Fig. 1). On the other hand, clear structuring was apparent in common grasshopper warbler and in Pallas's grasshopper warbler. Common grasshopper warblers from Caucasian alpine meadows (elevation ≥ 1950 m a.s.l.) differed by 1.85% sequence divergence from birds in grasslands of the Caucasian foothills and in Tyva. The three Pallas's grasshopper warblers from the two southeastern most localities (Primor'ye and Khabarovsk) differed by 0.95% from the other five individuals collected from across the species range. Our study also supports the status of Sakhalin warbler as a distinct species. The difference between Sakhalin warbler and its closest relative Gray's grasshopper warbler is 5.78% GTR+G+I corrected divergence.

Discussion

Our data indicate that the current classification of some grass and bush warblers does not reflect their evolutionary history at several levels. The basic dichotomy between grass and bush warblers noted by Baker (1997) is inconsistent with the mtDNA tree in that *Cettia* and *Bradypterus* do not group together. Our tree shows two broad groupings of species assigned to *Locustella*, with each grouping containing species currently assigned to other genera. However, our analysis supports Baker's (1997) conclusion that marsh grassbird belongs in *Locustella*, a taxonomic change that would remove some of the paraphyly evident in our tree. The little grassbird is distinct from *Locustella*, supporting Baker's (1997) view. The strong support for the sister taxon

relationship between common grasshopper warbler and Chinese bush-warbler, and the occurrence of chestnut-backed bush-warbler as sister to these two species, renders *Locustella* and *Bradypterus* paraphyletic. Thus, at least some Asian *Bradypterus* belong in *Locustella*. Lack of monophyly for genera other than *Locustella* seems clear but final determination awaits sampling of all relevant species.

Current classifications also might not reflect evolutionary divergence at the species level and below. The tree and genetic distance computation confirm that the easternmost population of the Gray's grasshopper warbler found on Sakhalin Island merits species status (Sakhalin warbler). Phylogeographic analyses are needed to corroborate the groupings (Fig. 1) of grasshopper warbler and Pallas's grasshopper warbler, each of which might merit species splits. Also, although Middendorff's grasshopper warbler and Pleske's grasshopper warbler appear to be very recently derived species, this conclusion merits testing with broader geographic sampling. Some phylogeographic surveys of widespread Eurasian species, such as bluethroat *Luscinia svecica*, show little phylogeographic structure (Zink et al. 2003), whereas species such as great tit *Parus major* do (Kvist et al. 2003).

A possible reason for the discrepancy between the current classification and the molecular tree is that the characters used to group species into genera reflect convergence to similar habits and lifestyles rather than shared ancestry. For example, species assigned to *Bradypterus*, but evolutionarily within *Locustella*, have likely converged in habits with species validly assigned to *Bradypterus*. Given our estimate of evolutionary history quantitative analysis of behavior and habitat preferences can reveal instances of convergence versus retained primitive states.

Discordance between molecular trees and classifications based on morphology and behavior is not unique to this study. The yellow wagtail *Motacilla flava*, a widely distributed and common Eurasian species, actually consists of three species-level groups that are not each other's nearest relatives (Voelker 2002, Pavlova et al. 2003). In fact, groups of yellow wagtail are sisters to groups of another non-monophyletic species, citrine wagtail *M. citreola*. In the New World flycatchers, a group consisting of many sibling species, molecular analysis resulted in several changes to the generic classification to make it consistent with evolutionary history (Cicero and Johnson 2002). In the case of *Locustella* and other genera analyzed here, the discordance between evolutionary patterns deduced from mtDNA and the classification is especially high, owing perhaps to the lack of change in morphology and habitat preference that is overlain on a molecular history that is as deep as that in other, morphologically distinct genera.

The phylogenetic hypothesis (Fig. 1) suggests several geographical patterns. The clade consisting of Sakhalin warbler, Gray's grasshopper warbler, Pallas's grasshopper warbler, Middendorff's grasshopper warbler, Pleske's grasshopper warbler and marsh grassbird are all predominantly found in the eastern Palearctic, suggesting that this area, especially its Pacific coast and islands (Sakhalin and small islands of Russia and Japan), was an active region of speciation over a considerable span of time. Of interest were the split of Sakhalin warbler from its sister Gray's grasshopper warbler a widespread Eastern Palearctic species, and the division of Pallas's grasshopper warbler into two distinct clades southeastern (to the south of Amur river in the Russian Far East) and a clade occupying the rest of the Eastern Palearctic. In other species, such as great spotted woodpecker *Dendrocopos major* the populations on Sakhalin and in the southern part of the Russian Far East (Primor'ye) are well differentiated from mainland populations (Zink et al. 2002a), whereas in yellow wagtail (Pavlova et al. 2003) and three-toed woodpecker *Picoides tridactylus* (Zink et al. 2002b), populations on Sakhalin are not differentiated.

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