Ibis (2001) **143**, 233–247

Cryptic species in the genus *Phylloscopus* (Old World leaf warblers)

DARREN E. IRWIN[1]*, PER ALSTRÖM[2], URBAN OLSSON[3] & Z.M. BENOWITZ-FREDERICKS[1]

[1]*Department of Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0116, USA*

[2]*Department of Systematic Zoology, Evolutionary Biology Centre, Uppsala University, Norbyv. 18 D, SE-752 36 Uppsala, Sweden*

[3]*Department of Zoology, University of Göteborg, Box 463, 405 30 Göteborg, Sweden*

In almost all ecological and evolutionary research, it is important to assess the number of species under study. Cryptic species, which are morphologically similar, present a special problem because they need to be identified through studies of behavioural or genetic variation. Here we review the important factors in the recent discovery of three species and elevation of nine previously known taxa to species status within the genus *Phylloscopus* (Old World leaf-warblers) and we examine the case of three morphologically similar taxa (*inornatus, humei* and *mandellii*) that until recently were considered to be members of the single species *Phylloscopus inornatus*. We have identified several locations at which *humei* and *inornatus* coexist at high density, and the results of playback experiments, observations of interactions, and spectrogram analysis of vocalizations at these sites all indicate that there are significant behavioural differences and a lack of recognition between *humei* and *inornatus*. Estimated relationships within the species complex based on mitochondrial control region DNA sequences show that there is little variation within the geographic ranges of either *humei* or *inornatus* compared with the deep split between the two taxa. These observations support the division of *humei* and *inornatus* into separate species. The third taxon, *mandellii*, which is geographically separated from both *humei* and *inornatus*, is behaviourally and genetically more similar to *humei*. Under the biological species concept, it is difficult to determine the species status of allopatric taxa. Alternative species concepts, such as the phylogenetic and recognition concepts, are more easily applied to allopatric taxa but lead to differing conclusions over whether *mandellii* and *humei* are separate species. This confusion arises from the fact that the two taxa are in an early stage of allopatric divergence. The review of other recently designated species reveals that song divergence is of primary importance in their designation and that song variation, playback experiments and genetic analysis lead to similar conclusions regarding species status.

There have been many attempts to estimate the total number of species, both globally and within geographic regions and taxonomic groups (Wilson 1988, May 1990, 1994, Grassle 1991). The estimates rely primarily on morphology to classify organisms into species, and hence morphologically similar species that differ in behaviour and physiology (cryptic species) are often not distinguished. In fields of research as diverse as community ecology, conservation biology and animal communication, inaccurate assessments of the number of species under study can lead to incorrect conclusions (Grassle 1991, Knowlton & Jackson 1994, Jones 1997). If cryptic species are not identified, measurements of range size, niche width and geographic variation may be overestimated, and measurements of species diversity, speciation rates and habitat specificity underestimated.

Here we examine the issue of cryptic species by focusing on a single genus, *Phylloscopus*, which has recently seen explosive growth in the number of known species. During the last decade three *Phylloscopus* species have been discovered and nine subspecies have been elevated to species status (Price

*Corresponding author. Present address: Department of Ecology, Lund University, Ecology Building, S-223 Lund, Sweden.
Email: dirwin@biomail.ucsd.edu

1996, present study). *Phylloscopus* species are small insectivorous warblers that are distributed throughout the Old World (Baker 1997) and are renowned for being morphologically similar and often difficult to identify. Ticehurst (1938) described 29 species of *Phylloscopus* in Eurasia excluding Australasia. Almost 50 years later, the number of species in this group had risen by only two (Mayr & Cottrell 1986) and in 1993 the number was reported to be 32 (Monroe & Sibley 1993). The most recent list (King 1997) includes no fewer than 42 species of *Phylloscopus* in Eurasia excluding the Philippines, Greater Sundas and Wallacea, an increase of at least 31% in less than a decade. We review the factors that have led to this rapid growth. We also present a detailed study of three morphologically similar *Phylloscopus* taxa that have often been considered subspecies of a single species.

The biological species concept defines species as 'groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups' (Mayr 1942). It has been very successful both conceptually and practically (Coyne 1994, Snow 1997) but has also been criticized (Paterson 1985, Cracraft 1989, Zink 1997). Much of the criticism has revolved around the words 'actually or potentially interbreeding' (Cracraft 1989, Hailman 1995, Zink & McKitrick 1995). When applying the concept to parapatric forms, it is crucial to study the two forms where they are in contact. When applying the concept to two disjunct populations it is necessary to determine whether they would interbreed if they came into contact. Researchers differ in their opinions over whether two allopatric populations would interbreed if brought together in the wild. In part to avoid the emphasis on sympatry in the assessment of species status, several other species concepts have been proposed. Two of these are the phylogenetic species concept *sensu* Cracraft (1983), which defines a species as 'the smallest diagnosable cluster of individual organisms in which there is a parental pattern of ancestry and descent', and the recognition species concept, which defines a species as 'that most inclusive population of individual biparental organisms which share a common fertilization system' (Paterson 1985).

Here, we apply the biological, phylogenetic and recognition species concepts in our assessment of species status. Two of the taxa in our case study come into contact with each other in a narrow overlap zone, while the third is geographically isolated. We use observations of interactions and behavioural experimentation to assess levels of reproductive isolation, and use mitochondrial DNA sequences to determine genetic differences between taxa. Finally we use song spectrogram analysis to examine a major component of the 'fertilization systems.'

We conclude that the species complex in our case study consists of at least two species. In this case, and indeed throughout *Phylloscopus*, song differences are of major importance in recent species designations. Cryptic species appear to be common in this genus, are often relatively old and are recognized under each of the species concepts discussed above.

MATERIALS AND METHODS

Study group

To identify the factors that have led to the high rate of species designation within *Phylloscopus* in the last decade, we reviewed the literature for information on the 11 presently known species that were not listed by Mayr and Cottrell (1986). We also reviewed one species (*P. sindianus*) which was listed by Mayr and Cottrell (1986) but whose taxonomic status has been debated (Martens 1982, Helbig *et al.* 1996).

The three taxa we examine as a case study (*inornatus, humei* and *mandellii*) were treated as conspecific under the name Yellow-browed Warbler *Phylloscopus inornatus* for most of the twentieth century (Ticehurst 1938, Vaurie 1959, Williamson 1967, Dement'ev & Gladkov 1968, Voous 1977, Mayr & Cottrell 1986, Cheng 1987, Cramp 1992, Monroe & Sibley 1993). The taxon *inornatus* breeds throughout most of Siberia south to the Sayan mountains (Baker 1997), where it overlaps with *humei* (Formozov & Marova 1991, Chrabryj *et al.* 1989) which breeds in central Asia from the Sayan mountains south to the northwest Himalayas (Baker 1997; Fig. 1). The third taxon, *mandellii*, breeds in central China and is separated geographically from the other two (Cheng 1987, Baker 1997). There are only minor differences in morphology between these three taxa (Ticehurst 1938, Williamson 1967, Baker 1997). Differences in vocalizations in particular have led some to suggest that there is a species boundary between *inornatus* and *humei* (Svensson 1984, 1987, 1992, Mild 1987, Formozov & Marova 1991, Beaman 1994, Inskipp *et al.* 1996, Baker 1997, BOURC 1997, Sangster *et al.* 1997). Different authors have described *mandellii* as most similar to *inornatus* based on morphology (Ticehurst 1938), most similar to *humei* based on morphology and song (Alström & Olsson 1988), or intermediate between *humei* and *inornatus* based on vocal characteristics (Madge 1985, 1987).

Research sites

We examined several sites in the West Sayan Mountains of southern Siberia at which both *inornatus* and *humei* occur at high density. At Maly Abakan (site 4, see Fig. 1 for locations of sites) the two taxa held territories in a dense forest of young willow *Salix* spp. and birch *Betula* spp. trees in a riverine valley at about 1000 m elevation. At Olenya Rechka (site 6) the two taxa occurred together at treeline in willow and sparse fir *Abies* spp. at about 1500 m elevation. At Adardash (site 5) and Uyukskiy Range (site 7) both taxa were present in forest dominated by larch *Larix* spp. and birch. We collected blood samples, recorded songs and conducted playback experiments on *inornatus* and *humei* at both Maly Abakan (site 4) and Olenya Rechka (site 6). We also collected blood from *humei* at two other sites in the Altai and Sayan Mountains of southern Siberia (Tayozhniy [site 2] and Teletsk Lake [site 3]). In addition, we studied the various taxa at other sites across their ranges. These included Ala Archa (site 10) and Manali (site 11) within the range of *humei*, Magadan (site 8) at the eastern edge of the range of *inornatus*, and Xining (site 12), Jiuzhaigou (site 13) and Panquengou (site 14) within the range of *mandellii*.

Song recordings, spectrogram analysis and playbacks

Recordings were made using a Telinga Pro parabolic microphone and a Sony WM-D6 cassette recorder or a Sony TCD-D3 DAT recorder (in China), or using Audio-Technica 815a shotgun microphones and Sony TCD-D7 DAT recorders (at all other locations). Spectrograms were produced using the program Canary 1.2 (Mitchell *et al.* 1995) on default settings.

Playback experiments took place just after the start of the breeding season. They were conducted by placing a speaker on the ground near a singing individual (usually directly below it), playing a recording (the 'source') and observing the behaviour of the bird (the 'target'). To ensure that playback experiments were independent, we played only one recording to each tar-

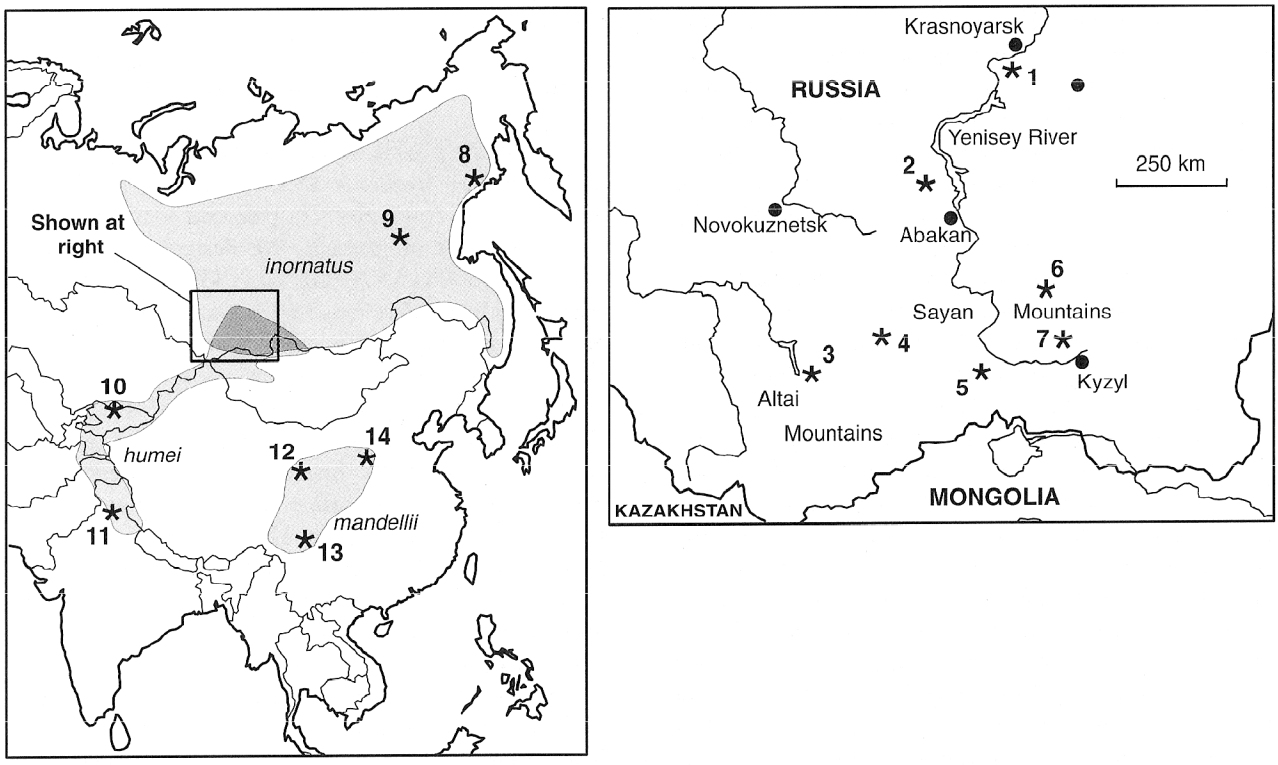

Figure 1. Maps of Asia and the Altai/Sayan Mountains region showing the ranges of *inornatus*, *humei* and *mandellii* and the locations of major study sites. Sites within Russia include: (1) Stolbi National Nature Reserve; (2) Tayozhniy, 60 km NW of Abakan; (3) Teletsk Lake, SE side; (4) Maly Abakan National Nature Reserve; (5) Adardash, 30 km E of Chadan; (6) Olenya Rechka, 40 km SE of Tanzibey; (7) Uyukskiy Range, 40 km NW of Kyzyl; (8) Magadan; and (9) Aldan. Sites in other countries include: (10) Ala Archa National Park, Kyrgyzstan; (11) Manali, Himachal Pradesh, India; (12) Xining, Qinghai province, China; (13) Jiuzhaigou, Gansu province, China; and (14) Panquengou, Shanxi province, China.

get bird before moving on to a new target, except in a few cases in which a second playback was conducted after several minutes of silence. In all playback experiments, responses were graded on a four-point scale (from 0 = no response to 3 = aggressively approaching the speaker).

For each target taxon (i.e. *inornatus*, *humei* and *mandellii*), mean responses were calculated for each source taxon. To estimate the statistical significance of the difference in mean response strength between two source taxa, we compared the observed difference with a distribution of differences from 10000 random permutations of the playback responses.

Genetic analysis

Sequences of mitochondrial DNA (part control region, part tRNA–Phe) were obtained. The mitochondrial control region is useful for estimating relationships within closely related taxa because of its high mutation rate (Baker & Marshall 1997), and has been used previously in phylogeographic studies in birds (Edwards 1993, Wenink *et al.* 1996, Baker & Marshall 1997). Bensch and Härlid (2000) recently reported that the *Phylloscopus* have a derived mitochondrial gene order resulting from a tandem duplication of a region including three genes (tRNA-Pro, NADH6 and tRNA-Glu) and the control region, followed by multiple deletions. The control region sequences obtained in this study are those from the NC (non-coding) region described by Bensch and Härlid (2000).

The following *inornatus*, *humei* and *mandellii* samples were used (see Fig. 1 for locations): three *inornatus* (two from Maly Abakan [site 4], one from Magadan [site 8; provided by T. Price]), eight *humei* (two from Maly Abakan [site 4], two from Olenya Rechka [site 6], one from Tayozhniy [site 2], one from Teletsk Lake [site 3], one from Ala Archa [site 10], and one from Manali [site 11]) and two *mandellii* (from Xining [site 12]). In addition, the following samples of other *Phylloscopus* species were used as outgroups: two *P. proregulus* (from Stolbi [site 1]), one *P. chloronotus* (from Manali [site 11; provided by T. Price]), one *P. trochiloides viridanus* (from Teletsk Lake [site 3]), one *P. trochiloides plumbeitarsus* (from Stolbi [site 1]). The Magadan *inornatus* sample consisted of several feathers; all other samples consisted of a few drops of blood collected in buffer (see Seutin *et al.* 1991).

To extract DNA from the feather sample, the tips of two feather shafts were cut lengthwise, mixed with 250 µl of 5% Chelex 100 (Biorad) and 2 µl of 10 mg/ml Proteinase K, vortexed, and incubated at 55°C overnight. DNA was extracted from the blood samples using the Blood Protocol of the QIAamp Tissue Kit (Qiagen).

We used the primers DLL3 (control region: 5′-TGATGCACTTTGACCCCATTCATGG-3′) and 12SH2 (12S: 5′-AGCAACAACCAACGGTAAG-3′) (Bensch & Härlid 2000) to amplify a DNA fragment approximately 1100 bp in length. The total reaction volume was 100 µl and contained 5 units *Taq* polymerase (Boehringer Mannheim), 1 × PCR buffer, 100 µM each dNTP, and 0.4 µM each primer. Reactions were carried out in an Ericomp Powerblock I System thermal cycler under the following cycling parameters: 1 × 94°C, 3 min; 35 × (94°C, 30 s, 53°C, 1 min, 72°C, 2 min). The PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen) and preliminary sequences from a subset of samples in the study were then obtained manually using the Gibco BRL dsDNA Cycle Sequencing System (Life Technologies) and ^{32}P-dATP (NEN), with each of the above primers. Sequagel polyacrylamide gels were used to separate the products of sequencing reactions and dried gels were exposed to Kodak Biomax MR film for 1–6 days. Sequences were read by eye. Using this method, approximately 400 bp of sequence were obtained from each primer.

Because the 12SH2 end of the fragment aligned well with sequence from the tRNA–Phe gene of chicken mtDNA (see Appendix 1), this end was chosen to sequence in all individuals. We designed the primer DLDI1 (5′-TGAAAAGCTRTCGTTACAAAA-3′) that could be used to sequence toward the 12SH2 end of the fragment from about 300–400 bp from that end. Sequences ranging 256–352 bp in length (variable because of insertions and deletions) were obtained for all samples in both directions. Final sequences were obtained using both manual cycle sequencing as described above (from primer DLDI1) and automated sequencing (from primer 12SH2) on an Applied Biosystems 373 Automated DNA Sequencer.

The possible amplification of nuclear sequences of mitochondrial origin (numts) is a potential problem when using PCR to amplify mitochondrial sequences (Sorenson & Quinn 1998). Numts are unlikely to be a problem in this study for several reasons. First, the region that corresponds to the tRNA–Phe gene is highly conserved compared with the control region part of the sequence (see Appendix 1). This is expected if the sequences are evolving in the mitochondria but would be unlikely if they are evolving in the nucleus. Secondly, sequences obtained from the feather sample

(the Magadan *inornatus*) were very similar to sequences obtained from blood samples of the same species. Feather samples are less problematic than blood samples with respect to nuclear contamination (Sorenson & Quinn 1998). Thirdly, the control region of two of the species studied here (*P. trochiloides* and *P. proregulus*) was also sequenced by Bensch and Härlid (2000), who obtained similar sequences. Bensch and Härlid found no evidence for numts of the control region from six *Phylloscopus* species.

Sequences were aligned using the program CLUSTAL W (Thompson *et al.* 1994) with reduced gap penalties (gap open penalty = 5.0, gap extension penalty = 2.5) because insertions and deletions are common in the control region. The aligned sequences revealed the presence of several large gaps at the 5′ end of the sequences, raising concern that the absence in some taxa of a large block of characters in a highly variable part of the sequence might cause a lack of rate constancy in the phylogenetic tree-building algorithms. Because our goal was in part to determine the relative timing of branch points in the reconstructed tree, the end containing these large gaps was excluded from the phylogenetic analysis presented in this paper (however, including this end resulted in similar estimated relationships). This reduced the length of the sequences used in the analysis to 197–219 bp (shown in Appendix 1). The aligned sequences were used to construct phylogenetic trees using parsimony (PAUP 3.1, Swofford 1991), neighbour-joining (PHYLIP 3.5c, Felsenstein 1993), and maximum likelihood (PHYLIP 3.5c). To test for a molecular clock, the two PHYLIP programs DNAMLK, which assumes a molecular clock when determining branch lengths, and DNAML, which does not, were used to evaluate the likelihood of trees of the topology produced in DNAML (Felsenstein 1993).

While many phylogenetic studies have used mtDNA restriction site variation or sequences of mtDNA genes such as cytochrome b (Moore & DeFilippis 1997), relatively few have used the control region, especially the end next to tRNA–Phe (Baker & Marshall 1997). The control region is known to be one of the most rapidly evolving parts of the mtDNA molecule (Baker & Marshall 1997). The rate of divergence throughout the rest of the mtDNA molecule in general, and the cytochrome b gene in particular, is thought to be about 2% per million years in a diverse array of avian taxa (Moore & DeFilippis 1997, Klicka & Zink 1997). Hence, to calculate the approximate rate of divergence in the sequences of DNA (part control region, part tRNA–Phe) used in this paper, genetic distances based on those sequences were compared with genetic distances based on cytochrome b sequences, which have been determined for three of the taxa (*humei, chloronotus* and *trochiloides*) by Richman and Price (1992).

RESULTS

Review of new species of *Phylloscopus*

In Table 1 we summarize information about 12 *Phylloscopus* species which have recently been elevated from the rank of subspecies (nine) or newly described (three; references in Table 1). While many of the nine taxa that were elevated were originally described as species in the nineteenth century, for most of the twentieth century they were treated as subspecies of polytypic species because of similarities in morphology. Most of them have again become well accepted as species only in the last ten to 15 years.

Of the 12 recently designated species, nine have songs that are audibly distinct from the songs of their likely sister taxa, and for these, song was an important factor in their designation as species. For the other three species, there may also be song differences that are distinguished by the birds. For *P. sindianus* and its close relative *P. collybita*, songs were described as almost indistinguishable acoustically, but thorough analyses of spectrograms revealed some differences (Martens 1982, Helbig *et al.* 1996). Such was also the case with *P. bonelli* and its sister species *P. orientalis* (Helb *et al.* 1982, Helbig *et al.* 1995). Songs were described as similar between *P. hainanus* and its likely sister taxon *P. davisoni*, although no detailed comparison was made (Olsson *et al.* 1993).

Song playbacks were conducted between ten of the newly designated species and their sister taxa, and in eight of these there was little or no recognition between taxa. In the two other cases, results were more complex. In the case of *P. sindianus*, Martens and Hänel (1981) and Martens (1982) reported some response between it and its close relative *P. collybita*. In the case of *P. brehmii*, males often react aggressively to songs of *P. collybita*, while males of *P. collybita* respond little to song of *P. brehmii*; in contrast, females of *P. brehmii* pay little attention to song of *P. collybita*, while females of *P. collybita* are often attracted by song of *P. brehmii* (Thielcke *et al.* 1978, Salomon 1989).

Morphological differences are in general slight and of little importance in the species designations, except in one case. For *P. hainanus*, morphology was of primary importance in its species designation (Olsson *et al.* 1993).

Studies of DNA sequence variation have been pub-

Table 1. The importance of various factors in the recent designations of 12 species of *Phylloscopus*. For each newly designated species/sister group pair, we show the distinctness of songs, the results of playback experiments between taxa, the similarity of morphology, the estimated divergence time between mitochondrial haplotypes and whether there is an area of contact between the taxa during the breeding season. Regarding song: 'similar' is defined as difficult to distinguish audibly (though differences may be evident in spectrograms); 'rather distinct' means basically similar but easily distinguishable audibly; 'highly distinct' means bearing little or no resemblance. Regarding morphology, 'very similar' is defined as identifiable with difficulty in the hand; 'similar' means distinguishable with difficulty in the field; 'distinct' means easily identified.

Species	Likely sister group	Former designation	Song	Play-backs	Morphology	Genetic distance and divergence time (approximate)	Area of contact	References
P. emeiensis	*P. reguloides* group	None	Highly distinct	No response	Similar	Not done	Yes	Alström & Olsson 1995
P. hainanus	*P. davisoni*	None	Similar	Not done	Distinct	Not done	No	Olsson *et al.* 1993
P. chloronotus	*P. proregulus/ P. kansuensis*	*P. proregulus chloronotus*	Highly distinct	No response	Similar	9.9% control region[2],1.2 MYA[3]	No	Alström & Olsson 1990, Alström *et al.* 1997, present study
P. sichuanensis	*P. proregulus* group	None	Highly distinct	No response	Similar	Not done	Yes	Alström *et al.* 1990, Alström *et al.* 1992
P. kansuensis	*P. proregulus / P. chloronotus*	*P. proregulus kansuensis, P. p. proregulus* (junior synonym) or *P. p. chloronotus* (junior synonym)	Highly distinct	No response	Very similar	Not done	Yes	Alström *et al.* 1997
P. brehmii	*P. collybita, P. canariensis and P. sindianus*	*P. collybita brehmii* or *P. c. collybita* (junior synonym)	Rather distinct	Some response	Very similar	4.3–5.4 % cytochrome b, 2.1–2.7 MYA[1]	Yes	Thielcke & Linsenmair 1963, Thielcke *et al.* 1978, Salomon 1987, 1989, Salomon & Hemim 1992, Helbig *et al.* 1993, 1996, Salomon *et al.* 1997, Clement & Helbig 1998
P. canariensis	*P. collybita / P. sindianus*	*P. collybita canariensis*	Rather distinct	No response	Very similar	3.4–4.1% cytochrome b, 1.7–2.1 MYA[1]	No	Ticehurst 1938, Williamson 1967, Helbig *et al.* 1996, Clement & Helbig 1998
P. sindianus	*P. collybita / P. canariensis*	*P. collybita sindianus*	Similar	Some response	Very similar	3.4–4.6 % cytochrome b, 1.7–2.3 MYA[1]	Yes	Ticehurst 1938, Williamson 1967, Cramp 1992, Martens & Hänel 1981, Martens 1982, Helbig *et al.* 1996, Clement & Helbig 1998
P. subaffinis	*P. affinis*	*P. affinis subaffinis*	Rather distinct	No response	Similar	Not done	Yes	Alström & Olsson 1992, 1994, Alström *et al.* 1993
P. orientalis	*P. bonelli*	*P. bonelli orientalis*	Similar	No response	Very similar	8.3–8.6% cytochrome b, 4.1–4.3 MYA[1]	No	Helb *et al.* 1982, Cramp 1992, Svensson 1992, Helbig *et al.* 1995, Page & Lewington 1999
P. borealoides	*P. tenellipes*	*P. tenellipes borealoides* or *P. tenellipes* (junior synonym)	Highly distinct	Not done	Very similar	Not done	No	Martens 1988, Veprintsev *et al.* 1990
P. humei	*P. inornatus*	*P. inornatus humei and P. i. mandellii*	Highly distinct	No response	Similar or very similar	15.1–19.2% control region[2], 2.4 MYA[3]	Yes	Svensson 1984, 1992, Mild 1987, Alström & Olsson 1988, Cramp 1992, Alström *et al.* 1991, Formozov & Marova 1991, present study

[1]Based on a divergence rate of 2% per million years applied to cytochrome b distances (Helbig *et al.* 1995, Helbig *et al.* 1996). [2]See Appendix 2. [3]Based on Figure 4.

lished for six of the species in Table 1. In each case, these studies showed pronounced genetic divergence between each species and its sister taxon (3.4–8.6% in cytochrome b, 9.9–19.2% in the control region), and in two taxa (*P. brehmii* and *P. canariensis*) the sample sizes were sufficiently large to indicate an absence of gene flow. The estimated divergence times vary from 1.2 to 4.3 million years ago (MYA).

Six of the species in Table 1 occur sympatrically with their likely sister taxon or with another close relative over at least part of their range, enabling an assessment of their species status under the biological species concept. For all of these, research conducted in the sympatric area was important in their taxonomic ranking. For the other newly designated species, which are all allopatric with respect to their sister taxa, subjective decisions had to be made as to whether the taxa differed enough morphologically, genetically, or behaviourally to be designated as separate species.

Yellow-browed Warbler complex

Song and call structure

The songs of *inornatus* and *humei* differ markedly from each other, while the songs of *humei* and *mandellii* are similar (Fig. 2). There is some individual variation in all three taxa (shown for *humei* in Martens 1980), but there is little geographical variation within each taxon over great distances (Fig. 2). The song of *inornatus* usually consists of three high-pitched, drawn-out elements separated by split-second pauses (Fig. 2a, 2b). Unlike *inornatus*, individual *humei* have two song types. Type 1 (Fig. 2c–e) consists of a double whistled note (sometimes given singly), while type 2 (Fig. 2f, 2g) is a drawn-out descending buzz. The taxon *mandellii* also has two song types (Fig. 2h–k), and these are similar to the respective types of *humei*, though the *mandellii* type 1 (Fig. 2h–j) is higher-pitched and covers a broader frequency range than that of *humei*. As in the case of song, call notes differ audibly between the three taxa and show little variation between sites within each taxon (Fig. 3).

Playbacks and interaction between the taxa

Playback results are summarized in Table 2. Within the three *inornatus* populations, playbacks of *humei* and *mandellii* never elicited a response, whereas playbacks of *inornatus* always brought on strong or medium responses (comparison of *inornatus* vs. *humei*, $P < 0.0001$; *inornatus* vs. *mandellii*, $P < 0.05$). Within the four *humei* populations, playbacks of *inornatus* never elicited a response, whereas playbacks of *humei* always resulted in strong or medium responses (*humei* vs. *inornatus*, $P < 0.0001$) and playbacks of *mandellii* brought on strong, medium or weak responses (*humei* vs. *mandellii*, $P < 0.05$). Within each of these taxa, there was little difference between locations in the response that playbacks elicited (Table 2). In the *mandellii* population, playback of *inornatus* never elicited a response, while playbacks of *mandellii* always brought on strong responses (*mandellii* vs. *inornatus*, $P < 0.001$).

Observations at Maly Abakan (site 4, Fig. 1) and Olenya Rechka (site 6), where *humei* and *inornatus* occur sympatrically, revealed that there is little interaction between them. They have overlapping territories and do not react to an individual of the other form that is singing near them. At a given location of the speaker, we were able to attract individuals of each taxon using playbacks of their respective songs, and individuals never responded to the song of the other taxon.

Phylogenetic analysis

Sequences range from 197–219 bp in size and include some of the control region (Baker & Marshall 1997, Bensch & Härlid 2000), as well as 49 bp of the adjacent tRNA–Phe gene, which is highly conserved and aligns well with the corresponding chicken tRNA–Phe (positions 1228–1279 of chicken mtDNA, Desjardins & Morais 1990, see Appendix 1). Excluding gaps, there are a total of 91 variable sites within the entire data set, 35 of which are variable within the complex being considered here (i.e. *inornatus*, *humei* and *mandellii*). A total of 11 haplotypes were found among the 18 samples that were sequenced (see Appendix 1), and only these 11 unique haplotypes were used in the tree-building algorithms. All six *humei* samples from the Sayan and Altai Mountains of southern Siberia had the identical haplotype, both *mandellii* samples from China had the same haplotype and the two *proregulus* samples had identical haplotypes.

Parsimony, neighbour-joining and likelihood all produced phylogenetic trees with identical topologies. In a comparison of likelihood trees assuming (DNAMLK), and not assuming (DNAML), a molecular clock, the assumption of rate constancy was not rejected ($\chi^2_9 = 12.48$, ns, Felsenstein 1993). The rate-constant maximum likelihood (DNAMLK) tree is shown in Figure 4, along with bootstrap support for both the parsimony and the neighbour-joining algorithms.

The tree based on cytochrome b sequences (Richman & Price 1992) is consistent with the tree based on control region/tRNA sequences (Fig. 4) and the relative placement of the two nodes that connect

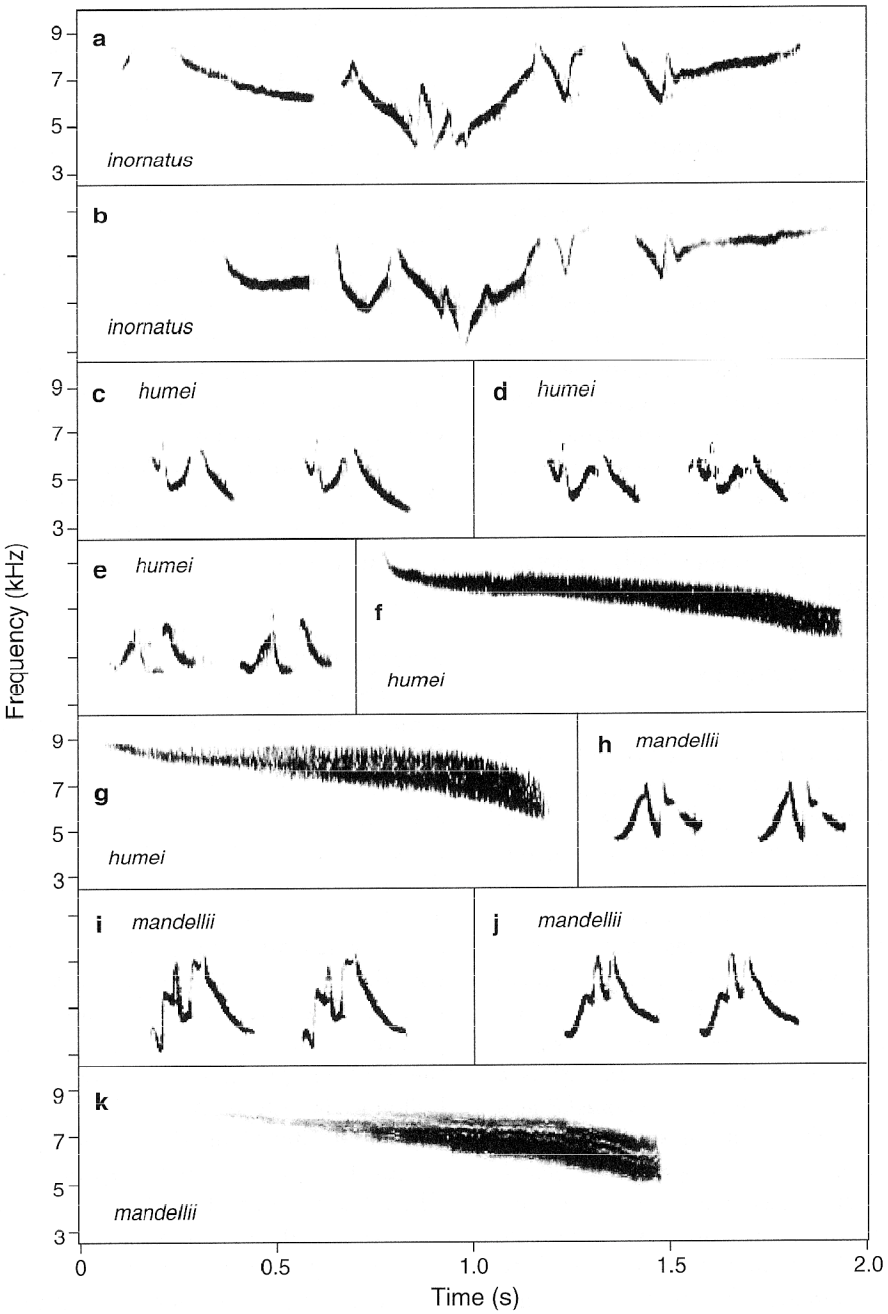

Figure 2. Spectrograms of songs from various populations of Yellow-browed Warblers. Taxa recorded and locations are: (a) *inornatus* at Maly Abakan (site 4, Fig. 1); (b) *inornatus* at Aldan (site 9); (c–e) *humei* type 1 songs from (c) Maly Abakan (site 4), (d) Ala Archa (site 10) and (e) Manali (site 11); (f–g) *humei* type 2 songs from (f) Maly Abakan (site 4) and (g) Manali (site 11); (h–j) *mandellii* type 1 songs from (h) Jiuzhaigou (site 13), (i) Panquengou (site 14) and (j) Xining (site 12); and (k) *mandellii* type 2 song from Panquengou (site 14). Songs were recorded by D.I. (a, d and f), B. Veprintsev (b), Z.B. (c and g), T. Price (e), and P.A. (h–k).

the three common taxa differs by only 3.6% between the two trees. The distances in pairwise comparisons between the three taxa is higher for the control region than for cytochrome b (2.52 ± 0.11 sd times higher for the entire sequence used here; excluding the tRNA region, 3.10 ± 0.17 sd times higher). If cytochrome b is assumed to diverge at 2% per million years (Moore & DeFilippis 1997, Klicka & Zink 1997), the control region diverges at about 6% per million years and the divergence rate for the sequences used in this paper (part control region, part tRNA–Phe) is about 5% per million years.

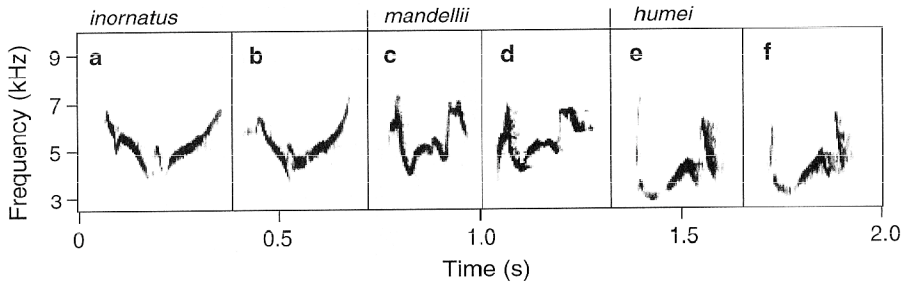

Figure 3. Spectrograms of calls from various populations of Yellow-browed Warblers. Taxa recorded and locations are: (a) *inornatus* at Maly Abakan (site 4, Fig. 1); (b) *inornatus* at Aldan (site 9); (c) *mandellii* from Jiuzhaigou (site 13); (d) *mandellii* from Xining (site 12); (e) *humei* from Maly Abakan (site 4); and (f) *humei* from Manali (site 1). Calls were recorded by Z.B. (a and f), B. Veprintsev (b), P.A. (c and d) and D.I. (e).

The phylogenetic analysis shows that the haplotypes of *humei*, *inornatus*, and *mandellii* each form monophyletic groups. Within each group, there is relatively little variation, even between haplotypes obtained on opposite sides of the range of a taxon (also see Appendix 2). Given the rate of 5% divergence per million years, the constant-rate likelihood tree (Fig. 4) indicates that the various haplotypes within each of the *humei* and *inornatus* groups split from common ancestors about 0.23 MYA, that *humei* and *mandellii* had a common ancestor about 1.0 MYA, and that the split between the *inornatus* and *humei/mandellii* groups occurred about 2.4 MYA.

Based on song variation, response to playbacks, behavioural interactions and genetic variation this study strongly supports the recent division of the three taxa into Hume's Leaf Warbler *Phylloscopus humei* (including *P. h. mandellii*) and Yellow-browed Warbler *Phylloscopus inornatus* (Svensson 1984, 1987, 1992, Mild 1987, Formozov & Marova 1991, Beaman 1994, Inskipp *et al.* 1996, Baker 1997, BOURC 1997, King 1997, Sangster *et al.* 1997).

DISCUSSION

Taxonomic classifications that rely on morphology or appearance alone will often, as in the case of the *Phylloscopus* in general and the *P. inornatus* and *P. humei* complex in particular, lead to mistakes in the estimation of species number. This is not a trivial problem, as cryptic species can be relatively old, and ignorance of them can affect research projects in many ways. In taxa as diverse as bats (Jones & Van Parijs 1993, Jones 1997), corals (Knowlton *et al.* 1992), frogs (Heyer *et al.* 1996), pseudoscorpions (Zeh & Zeh 1994), skinks (Bruna *et al.* 1996) and wasps (Bower & Brown 1997), similar morphology has concealed species that have now been shown to be well differentiated behaviourally and/or genetically. In birds, recently invented techniques such as playback experi-

Table 2. Response strength of eight populations ('targets') to playbacks of recordings from five populations ('sources'). Each trial was graded on a four-point scale (from 0 = no response to 3 = strong response). Average scores ± standard errors are shown, along with sample sizes in parentheses. See Figure 1 for locations of sites.

	Sources				
Target	*inornatus* Maly Abakan	*inornatus* Magadan	*humei* Maly Abakan	*humei* Ala Archa	*mandellii* Jiuzhaigou
inornatus					
Maly Abakan	3.0 ± 0.0 (6)		0.0 ± 0.0 (6)		
Olenya Rechka	2.7 ± 0.3 (3)		0.0 (1)		0.0 ± 0.0 (2)
Magadan		3.0 ± 0.0 (3)		0.0 ± 0.0 (3)	
humei					
Maly Abakan	0.0 ± 0.0 (6)		3.0 ± 0.0 (6)		
Olenya Rechka	0.0 ± 0.0 (4)		3.0 ± 0.0 (2)		2.0 (1)
Ala Archa	0.0 ± 0.0 (3)		3.0 ± 0.0 (3)		2.0 ± 0.6 (3)
Manali	0.0 ± 0.0 (5)		2.7 ± 0.2 (6)		
mandellii					
Jiuzhaigou		0.0 ± 0.0 (7)			3.0 ± 0.0 (7)

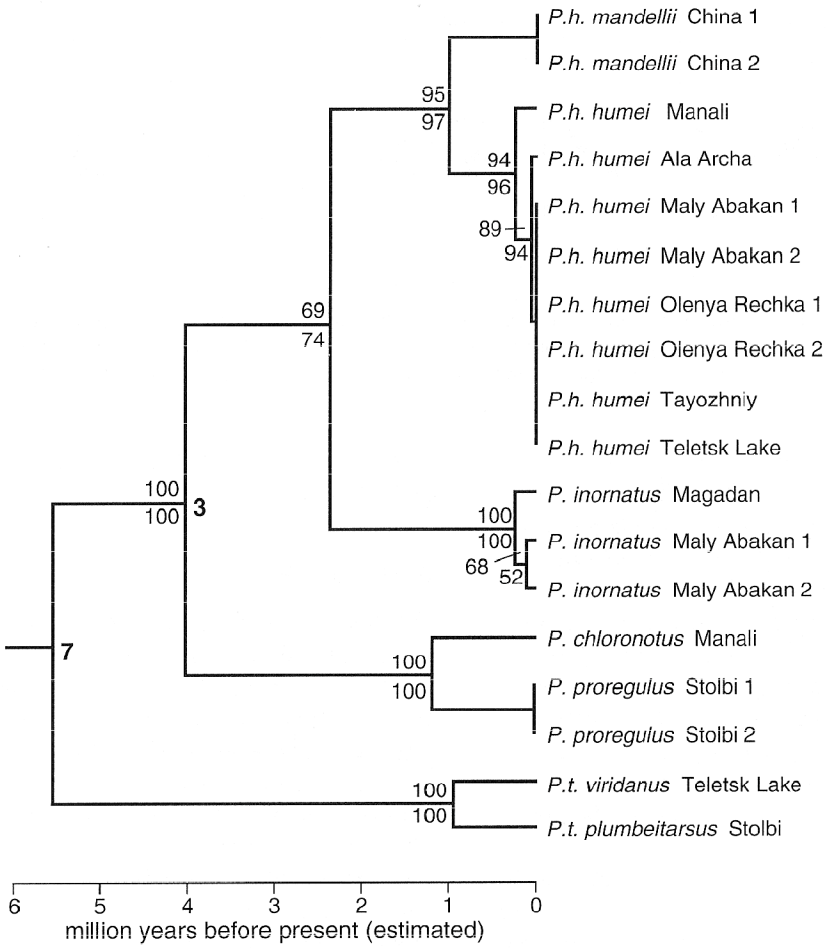

Figure 4. Phylogenetic tree based on mitochondrial control region sequences. Abbreviations are as follows: '*P.*' is *Phylloscopus*, '*h.*' is *humei*, and '*t.*' is *trochiloides*. The tree was produced using maximum likelihood by the DNAMLK program of PHYLIP (Felsenstein 1993), in which branch lengths are proportional to time. The identical tree topology was produced using parsimony (PAUP; Swofford 1991) and neighbour-joining (NEIGHBOR in PHYLIP). Numbers above the nodes are parsimony bootstrap percentages based on 1000 random-addition heuristic searches, and numbers below the nodes are bootstrap percentages based on 500 neighbour-joining replicates. The estimated timeline was obtained by applying a divergence rate of 5% per million years (see Methods). The numbers '7' and '3' designate nodes which are represented in the cytochrome b tree of Richman and Price (1992).

ments, spectrogram analysis and genetic analysis can supplement the well-used and important technique of simple field observation in the task of uncovering cases of cryptic species.

The *P. inornatus* complex provides good examples of the roles that sympatry and allopatry play in the assessment of species status. Previous authors have suggested that *inornatus* and *humei/mandellii* are separate species based on vocalization differences (Svensson 1984, 1987, 1992, Beaman 1994, Baker 1997, BOURC 1997) or playback experiments in allopatry in combination with vocalization differences (Mild 1987). This was questioned largely because *mandellii* was thought to be somewhat intermediate between *inornatus* and *humei* vocally (Madge 1985), or because these three taxa were considered entirely allopatric and only very few playback tests had been conducted (Alström & Olsson 1988). It was possible that there were clines in song structure between the song of *humei* in the western Himalayas and the song of *inornatus* in eastern Siberia by way of intermediate song types in geographically intermediate locations, or that these intermediate locations contained populations of mixed singers, in which individuals could sing both *humei* and *inornatus* types of song. It was also possible that playback experiments in allopatry were not a good indicator of the playback response of birds in sympatry (Irwin & Price 1999). Studies in sympatry have been

crucial in resolving these issues. Formozov and Marova (1991) studied *inornatus* and *humei* in sympatry in the Tuva region of southern Russia, and concluded that they are separate species because they found no birds with intermediate vocalizations, nor any individuals with both types of vocalization. Their results are corroborated by this study, which provides proof that *inornatus* and *humei* behave as separate species at two sites of sympatry.

Difficulties of assigning species status to allopatric taxa are exemplified by a consideration of *mandellii*. Given the present evidence, it should be treated as conspecific with *humei* because of their obvious similarities in song, the fairly strong response of *humei* to playbacks of *mandellii* song, its lack of playback response to *inornatus* and its closer genetic relatedness to *humei* compared with *inornatus*. But its disjunct geographic distribution (with a separation of about 1500 km from *humei*), its deep genetic split with *humei* compared with the genetic diversity within the *humei* group and the *mandellii* group, and its distinct call note, indicate that it may be well on its way towards species status. The lack of any sympatric area between *humei* and *mandellii* precludes any direct observation of their interactions during the breeding season, making it difficult to apply the biological species concept. We could argue that the similar songs and mutual responses to playbacks indicate that *humei* and *mandellii* would interbreed if they came into contact, making them a single species. Other species concepts are more clearly applied to allopatric taxa but in this case result in conflicting conclusions. The phylogenetic species concept implies that *humei* and *mandellii* are separate species because they are diagnosably different (by call notes as well as by mtDNA). Applying the recognition concept leads to the conclusion that *humei* and *mandellii* are the same species because of their similar songs and mutual response to playbacks. Uncertainty of the taxonomic rank of these taxa is a necessary consequence of the fact that they are in an early stage of allopatric divergence and the speciation process takes time for completion (Avise & Walker 1998).

A surprising result that arose from the DNA sequencing is the old age of the branch point between *inornatus* and *humei/mandellii*, about 2.4 MYA or 42% as far back in time as what is probably the oldest branch point in the entire Eurasian *Phylloscopus* (estimated at 5.6 MYA, node 7, Fig. 4 and Richman & Price 1992, Fig. 1). It is about twice as old as both the branch point between *P. chloronotus* and *P. proregulus* (estimated at 1.2 MYA, Fig. 4), which are now considered separate species (Alström & Olsson 1990, Monroe & Sibley 1993, Beaman 1994, Inskipp *et al.* 1996, King 1997), and the branch point between *P. trochiloides viridanus* and *P. t. plumbeitarsus* (estimated at 0.9 MYA, Fig. 4), which are often treated as different species (Williamson 1967, Mayr & Cottrell 1986, King 1997). The extreme similarity in morphology of *inornatus, humei* and *mandellii* has concealed the existence of two relatively old separate species. All of the other newly designated species which have been studied genetically (n = 5) are old, between 1.2–4.3 million years (Table 1). Although it has been suggested that in one case at least the divergence time may be overestimated (Helbig *et al.* 1995), the data suggest that very little morphological differentiation has taken place in a considerable time span, while vocalizations have generally diverged much more.

We make several generalizations regarding recent species designations in *Phylloscopus* (see Table 1).

1. All species except one (*P. hainanus*) are morphologically similar to their nearest relatives, and several (especially *P. kansuensis* and *P. borealoides*) cannot always be safely identified by morphological characters alone.
2. Compared with the songs of their closest relatives, songs of all but three species (*P. hainanus, P. sindianus* and *P. orientalis*) are easily distinguishable to the human ear. For two of these (*P. sindianus* and *P. orientalis*), playback experiments have shown that the birds respond less to heterospecific song than to conspecific song. The same holds true for the other species on which playbacks have been carried out (all except *P. hainanus* and *P. borealoides*).
3. Every species that has been analysed genetically differs markedly from its nearest relatives. All of the estimated divergence times are at the upper end or well beyond typical divergence times for subspecies of birds (Helbig *et al.* 1995, Avise & Walker 1998). In no case have results from genetic studies been the sole reason for the elevation of a taxon from subspecies to species; rather, molecular research has provided additional evidence for the distinctness of the taxon under study.
4. Seven of the species come into contact with close relatives during the breeding season in at least a narrow overlap zone. In every one of these cases, research in the overlap zone played an important role in the taxonomic ranking.

These generalizations indicate that song divergence

is often a major factor in species-level divergence in *Phylloscopus* (see also Irwin 2000), and that two taxa that have diverged significantly in song will not recognize each other's songs and will have a deep genetic separation. This suggests that two allopatric taxa that differ significantly in song, such as *P. borealoides* and *P. tenellipes* (Table 1), are best treated as distinct species even though playbacks and genetic analyses have not been performed.

The rate at which new species have been discovered in various taxonomic groups has varied tremendously through time and has slowed over the last century in well-studied taxa such as birds (May 1990). What then accounts for the recent burst of species designations within *Phylloscopus*? First, the relevance of song and other behaviours as a complement to traditional morphological studies has been more widely acknowledged among taxonomists in recent years (Monroe & Sibley 1993). Secondly, the increasing availability of quality lightweight recording and playback equipment, and software to produce and analyse spectrograms, has increased our ability to conduct playback experiments and examine song variation. Thirdly, political changes in countries such as Russia and China have facilitated internal and international travel, thus fostering research on species that span international borders.

As our investigation of the genus *Phylloscopus* makes clear, many morphologically similar species would be clearly recognized under any species concept if enough data were gathered. Of the 12 recently designated species (Table 1), 11 are morphologically similar to their sister taxa, but all of them are clearly separate species under all of the major species concepts, given recently obtained spectrogram, playback and genetic data. Much of the debate over species concepts (Cracraft 1989, Zink & McKitrick 1995, Avise & Wollenberg 1997) arises out of situations such as *humei* and *mandellii*, where the speciation process is ongoing. It is ironic that the biological species concept is only directly applicable to sympatric taxa (Mayr 1963), because speciation usually begins when a single species is divided into two allopatric groups. Thus, for two reasons species status is often more difficult to assess for taxa that occur only in allopatry than for those that occur in sympatry. First, it is unclear how to apply the biological species concept to allopatric taxa. Secondly, a pair of diverging taxa usually pass through an allopatric phase before they become sympatric, and hence allopatric taxa are often at an earlier stage of divergence when species status is not clear. In *Phylloscopus*, there are many taxa that can be considered to be at the subspecies/species boundary (such as *humei* and *mandellii*). The presence of such taxa, as well as others that have clearly reached species status (for example those in Table 1), should be assessed in studies of species diversity, especially over large spatial scales.

We are grateful to Joshua Kohn for generously providing laboratory space and equipment for DNA sequencing, and thank Staffan Bensch, Nicholas Mundy, Kenneth Petren, Ray Radtkey and Adam Richman for technical advice. Staffan Bensch provided primers for the DNA sequencing. Assistance in Russia was provided by Jessica Irwin, Gennady Kelberg and the Stolbi National Nature Reserve, and in Kyrgyzstan by Sasha Vorobyev and Ala Archa National Park. Karen Marchetti and Trevor Price provided assistance in India. For financial assistance, we thank the American Ornithologists' Union, The Explorer's Club Youth Activity Fund and Exploration Fund, Helge Axson Johnsons Stiftelse, the Jeanne Messier Memorial Fund, Kungliga & Hvitfeldtska Stipendieinrättningen, the Lucille P. Markey Charitable Trust, Olle & Signhild Engkvists Stiftelser, Sigma Xi, the US National Institutes of Health (CMG training grant T32GM-07240) and the US National Science Foundation (grant INT-9724817). We thank Marc Dantzker, Jessica Irwin, Karen Marchetti, David Parkin, Trevor Price, Kaustuv Roy, Christoffer Schander, Andrew Suarez and three anonymous reviewers for helpful comments and discussion. We are grateful for the advice of Trevor Price throughout this project.

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Received 11 August 1999; revision accepted 18 April 2000

APPENDIX 1

Aligned mtDNA haplotype sequences used in the phylogenetic analyses. Dots indicate identity with the first sequence, and dashes indicate gaps. Part of the chicken tRNA–Phe gene is shown at the bottom (corresponding to positions 1228–1279, Desjardins & Morais 1990). The total length of each sequence (excluding gaps) is shown in brackets at the end of each sequence.

```
P. h. humei Manali          CTTTTTATCTTGACATTTCAT-CCTC--CACACACAACCAATA-TCCC--CCTACCCATT-T------CCCCATCAATAA
P. h. humei Ala Archa       ....................-....--..................-...T--..........-......-..........
P. h. humei Maly Abakan     ....................-....--..................-...T--..........-......-..........
P. h. mandellii             ..............C..T..T..C.--.C..................-....--T..G.....-......-.....C.....
P. inornatus Maly Abakan 1  ..................T.C-.....--AC.......T..GC.C.T.T--.......CC.A.------.T...C...C..
P. inornatus Maly Abakan 2  ..................T.C-.....--AC.......T...C.C.T..--.......CC.A.------.T...C...C..
P. inornatus Magadan        ..................T.C-..C.--AC.......T..GC.C.T..--.......CC.A.------.T...C...C..
P. chloronotus              .C................T..-T.CG--TT...........C.-CT.T--......T.C.T.------A.TTCA.C.C..
P. proregulus               .C................T..-T.CG--.T...........C.-CT..--......T.C.T.------..T.CA.C.C..
P. t. viridanus             T....A...G...TT...TCAC..C.CCACA..TA..TG....A....AA...-.T..CACCCAATCA.T.ACC...C..
P. t. plumbeitarsus         T....A...G...TT...TCAT..A.C-ACA..TA..TG....A....AG...-....CACCCAATCA.T.ACA...C..

P. h. humei Manali          CCCAC--CTAATCTAACCCAAATCTCC-TCCCACTAAAAAA-CAAACAAAAATACAATCCATT-CACCACAACCAAAAAC
P. h. humei Ala Archa       .....--..............C..-...........-...................-.........T......
P. h. humei Maly Abakan     .....--..............C..-...........-...................-.....................
P. h. mandellii             .T...--.A.....G..........-.A.........-...................-.....................-.
P. inornatus Maly Abakan 1  .A...--.A..CA..G..........-.A...A.....-.........C........CC-....----------.
P. inornatus Maly Abakan 2  .A...--.A..CA..G..........-.A...A.....-.........C........CC-....----------.
P. inornatus Magadan        .A...--.A..CA..G..........-.A...A.....-.........C........CC-....----------.
P. chloronotus              .....--.A....G.....C.C..C.....C.....-.........C..G.A...CC-...AC.C--------A
P. proregulus               ..A..--.A..C...T.....C.C..C...........-.........C....A....C-.G.AC.CC-------A
P. t. viridanus             .A...GT.A....C.....G..CTC..C.A..C.C.....G.........C....CA....A..TA.TC.------A
P. t. plumbeitarsus         .A..TGT.A....C.....G..CTC..C.A..C.C.....G.........C.....A.G.CG...A.TC.------A

                                                  ----------tRNA-Phe----------
P. h. humei Manali          AACCAAAAC--CACCTTTGTCCCCGTAGCTTA---CACAAAGCATGACACTGAAGATGTCAAGACGGCCG  [207]
P. h. humei Ala Archa       ..........-.........T.........---.....................................  [207]
P. h. humei Maly Abakan     ..........-.........T.........---.....................................  [207]
P. h. mandellii             ..........-.........-.........---.....................................  [207]
P. inornatus Maly Abakan 1  ..........---.A.CC.....T.........---.....................................T..  [197]
P. inornatus Maly Abakan 2  ..........---.A.CC.....T.........---.....................................T..  [197]
P. inornatus Magadan        ..........---.A.C......T.........---.....................................T..  [197]
P. chloronotus              .G........--TGA.C......T.........---T.....................................T...T.  [201]
P. proregulus               ..........---.A.C......TT........---T.....................................T...T.  [202]
P. t. viridanus             .C.......TA-...CAC.....T.........---A.....................................T...T.  [219]
P. t. plumbeitarsus         .C.......CA-....AC.....T.........---A.....................................T...T.  [218]
chicken tRNA-Phe                                .C....A.......ACC..........G..........C.....T..TAC
```

APPENDIX 2

Pairwise numbers of transition/transversion substitutions (above the diagonal) and uncorrected sequence divergences (below the diagonal) for partial mtDNA control region haplotypes from *Phylloscopus* warblers (see Appendix 1). The tRNA–Phe region was excluded from this analysis.

	1	2	3	4	5	6	7	8	9	10	11
1. *P. h. humei* Manali	–	3/0	2/0	8/3	18/9	16/9	17/9	25/14	18/15	23/25	20/27
2. *P. h. humei* Ala Archa	0.019	–	1/0	11/3	18/9	18/9	19/9	23/14	18/15	23/25	20/27
3. *P. h. humei* Maly Abakan	0.013	0.006	–	10/3	18/9	18/9	19/9	23/14	18/15	23/25	20/27
4. *P. h. mandellii*	0.070	0.089	0.083	–	19/6	17/6	16/6	29/13	21/14	24/22	21/24
5. *P. inornatus* Maly Abakan 1	0.185	0.185	0.185	0.171	–	2/0	3/0	21/16	17/16	22/24	20/26
6. *P. inornatus* Maly Abakan 2	0.171	0.185	0.185	0.158	0.135	–	3/0	21/16	15/16	20/24	18/26
7. *P. inornatus* Magadan	0.178	0.192	0.192	0.151	0.020	0.020	–	20/16	14/16	20/24	19/26
8. *P. chloronotus*	0.260	0.247	0.247	0.280	0.252	0.252	0.245	–	11/4	25/27	25/27
9. *P. proregulus*	0.219	0.219	0.219	0.232	0.224	0.211	0.204	0.099	–	25/28	25/28
10. *P. t. viridanus*	0.318	0.318	0.318	0.303	0.313	0.299	0.299	0.344	0.349	–	11/2
11. *P. t. plumbeitarsus*	0.311	0.311	0.311	0.303	0.313	0.299	0.306	0.344	0.349	0.077	–