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A molecular phylogeny of the *Sylvia cantillans* complex: Cryptic species within the Mediterranean basin

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ABSTRACT

The subalpine warbler *Sylvia cantillans* is formally considered a polytypic species, with four subspecies, European *S. c. cantillans*, *albistriata*, *moltonii* (recently resumed name: *subalpina*) and North African *S. c. inornata*. They are very similar in external morphology but clearly differ in their vocalizations. We evaluated their uncertain taxonomic status reconstructing the phylogenetic and phylogeographic relationships among populations sampled across major biogeographical areas in the European species' range, using nucleotide sequences of the mitochondrial cytochrome *b* gene (mtDNA cyt *b*). A variety of phylogenetic analyses concordantly led to identify four major groups, only partially corresponding to the three European nominal subspecies. Phylogenetic trees showed a monophyletic group including all *moltonii* individuals, well diverged from all other taxa. Populations taxonomically assigned to *cantillans* were polyphyletic being split into two distinct clades (western and southern *cantillans*), with monophyletic *albistriata* closely related to southern *cantillans*. Individuals of *moltonii* and southern *cantillans* sampled in sites of sympatry in central Italy were assigned to their respective groups, with perfect concordance between phenotypic and genetic identifications. All findings indicate that *moltonii* should be ranked as a distinct species. Former subspecies *cantillans* is polyphyletic, but additional data are needed to define the taxonomic status of its two clades. *Albistriata* is phylogenetically related to southern *cantillans* and should be provisionally kept as a subspecies of *S. cantillans*. The *cantillans* complex thus provides an interesting case-study illustrating geographical structuring across small geographical ranges, and it exemplifies speciation through differentiation in allopatry leading to reproductive isolation after a secondary contact.

1. Introduction

Morphological similarities between closely related avian species often generated taxonomical errors or uncertainties (see e.g. Thomassen et al., 2003, and references therein). The conservative evolution of external morphological traits in some genera and families within the Passeriformes, which have many sibling species showing only slight morphological differences, may pose problems in defining species limits and in the taxonomical assignment of individual specimens (Martens et al., 2004). Careful re-evaluation of ecological or ethological distinctions, and the use of modern molecular systematic approaches, have led to deep taxonomic revisions in a variety of passerine groups in the Palaearctic (Irwin et al., 2001a; Martens et al., 2002, 2004; Päckert et al., 2003; Salzburger et al., 2002). Accordingly, a number of subspecies have been upgraded to full biological species (see e.g. Martens et al., 2004; Olsson et al., 2005). In particular, recent revisions have deeply questioned and dramatically modified currently accepted taxonomic arrangements of species (often belonging to species complexes) in several genera of the superfamily Sylvioidea (Alström et al., 2006; Martens et al., 2004; Olsson et al., 2005), such as *Phylloscopus*, *Hippolais* (see AERC TAC, 2003) and *Sylvia* (Shirihai et al., 2001).

The taxonomy of the Sylvioidea is particularly puzzling, and has repeatedly been revised in the last decade (see AERC TAC, 2003; Shirihai et al., 2001). Several species are poorly differentiated in external morphology, and yet appear to be subdivided into distinct and mainly, but not exclusively, allopatric population systems (such as e.g., *Sylvia curruca* or *Phylloscopus proregulus*; Martens et al., 2004; Shirihai et al., 2001), which differ in their ecology or for a number of acoustic or genetic characters (Alström and Ranft, 2003; Olsson et al., 2005; Päckert et al., 2004). Vocalizations, or other behavioural traits, may act as effective isolation mechanisms,

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resulting in incompatible mating systems, which could prevent interspecific hybridisation and introgression in nature (Irwin et al., 2001b). Consequently, such differentiated populations have been recently designed as distinct species (Irwin et al., 2001b; Martens et al., 2004).

The subalpine warbler *Sylvia cantillans* has been considered as a polytypic species, including four subspecies widely distributed around the Mediterranean basin: (i) the nominate *S. c. cantillans*, distributed in Spain, France and Italy; (ii) the eastern *S. c. albistriata*, distributed in north-eastern Italy, Balkans, Turkey; (iii) the north-African *S. c. inornata*; and (iv) the insular subspecies *S. c. moltonii* (Corsica, Sardinia and Balearics), which was only recently re-proposed as distinct taxon (Gargallo, 1994; Shirihai et al., 2001), after being included within the nominate/*S. c. inornata* group (Vaurie, 1954; see also Cramp, 1992). *S. c. moltonii* should be named *S. c. subalpina* according to Baccetti et al. (2007). However, we used its former name in this paper, for consistency with all recent publications. *S. c. moltonii* occurs also in few regions in mainland Italy (Brambilla et al., 2006, 2007; see also Fig. 1 for subspecies distributions, and Baccetti et al., 2007, for historical mainland records). These four subspecies show only slight differences in their external morphology, mainly in a few male plumage traits, while females are nearly indistinguishable in the field and often also in the hand (Shirihai et al., 2001). *S. c. albistriata* individuals are usually bigger than the other subspecies. However, all subspecies but *inornata*, which has vocalizations very similar to *cantillans*, clearly differ by their contact/alarm calls and, partially, also by their songs (Brambilla and Guidali, 2005; Cramp, 1992; Shirihai et al., 2001). According to their external morphology (Cramp, 1992; Shirihai et al., 2001) and vocalizations (Brambilla and Guidali, 2005) *S. c. albistriata* and *moltonii* seem to be the most diverging subspecies and were described as potentially distinct phylogenetic species, separated from *cantillans* (Shirihai et al., 2001).

The polytypic subalpine warbler species group thus represents an interesting case-study to investigate the relationship between phylogeographic structuring and phylogenetic divergence. In fact, the two taxa *moltonii* and *cantillans* are syntopic in mainland Italy where they breed at the same sites, apparently without interbreeding (Brambilla et al., 2006). This is a first indication that pre-mating barriers might have already developed and acting in sympatric condition, and that the two forms are candidate distinct species, as suggested also by different song perception (Brambilla et al., 2008). Molecular data could reveal whether the two taxa are represented by mutually exclusive lineages in the contact zones, and hence if reproductive isolation is indicated also by genetic data (Helbig et al., 2002). On the other side, *cantillans* and *albistriata* co-occur in the northern Adriatic (Croatia; Brambilla, 2007) where they hybridise, as shown by the occurrence of *cantillans* mtDNA haplotypes in phenotypically *albistriata*-like individuals (Brambilla, 2007).

Aim of this study is to develop a mtDNA phylogeography of subalpine warbler populations, infer their phylogeny and, where necessary, re-define the taxonomic status of former subspecies.

2. Materials and methods

2.1. Sampling design and collection

Subalpine warblers were sampled during the 2005 and 2006 breeding seasons (May–July) aiming to collect individuals representing the different geographical groups throughout the range of the complex (see Fig. 1 for location and Table 1 for name of sampling sites), that is: western (France and Spain) and southern *cantillans* (central and southern Italy, including Sicily), insular (Corsica, Balearics) and mainland (central and northern Italy) *moltonii*, western (Croatia: Dalmatia) and eastern (Greece: Lesvos) *albistriata*. The putative contact zone between southern *cantillans* and *moltonii* (which includes known sites of sympatry and covers a large sector of the main *moltonii* range; Brambilla et al., 2006), was intensively sampled, in order to investigate phenotype/genotype segregation in contact sites and other areas of close proximity (see Fig. 1). Eight breeding individuals (four *moltonii* and four southern *cantillans*) were sampled in sympatric sites in June 2006. The colour of male underparts and contact calls were used to identify the (sub)species (see Brambilla et al., 2006; Shirihai et al., 2001). We could not obtain samples from the North African populations of the ssp. *inornata*. According to literature, this subspecies seems poorly differentiated from the nominate subspecies and a broad transi-

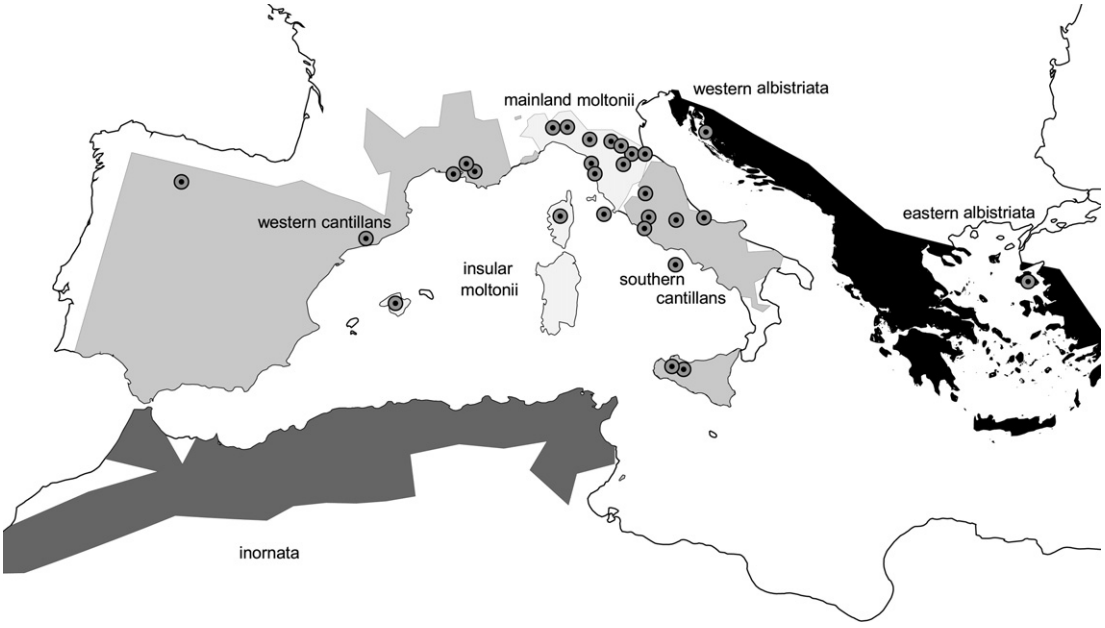

Fig. 1. Sampling points (grey dots with black centre) in the areal of the *S. cantillans* complex (re-drawn from Shirihai et al. (2001) and Brambilla et al. (2006); pale grey: *moltonii*; intermediate grey: *cantillans*; dark grey: *inornata*; black: *albistriata*).

tional zone between the western populations of *cantillans* and *inornata* is known to occur in southern Iberia (Cramp, 1992; Shirihai et al., 2001). Therefore, we should have obtained adequate and representative samples from all the main taxa of the *S. cantillans* complex (see Fig. 1).

2.2. Laboratory procedures

Birds were trapped with mist-nets and feathers where collected and stored at –20 °C in test tubes containing 95% ethanol. Total DNA was extracted using a guanidinium thiocyanate and diatomaceus earth protocol (Gerloff et al., 1995; Randi et al., 2003). Two pairs of primers were specifically designed to amplify the cytochrome *b* gene in *S. cantillans* (GenBank accession no. AJ534543 Boehning-Gaese et al., 2003) using Primer3 v.0.3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). They split the cytochrome *b* gene (1143 bp) into two sub-units: the first half was amplified with primers 4L (5′GCTCCCAATCTAC GCAAAAA3′) and 662H (5′AATGGGATTTTGTCGCAGTC3′), and for the second half we used primers 538L (5′TTCGCCCTTCACTTCCTC3′) and 1137H (5′TTTGAGTATTTTGTTTTCTAGGATGG3′). Amplifications were carried out in 10 μl reactions using 4 μl of template DNA solution, buffer 1.5 mM $MgCl_2$, 1 μg BSA, 0.2 mM of each primer, 0.04 mM of each dNTP, 0.25 U of *Taq* polymerase (Eppendorf AG, Hamburg, Deutschland) and the following thermal profile: 94 °C for 2 min; 45 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min. Excess of primers were digested enzymatically (ExoSAP–IT, USB Corporation, Cleveland, OH, USA) and

Table 1
Geographical distribution and relative occurrence of 50 haplotypes detected in a contiguous fragment of 1090 bp of the cytochrome *b* gene from 137 subalpine warblers

Haplotype	*n*	Localities
*albistriata*2	2	Lesvos (2)
*albistriata*3	1	Lesvos (1)
*albistriata*4	2	Lesvos (2)
*albistriata*5	3	Lesvos (3)
*albistriata*6	1	Lesvos (1)
*albistriata*1	1	Dalmatia (1)
*moltonii*11	1	Corsica (1)
*moltonii*19	1	Balearics (1)
*moltonii*1	1	Toscana (Faeto, 1)
*moltonii*2	1	Toscana (Faeto, 1)
*moltonii*3	15	Emilia-Romagna (Settefonti, 1), Lombardia (Lumello, 1), Toscana (Migliarino, 2), Emilia-Romagna (Brisighella, 4), Emilia-Romagna (Casola Valsenio, 2), Piemonte (Villalvernia, 1), Emilia-Romagna (Taro, 1), Emilia-Romagna (Dovadola, 2), Toscana (San Rossore, 1)
*moltonii*4	15	Emilia-Romagna (Settefonti, 2), Emilia-Romagna (Casola Valsenio, 3), Emilia-Romagna (Taro, 5), Emilia-Romagna (Dovadola, 2), Emilia-Romagna (Brisighella, 1), Toscana (San Rossore, 1), Toscana (Giglio Island, 1)
*moltonii*5	2	Lombardia (Lumello, 1), Emilia-Romagna (Casola Valsenio, 1)
*moltonii*6	7	Toscana (San Rossore, 6), Emilia-Romagna (Taro, 1)
*moltonii*7	2	Toscana (San Rossore, 2)
*moltonii*8	3	Toscana (San Rossore, 1), Emilia-Romagna (Casola Valsenio, 2)
*moltonii*9	1	Emilia-Romagna (Brisighella, 1)
*moltonii*10	1	Emilia-Romagna (Casola Valsenio, 1)
*moltonii*12	1	Emilia-Romagna (Taro, 1)
*moltonii*13	1	Emilia-Romagna (Taro, 1)
*moltonii*14	5	Emilia-Romagna (Dovadola, 1), Emilia-Romagna (Brisighella, 1), Toscana (San Rossore, 3)
*moltonii*15	1	Emilia-Romagna (Brisighella, 1)
*moltonii*16	2	Toscana (San Rossore, 2)
*moltonii*17	1	Toscana (San Rossore, 1)
*moltonii*18	1	Toscana (San Rossore, 1)
*cantillans*1	4	Lazio (Veio, 1), Umbria (Todi, 1), Abruzzo (Pescara, 1), Emilia-Romagna (Casola Valsenio, 1)
*cantillans*2	2	Lazio (Veio, 1), Umbria (Todi, 1)
*cantillans*3	5	Lazio (Castel di Guido, 1), Abruzzo (Pescara, 1), Abruzzo (L'Aquila, 1), Emilia-Romagna (Dovadola, 1), Sicilia (Vicari, 1)
*cantillans*4	2	Lazio (Castel di Guido, 1), Umbria (Todi, 1)
*cantillans*5	9	Lazio (Castel di Guido, 2), Umbria (Todi, 4), Lazio (Ventotene Island, 1), Lombardia (Lumello, 1), Toscana (Migliarino, 1)
*cantillans*7	1	Lazio (Ventotene Island, 1)
*cantillans*8	2	Lazio (Ventotene Island, 1), Sicilia (Ficuzza, 1)
*cantillans*9	1	Toscana (Migliarino, 1)
*cantillans*10	1	Toscana (Migliarino, 1)
*cantillans*19	1	Sicilia (Vicari, 1)
*cantillans*20	1	Sicilia (Vicari, 1)
*cantillans*21	1	Sicilia (Vicari, 1)
*cantillans*22	1	Sicilia (Vicari, 1)
*cantillans*23	1	Sicilia (Vicari, 1)
*cantillans*24	1	Sicilia (Vicari, 1)
*cantillans*25	1	Sicilia (Ficuzza, 1)
*cantillans*6	22	Leon (3), Provence (Villars, 1), Cataluña (Vacarisses, 8), Provence (Aix-en-Provence, 10)
*cantillans*11	1	Provence (Villars, 1)
*cantillans*12	1	Provence (Villars, 1)
*cantillans*13	1	Cataluña (Vacarisses, 1)
*cantillans*14	1	Cataluña (Vacarisses, 1)
*cantillans*15	1	Cataluña (Vacarisses, 1)
*cantillans*16	1	Provence (Aix-en-Provence, 1)
*cantillans*17	2	Provence (Aix-en-Provence, 2)
*cantillans*18	1	Provence (Aix-en-Provence, 1)

Within parentheses are given the following information: specific sampling locality (only for region including more than a single locality), and number of individuals showing that haplotype at that given locality. See Fig. 1 for geographical location of sampling sites and populations.

both strands of the cytochrome *b* were cycle-sequenced using BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA, USA). Sequencing analysis were performed using an ABI 3130xl Genetic Analyser automated sequencer and the software SeqScape v2.5 (Applied Biosystems). Finally, a sequence of ~500–600 bp was obtained for both fragments of the gene. We concatenated these two halves for all the samples for which they were both available, obtaining a fragment of 1090 bp. For some samples we could not amplify both fragments due to DNA scarcity (see Section 3). We aligned the sequences using Clustal W (Thompson et al., 1994). Sequences have been deposited in GenBank under Accession Nos. EU760644-EU760694.

2.3. Genetics

Unique haplotypes were identified using Collapse 1.2 (D. Posada) without considering missing data as differences. Estimators of molecular diversity, i.e. haplotype number (*h*), haplotype diversity (hd), average number of nucleotide differences (*k*) and nucleotide diversity (*p*), were estimated as implemented in the program DnaSP 4.1 (Rozas et al., 2003). Tajima's D (Tajima, 1989) and Fu's Fs (Fu 1997) statistics were estimated for the main haplotype groups (Table 2) using DnaSP 4.1 (see also Mäkinen and Merilä, 2008). These statistics compare the observed nucleotide diversity and number of haplotypes with their expectations in a population at mutation-drift equilibrium. Negative values indicate an excess of low frequency alleles, which is typical for expanding populations and as well as for loci under directional selection. A haplotype network was constructed based on the 95% parsimony criteria using the TCS 1.2 software (Clement et al., 2000) to illustrate the mutational connections among haplotypes of the three major clades (*albistriata* and southern *cantillans*, western *cantillans*, *moltonii*; see Section 3).

The proper selection of an outgroup is a critical step in reconstructing phylogenetic trees (Swofford et al., 1996). The outgroup should be selected among the most closely related taxa of the ingroup (Klicka et al., 2005; Smith, 1994; Wheeler, 1990). Therefore, we selected other *Sylvia* species, namely the blackcap *S. atricapilla*, belonging to the same genus of our study species (group) but not to the same Mediterranean clade, and *S. mystacea* and *S. melanocephala*, the species most closely related to the *S. cantillans* group (Blondel et al., 1996; Shirihai et al., 2001).

Gene trees were obtained according to five different approaches. MEGA 3.1 (Kumar et al., 2004) was used to build a gene tree with the Neighbour-Joining (NJ) method; a Maximum Parsimony (MP) tree was obtained in PAUP* 4.08b (Swofford, 2001), according to the following procedure: heuristic search strategy, with exclusion of all uninformative nucleotide positions, unordered and equally weighted characters, starting trees obtained via random stepwise addition, random haplotype additions with 10 replicates, multiple minimal trees swapped by TBR (tree–bisection–reconnection) branch swapping, collapsed zero length branches; MulTrees option in effect; Steepest descent option not in effect, gaps treated as missing data, 1000 replicates for assessing bootstrap values (7.04×10^6 rearrangements tried). A Maximum Likelihood (ML) tree was obtained in PHYLIP 3.67 (J. Felsenstein, University of Washington) and bootstrap assessed on 100 replicates.

Molecular phylogenies were estimated also by Bayesian inference using MrBayes 3.1.2. Posterior probabilities were calculated in MrBayes for cytochrome *b* under a Hasegawa–Kishino–Yano with gamma-shaped rate variation across sites (HKY + G) model (as in the Bayesian analysis the Markov chain is integrating over the uncertainty in parameter values, we did not use the parameter values estimated by the commands in MrModeltest, but only specified the general "form" of the model; Nylander, 2004). The choice of this model ($K = 5$, base frequencies: $A = 0.2606$, $C = 0.3597$, $G = 0.1374$, $T = 0.2422$; Substitution model: ti/tv ratio = 8.1612; among-site rate variation: proportion of invariable sites = 0; Gamma distribution shape parameter = 0.0159) was based on a hierarchical likelihood ratio test (Posada and Crandall, 1998), calculated in MrModeltest 2.2 (Nylander, 2004). Four metropolis-coupled MCMC chains were run for 10^6 generations and sampled every 100 generations. The temperature was set to 0.1 to improve the mixing of the chains, given that it was found to be poor at the default temperature 0.2 (cf. Olsson et al., 2005). Other settings were kept as default values. The first 370,000 generations before the chain reached apparent stationarity (burn-in), were discarded and the posterior probability estimated for the remaining 630,000 generations. Stationarity was confirmed by potential scale reduction factors (all the parameters equal to 1.000, except for kappa = 1.003) and by the plot looking like 'white noise', with no tendency to increase or decrease over time. The use of Bayesian posterior probabilities to evaluate strength of nodes has recently come under criticism (e.g., Douady et al., 2003; Erixon et al., 2003; Suzuki et al., 2002). Posterior probabilities are usually higher than corresponding non-parametric bootstrap frequencies and are typically considered too "liberal", while the latter are viewed as conservative (Klicka et al., 2005). In our data set, we noticed little discrepancy among these methods, and support values for nodes are rather similar (see also comparison between the two values in Klicka et al., 2005).

Finally, a further Maximum Parsimony (MP) tree was obtained in PAUP* 4.08b (Swofford, 2001), according to the HKI + G model with the following settings: base frequencies: $A = 0.2606$, $C = 0.3597$, $G = 0.1374$, $T = 0.2422$; ti/tv ratio = 8.1612; proportion of invariable sites = 0; Gamma distribution shape parameter = 0.0159. In all cases, trees were collapsed to obtain strict and 50% majority rule consensus trees.

Genetic distances (within and among groups, plus standard error calculated on 1000 bootstrap replicates; Table 3) were estimated in MEGA 3.1, according to the Tamura–Nei correction (Kumar et al., 2004; see also García et al., 2008), to allow comparison with other values among *Sylvia* species as calculated by

Table 2
Molecular diversity indices, and Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) statistics

Lineage (*n*)	π	Hd	*k*	*D*	Fs
Western *cantillans* (31)	0.00065	0.501	0.701	−2.12**	−7.25***
Italian *cantillans* (34)	0.00197	0.679	2.121	−1.84**	−10.35****
moltonii (62)	0.00242	0.869	2.606	−1.55	−8. 68****
albistriata (10)	0.00307	0.889	3.333	−0.25	−0.56

* 0.1 < P < 0.05.
** 0.05 < P < 0.01.
*** 0.01 < P < 0.001.
**** P < 0.001.

Table 3
Genetic distance (Tamura-Nei correction) within and among different groups for 1090 bp of the cytochrome *b* (*n* = 50 haplotypes). Standard error is calculated on bootstrap methods with 1000 replicates

	Genetic distance within groups	Genetic distance among groups		
Group		Southern *cantillans*	*albistriata*	*moltonii*
Western *cantillans*	0.002 ± 0.001	0.037 ± 0.006	0.0035 ± 0.006	0.043 ± 0.006
Southern *cantillans*	0.003 ± 0.001		0.017 ± 0.003	0.050 ± 0.007
albistriata	0.003 ± 0.001			0.044 ± 0.006
moltonii	0.005 ± 0.001			

Shirihai et al. (2001). Note that results remained practically unchanged using TN93 whether HKYG as molecular evolutionary models, for both building the trees and calculating distances (details not shown), so we presented distances calculated only under the simplest (TN93) model.

3. Results

3.1. Cytochrome b sequence description and variability

We got feather samples from approximately 200 breeding individuals. For 181 individuals we got a fragment of 576 bp corresponding to the first half of the cytochrome *b* gene; for a subsample of 137 samples (31 western Europe *cantillans*, 10 *albistriata*, 34 southern *cantillans*, 62 *moltonii*) we obtained also the second half of the gene (which proved to be more difficult to obtain) and thus a total fragment of 1090 contiguous base pair of the gene. In the alignment of the 181 shorter (576 bp) fragments we detected 41 different haplotypes; in the alignment of the longer (1090 bp) fragment, we detected 50 different haplotypes defined by 108 variable sites, 84 of those were parsimony-informative. We decided to use the longer alignment, which is more representative of the overall variation. The 50 different haplotypes were partitioned as follows: 16 were unique in southern *cantillans*, nine in western *cantillans*, six in *albistriata* and 19 in *moltonii*.

A potential problem with phylogenetic analyses based on mtDNA data is the occurrence and amplification of mitochondrial DNA homologues from the nuclear genome (numts) (e.g. Kidd and Friesen, 1998; Sorenson and Fleischer, 1996). All the cytochrome *b* sequences we obtained could be functionally translated without premature terminations. Furthermore, amplification of nuclear sequences from feather samples is unlikely, given that mitochondrial DNA is much more abundant than nuclear DNA. Therefore, there was no evidence suggesting that the presence of numts may bias our analyses on mtDNA.

3.2. Phylogenetic analyses of the cytochrome b alignment

Haplotypes are subdivided into four major groups, which differ from each other by a minimum of 13 fixed differences. Therefore, network analyses was carried out separately for (i) western *cantillans*, (ii) southern *cantillans* and *albistriata*, and (iii) *moltonii*,

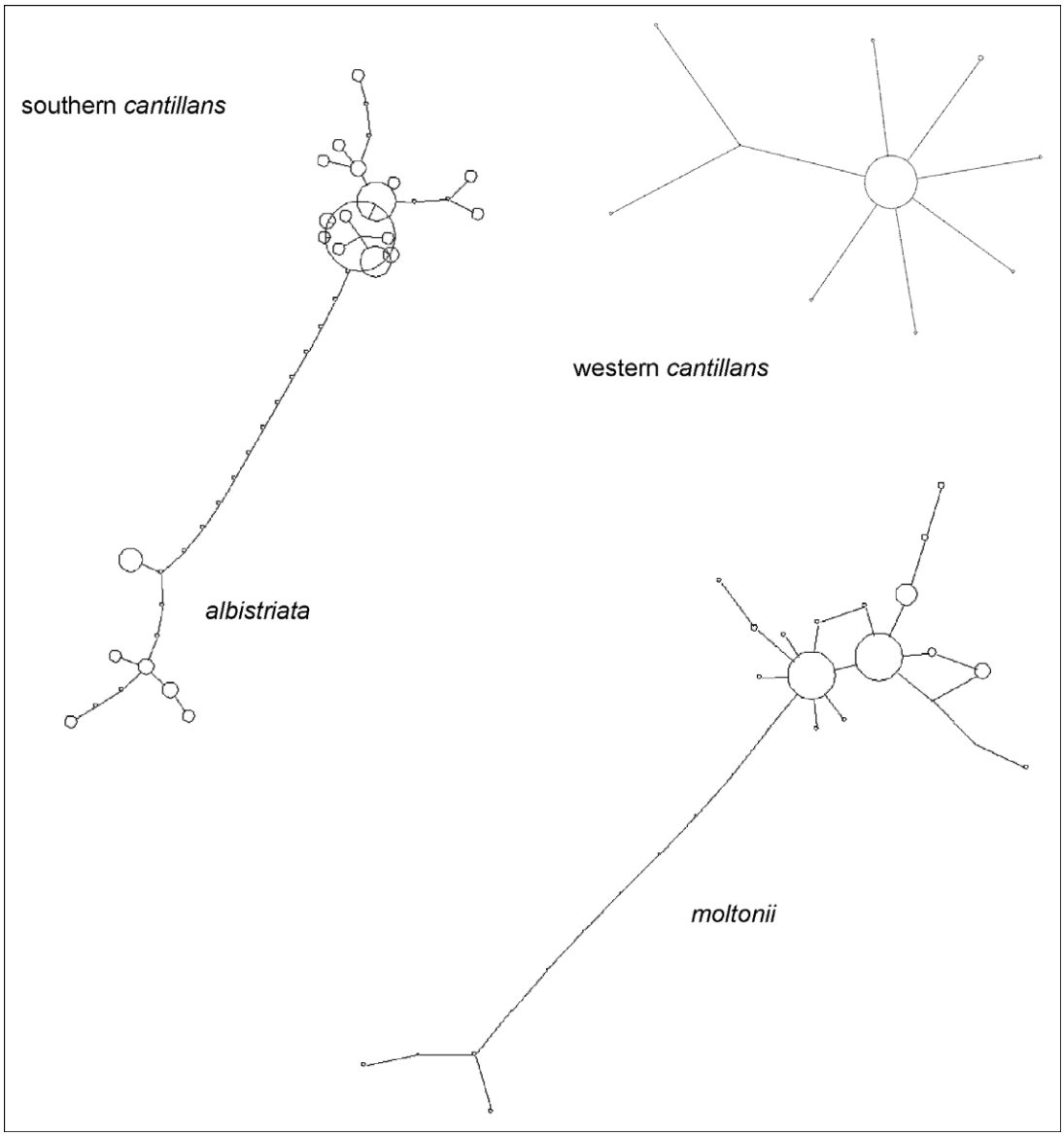

Fig. 2. Haplotype network based on the 95% parsimony criteria illustrating the mutational connections and the frequency of different haplotypes among the three major clades.

respectively, because the large divergence found among these three groups prevented a unique treatment. The divergence between southern *cantillans* and *albistriata* is relatively low and allowed simultaneous inclusion in the network analysis. The main mitochondrial lineages were mainly characterised by star-like patterns clustered around the most common haplotypes and perfectly matched with phylogenetic reconstructions (Fig. 2).

The two trees obtained according to the MP method provided with nearly identical results, identifying exactly the same clades and sub-clades, with bootstrap values differing no more than 2%. Overall, the phylogenetic relationships identified by all trees obtained with different methods (see Figs. 3–6) showed the occurrence of three well-distinct clades: southern *cantillans* and *albistriata*, which appeared as sister taxa, genetically diverged from western *cantillans* and *moltonii*. The first two clades were grouped in some of the trees, but almost invariably with very low statistical support: this node received poor bootstrap support in the two MP (49% and 51%) and in the NJ (68%) trees, and also a poor posterior probability (<0.5) in the Bayesian estimate; only the ML tree provided strong support for grouping the two clades (Fig. 5). Within the clade including all *moltonii* haplotypes, a clear subdivision was found between a larger group of mainland haplotypes and a smaller group including two insular (Corsican and Balearic) and one mainland (Tuscan) haplotypes.

All the eight individuals of *moltonii* and (southern) *cantillans* sampled in sites of sympatry (Lumello, Lombardia, and Migliarino, Toscana) were assigned to their respective groups with a perfect concordance between phenotypic and genetic identification. Moreover, two juveniles showing (southern) *cantillans* haplotypes were sampled in two different sites in Emilia–Romagna (Casola Valsenio and Dovadola, respectively), within the *moltoni*'s breeding range. Unfortunately, they were not phenotypically diagnosable (no call heard). They were trapped in July and August 2006, respectively, in sites where there were not *cantillans*-like breeders and where no *cantillans* haplotypes had been sampled before these two juveniles in that year. Therefore, we supposed their occurrence was due to juvenile dispersion from the neighbouring breeding grounds of *cantillans* or from sympatric sites, both only few tens of kilometres away from the sampling sites. It should be noted that both *cantillans* and *moltonii* perform post-breeding summer dispersion (pers. obs.).

The genetic distance within groups was extremely lower than the distance among groups (Table 3), thus further confirming the validity of the clades found in the phylogenetic analyses and the importance of genetic divergence among them.

Genetic distance between groups showed a large divergence between *moltonii* and all other groups ($\geqslant$0.043 for all possible combinations; see Table 3). A rather large distance was found also

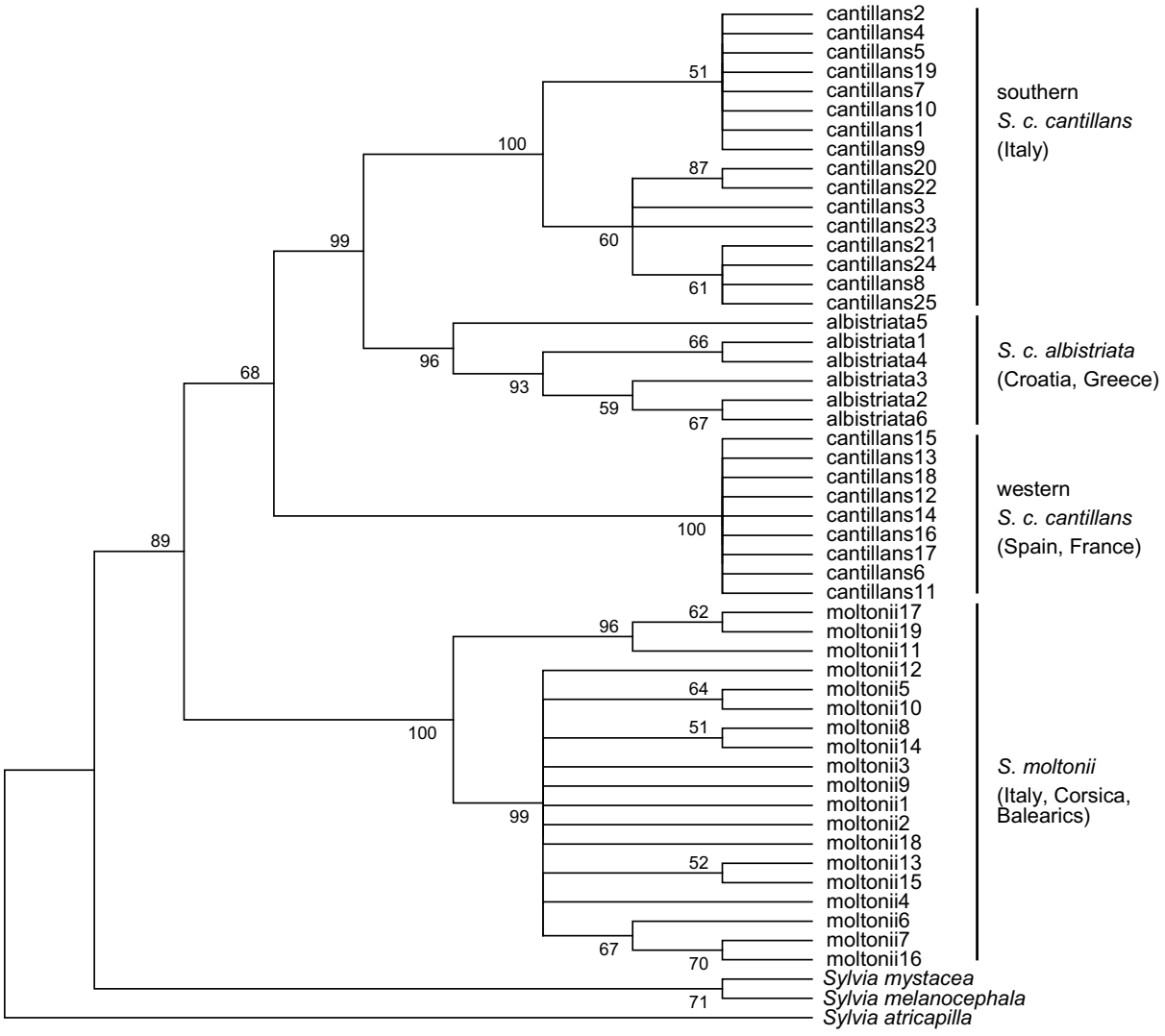

Fig. 3. NJ tree based on the number of differences for 50 haplotypes (50% majority rule); bootstrap values (>50%; 1000 replicates) are indicated.

between western *cantillans* and all the other groups (⩾0.035) while, on the other side, the distance between southern *cantillans* and *albistriata* was relatively low (0.017). Therefore, the phylogenetic structuring suggested by all the gene trees (Figs. 3–6) was confirmed by values of genetic distance.

Tajima's D and Fu's Fs showed non-significant values for *albistriata*, suggesting equilibrium, while the negative values obtained for the other three groups (with the only exception of Tajima's D for *moltonii*) suggested non neutrality or a demographic explanation such as population expansion.

4. Discussion

The *S. cantillans* complex showed a strong phylogeographic structure within a relatively small area, entirely comprised within the Mediterranean region. Such a strong structuring among conspecific avian populations is usually found over much larger areas (e.g. Martens et al., 2004; Olsson et al., 2005). The phylogeographic analysis of the *cantillans* complex in Europe revealed three main mitochondrial lineages (*moltonii*; western *cantillans*; Italian *cantillans* and *albistriata*), which have diverged from each other presumably before and at beginning of the Pleistocene, ca. between 2.5 and 1.7 Mya (calculated by applying a rate of 2% sequence divergence per million years, as estimated for many passerine birds; Qu et al., 2006, and references therein).

The existence of these lineages indicates that taxa of the subalpine warbler complex were able to survive the unfavourable climatic conditions of the Pleistocene in different refugia (likely Iberian for western *cantillans*, Tyrrhenian for *moltonii* and Balkans and/or southern Italy for Italian *cantillans* and *albistriata*). More recent isolation during the Pleistocene lead to further divergence between Italian *cantillans* and *albistriata* and, even more recently, between the two *moltonii* lineages.

Mismatch distributions (Fig. 7) and Tajima's D and Fu's Fs tests provided evidence for population expansion events for *moltonii* and the two *cantillans* clades, but they suggested more constant long-term effective population size for *albistriata*.

The large genetic distance found between *moltonii* and all other clades (⩾0.043) suggests a level of divergence definitely higher than what is expected for 'subspecies' of the same species. Similarly, also western *cantillans* appear to be strongly diverged (⩾0.035) from all other groups and all phylogenetic trees except for the ML one identified it as an independent lineage. The genetic divergence observed among the three main lin-

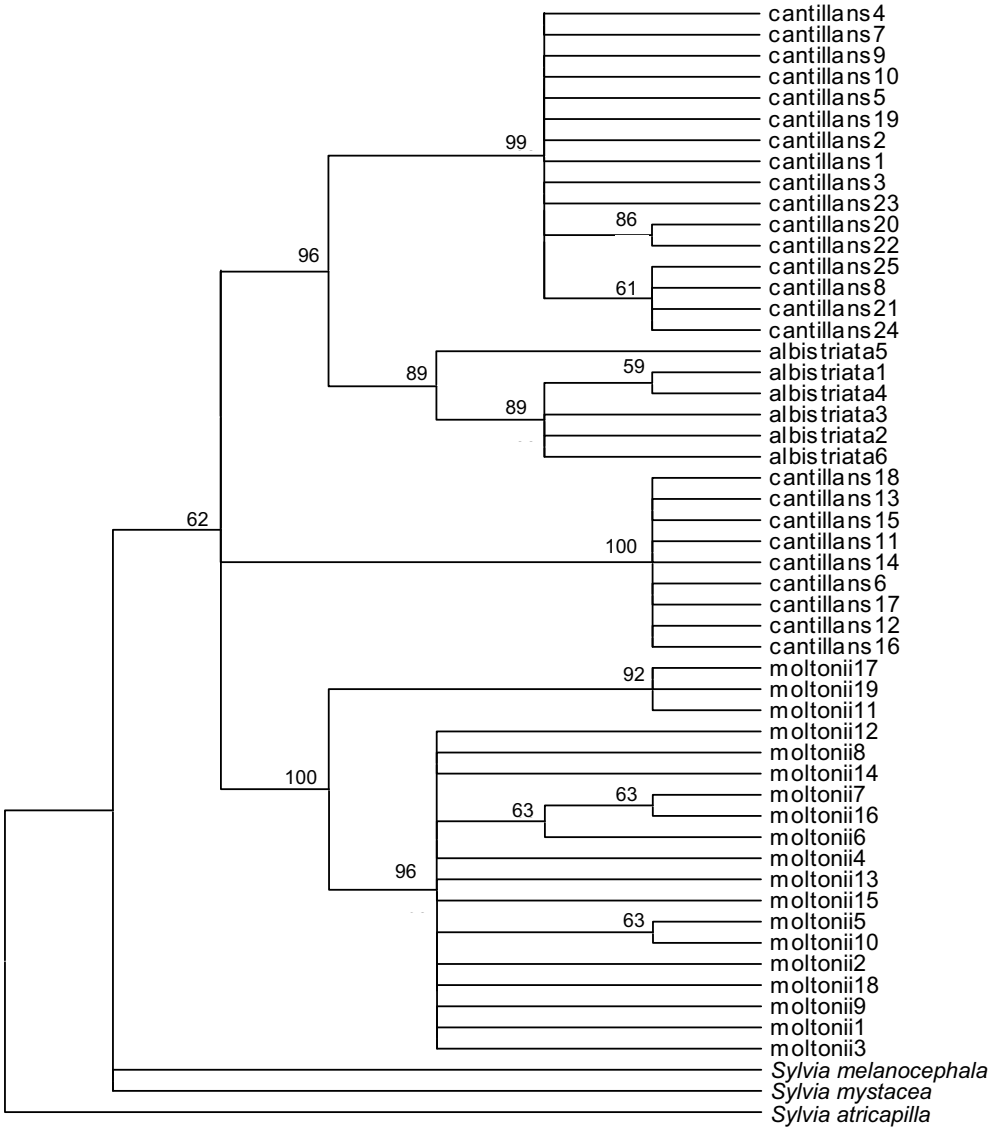

Fig. 4. Phylogenetic tree (50% majority rule) resulting from the MP analysis; bootstrap values (>50%; 1000 replicates) are indicated.

eages falls well within the range observed among pairs of closely related (but unambiguously distinct) *Sylvia* species (*S. deserticola* and *S. undata*: 2.5%; *S. undata* and *S. (sarda) balearica*: 4.4%; *S. deserticola* and *S. (sarda) balearica*: 4.6%; distances calculated with the same method on the same fragment used in this study, from cytochrome *b* sequences deposited in GenBank). In many cases, taxa showing similar level of divergence have been ranked as different species (e.g. Li et al., 2006; Martens et al., 2004; Qu et al., 2006).

Two clades, *moltonii* and (southern) *cantillans*, occur in geographical contact and still maintain their diagnosability (Brambilla et al., 2006, 2008). Moreover, they are represented by mutually monophyletic lineages in the gene trees. In details, they breed (and were sampled in this study) in syntopy and parapatry in mainland Italy, maintain their diagnostic calls (Brambilla, 2007) and do not share any haplotype. This suggests that the two forms are undergoing a secondary contact and are sufficiently diverged to maintain their respective integrity. This is fully consistent with evidences provided by the pattern of distribution (Brambilla et al., 2006) and by playback experiments (Brambilla et al., 2008), both clearly indicating reproductive isolation. *S. moltonii* is completely allopatric with respect to *albistriata*, but these are the most phenotypically different taxa (see Shirihai et al., 2001); the genetic distance is still notable and, thus, the lack of direct assessment of

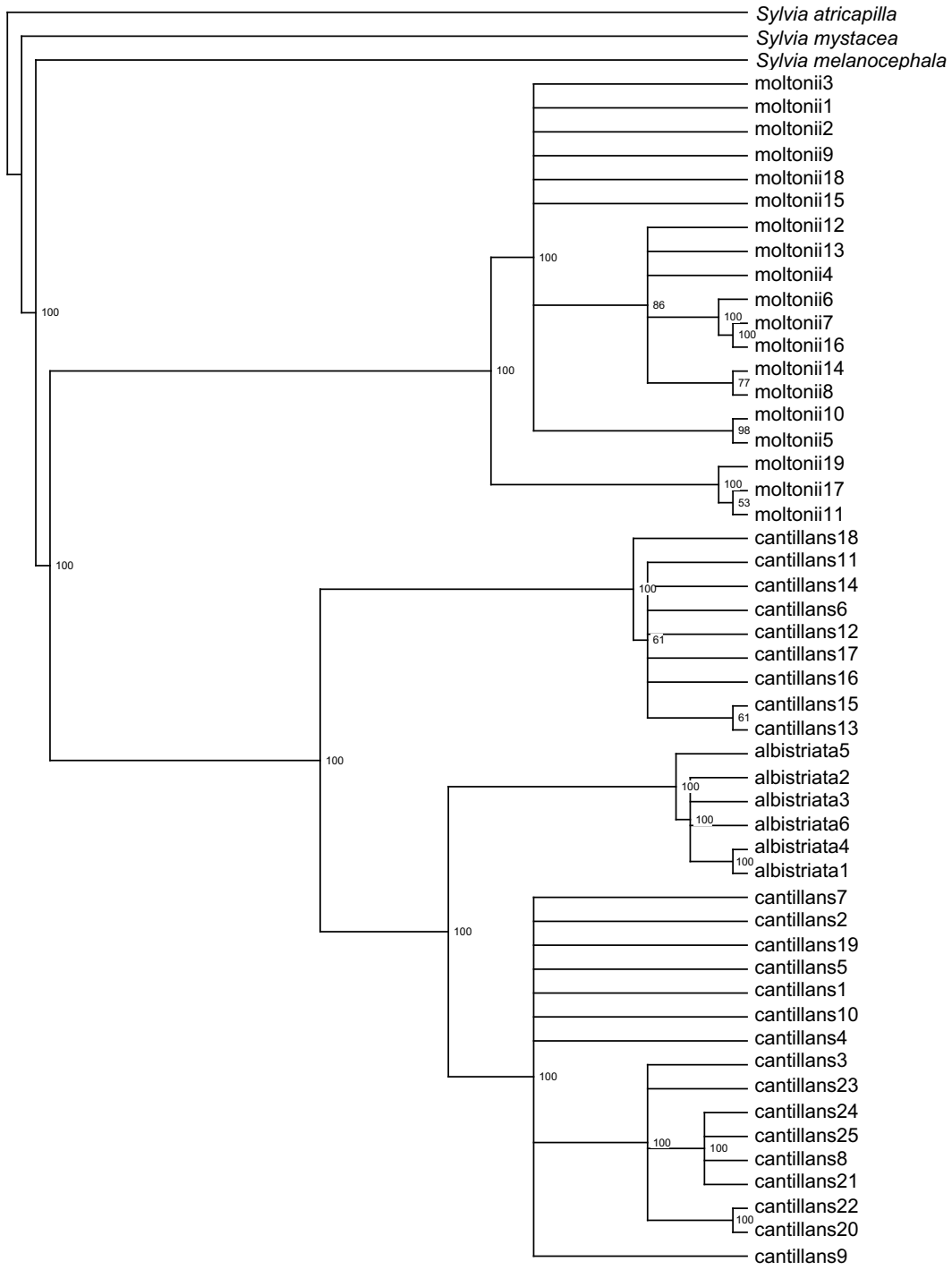

Fig. 5. Phylogenetic tree (50% majority rule) resulting from the ML analysis; bootstrap values (>50%; 100 replicates) are indicated.

reproductive isolation does not create problematic circumstances for the taxonomy of the group. The same applies to the relationship between *moltonii* and western *cantillans*. Therefore, *moltonii* should be unambiguously ranked as full species (see Helbig et al., 2002), *Sylvia moltonii* (*Sylvia subalpina* according to the change recently proposed by Baccetti et al. (2007), which is invariably diagnosable on the basis of the exclusive and very characteristic contact/alarm call, wholly different from calls of the other taxa (Fig. 8). Notably, within *moltonii*, a further subdivision may be observed (Figs. 3–6), with two groups including mainly 'insular' haplotypes (Corsica, Balearics and a single bird breeding at San Rossore, a coastal site in Tuscany) and 'mainland' haplotypes (all the others, sampled in central–northern Italy and in the Giglio Island too), respectively. Both groups showed strong statistical support (Figs. 3–6). Though only two samples from insular populations were included, it is plausible that *moltonii* underwent a long geographic isolation on

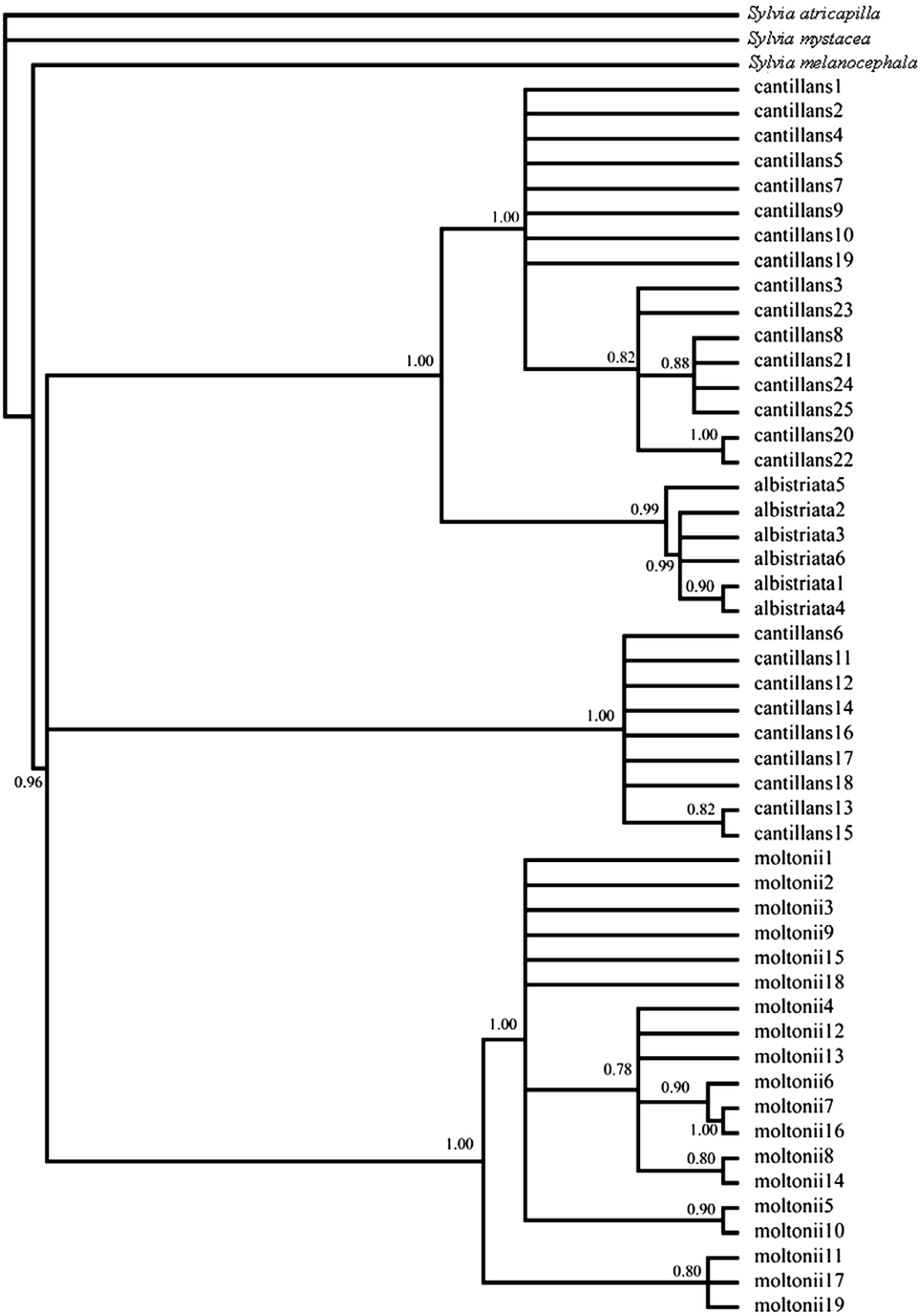

Fig. 6. Phylogenetic tree (50% majority rule) resulting from the Bayesian analysis (see text). Posterior probabilities (>0.50) are indicated.

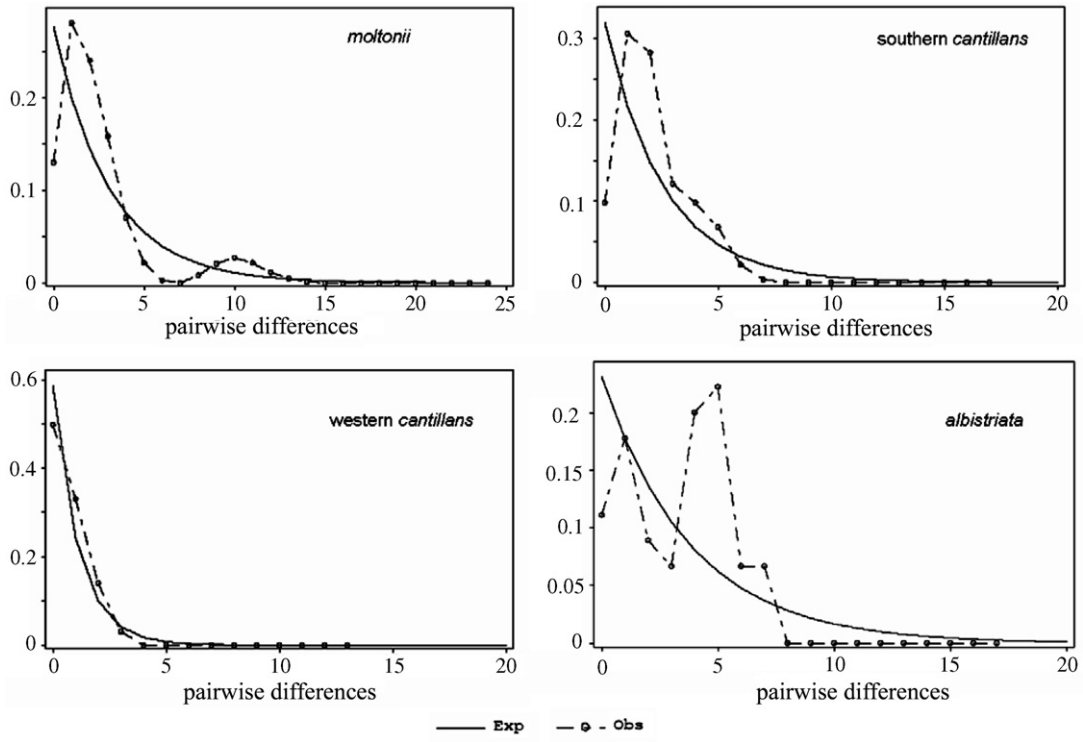

Fig. 7. Diagram of mismatch distributions for the four clades.

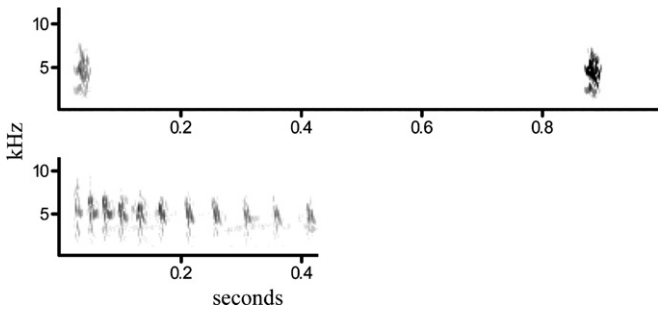

Fig. 8. Contact/alarm calls of (southern) *cantillans* (upper half; two different calls. Rome, Italy, May 2005) and *moltonii* (lower half; a single call. Pavia, Italy, June 2005).

the Tyrrhenian, where Sardinia and Corsica served as a refuge during the Pleistocene. The interior split among the two *moltonii* clades is younger than other splits among groups and presumably the mainland populations of this species originated from a recent Pleistocenic or post-Pleistocenic invasion.

More surprising is the large difference between western and southern *cantillans*. This former subspecies clearly appeared non-monophyletic (Figs. 3–6). According to the current literature, all *cantillans* populations should share the same features (Cramp, 1992; Shirihai et al., 2001). Although the Italian populations may be more approaching *albistriata*, being on average paler on the vent and with a larger submoustachial stripe, and are often more orange than red with respect to Spanish breeders (but large overlap; Brambilla, 2007), they are extremely similar to western breeders according to their external morphology and contact calls (Brambilla and Guidali, 2005), yet genetically well distinct (see Section 3 and above). Unfortunately, nothing is known about possible mechanisms of reproductive isolation and about the phenotype/genotype segregation, especially because currently there is no indication of divergent mating systems. Accordingly, western and southern *cantillans* could not presently be ranked as separate species (although they could qualify as phylogenetic species), as mtDNA sequence is the only character proved to be diagnostic among the two (but see above) (Helbig et al., 2002). Moreover, Italian populations may be connected with Spanish ones through intermediate African populations (see Brambilla et al., 2006; Shirihai et al., 2001) but, unfortunately, we were unable to obtain sequences from African samples until now.

On the other side, *albistriata* was regarded as a possible (allo)species/phylogenetic species (Shirihai et al., 2001); however, its divergence from the Italian *cantillans* (1.7%) is not so large as recorded for *moltonii* and is also much weaker than the divergence found between Spanish/French and Italian *cantillans* populations. Moreover, some migrants caught in central Italy are phenotypically *albistriata* but have southern *cantillans* haplotype (Brambilla, 2007), this possibly indicating mixed mating (a contact zone occurs between *cantillans* and *albistriata* in the northern Adriatic in Croatia; see Section 1 and Brambilla, 2007). This is a further confirmation of the closely relatedness of these two forms, actually evident also from the gene trees, where *albistriata* invariably appears as the sister group of southern *cantillans* (see Figs. 3–6). Consequently, we suggest to keep *albistriata* as a subspecies of *S. cantillans*, until new contrasting evidences are provided (e.g. the contact zone is a hybrid belt, and the two taxa could be considered semispecies; Helbig et al., 2002).

In conclusion, we suggest treating the *Sylvia cantillans* complex as two species, *Sylvia cantillans*, polytypic, and *Sylvia moltonii* (*S. subalpina*; see above and Baccetti et al., 2007, for nomenclatural proposed changes), spread over CN Italy, Sardinia, Corsica and Balearic Islands.

We are aware that many questions remain unsolved. Firstly, further data are required to determine whether *inornata* is closer to western or southern *cantillans* or if it is an intermediate race, although it cannot be excluded that it can be divergent from the others, representing another independent lineage originating from a further African refuge during the Pleistocene. Also the transition

zone between western *cantillans* and *inornata* (cf. Shirihai et al., 2001) requires further study. Secondly, we are aware that future splits in *Sylvia cantillans* cannot be excluded when more data are provided; in fact, a further subdivision into two branches, i.e. southern *cantillans* and *albistriata* on the one side and western *cantillans* on the other, representing two different (allo)species, could be expected.

Finally, the fact that *moltonii* and *cantillans* are still 'more parapatric than sympatric' (see Brambilla et al., 2006), likely due to ecological similarity among the two, could be regarded as a further indication of the power of sexual selection, which often plays a major role in speciation (Irwin et al., 2001a): the two species are still very similar in their ecological requirements, but they are clearly reproductively isolated, thanks to diverged mating systems and consequent different song perception (Brambilla et al., 2008).

In conclusion, the *Sylvia cantillans* complex provides a perfect study case for illustrating the potential role of geographical structuring even across small geographical ranges and exemplifies how speciation is usually a gradual process in birds, typically involving differentiation in allopatry leading to reproductive isolation after a secondary contact, when the divergence accumulated in allopatry is large enough to prevent reproductive compatibility (Brambilla et al., 2008; Haavie et al., 2004; Helbig et al., 2002).

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