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MOLECULAR DIVERGENCE IN TWO MEDITERRANEAN SONGBIRD SPECIES OF THE GENUS *Sylvia* (AVES: SYLVIIDAE): IMPLICATIONS FOR TAXONOMY AND RESEARCH

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CHAPTER 1
General introduction and outline of the thesis

The Mediterranean region is a major biodiversity hotspot within the Western Palearctic, with many taxa showing a geographical distribution restricted to this area, where they probably originated (Myers, 2000). The past geological and climatic dynamics of this region (such as glacial cycles and salinity crises) gave rise to multiple patterns of population isolation, leading to divergence accumulation and speciation (Hewitt 1996; Taberlet et al. 1998). However, in spite of the large number of bird species breeding in the Mediterranean region (366 recognized taxa), the number of endemic species is much reduced with respect to other classes of terrestrial vertebrates (e.g. amphibians, with 65% of the species breeding in the Mediterranean being endemic to this region): only 64 species are believed to have originated in the Mediterranean region and only 36 are regional endemisms (of which 9 are insular endemisms; Covas & Blondel 1997).

This difference has been traditionally attributed to the higher large-scale mobility of birds due to flight, which could have hypothetically limited the effects of isolation, both reducing the extent of isolation periods to only the worst geo-climatic conditions and favouring the mixing of former isolates in post-isolation periods (Covas & Blondel 1997, Pons et al. 2015a). However, whole ornithological clades evolved within the Mediterranean region (e.g. the genus *Sylvia*) and, whereas endemism to the species level seems reduced, several endemic subspecies have been recognized within the region (Pons et al. 2015a). Another hypothesis, therefore, may be needed to explain such a pattern. Taxonomy, as well as the investigation of divergences among populations in bird species, have traditionally been based upon anatomical and morphological differentiation of individuals belonging to different geographical populations of the same species (Watson 2005). The traditional taxonomic approach based on morphology had an irreplaceable role in contributing to defining the general taxonomical structure we still rely on, but obviously it does not take into account the whole traits and biological attributes of the organisms. In several cases it could have, therefore, led to the underestimation of the degree of divergence between geographical populations (traditionally ascribed to the same biological species, with at best subspecies status recognition), which may have markedly diverged from e.g. a genetic point of view and may have evolved reproductive isolation mechanisms (mainly mediated by vocalizations, in passerines), while maintaining a conservative external aspect (e.g. Brambilla et al. 2007, Illera et al. 2008, Toews & Irwin 2008, Pons et al. 2015a). Under this hypothesis, speciation in several Mediterranean bird taxa would be in progress or even already completed, and it has been under-appreciated due to only methodological issues.

In fact, several songbirds (Aves: Passeriformes), traditionally regarded as single biological species, have been recently proved to consist of populations which are morphologically undistinguishable but largely diverged in terms of genetics and/or vocalizations; these populations reached such levels of divergence to deserve an elevation to allospecies (Amadon 1966) or to 'full' biological species levels (Shirihai et al. 2001, Martens et al. 2004, Brambilla et. al 2008a, Reddy 2008, McKay et al. 2010).

These changes in taxonomic level attribution, made possible by a better understanding of divergence and isolation mechanisms even in well explored areas, triggered a growing interest for the analysis of the actual intraspecific geographical structuring in passerine species showing geographical variations in some aspects (such as genetics and vocalizations) in the face of an extraordinary spatial uniformity in terms of morphology. Subsequently, many taxa traditionally considered as single polytypic species have been shown to be in an advanced phase of divergence and speciation, and the populations belonging to these taxa may in fact deserve to be treated as allospecies or separate sister species (e.g. Spotted Flycatcher, *Muscicapa striata*; Woodchat Shrike, *Lanius senator*; Black-eared Wheatear, *Oenanthe hispanica*; Cramp & Simmons 2006, Pons et al. 2015a).

A complete understanding of these phenomena would allow the identification of divergent lineages and Mediterranean endemic subspecies/species, and in turn promote their conservation. In many cases, in fact, the level of conservation concern depends on taxonomic decisions (Collar 1997, Frankham et al. 2002, Newton 2003, Gamauf et al. 2005). Subspecies are included in conservation programs only exceptionally, even if population numbers are small, breeding areas restricted and subspecies well differentiated (Gamauf et al. 2005).

The advent of the Biological Species Concept (BSC; Mayr 1942) led ornithologists to an indiscriminate lumping of thousands of species during the first half of the twentieth century. Many allopatric taxa, originally described as “full” species, were downgraded to subspecies of newly defined polytypic species, without proper assessment of their real degree of phylogenetic divergence (Sangster 2014).

This historical issue may have led to reduced conservation efforts for evolutionary independent taxa, which would merit protection. The situation may have been even more problematic for insular taxa: their allopatric distribution with respect to other populations of the same polytypic species would have prevented their successive re-elevation to species rank, because of the impossibility of evaluation of their real degree of reproductive isolation based on BSC. Endemic island populations

may even be more prone to extinction: less than 20% of the world's bird species are restricted to islands, but over 90% of documented avian extinctions are island endemics (Johnson and Stattersfield 1990). This is because islands generally support smaller populations that are more prone to inbreeding and more susceptible to natural disasters and habitat loss (Duncan and Blackburn 2007).

The aim of the present study is the description of molecular phylogeography of two polytypic songbird species, endemic of the Mediterranean area, characterized by a conservative external morphology but likely concealing deeply diverged taxa, probably deserving elevation to species rank and dedicated conservation actions.

TAXONOMY AND SPECIES CONCEPTS

In-depth analyses of the intraspecific molecular divergence of species previously defined only by morphological characters often suggest a revision of the taxonomy at both species and/or subspecies level (e.g. Klicka et al. 2001, Shirihai et al. 2001, Brambilla et al. 2008a, Forschler et al. 2010). However, at least 22 different species concepts exist (Mayden 1997), which differ both in terms of theoretical definition and operational criteria for species delimitation, so that taxonomic hypotheses always require a certain degree of subjectivity (at least in the choice of the criterion; Sansgter, 2014).

The two most popular species concept in ornithology are the Biological Species Concept (BSC; Mayr 1942) and the Phylogenetic Species Concept (PSC; Cracraft 1983, 1989). The BSC defines species on the basis of complete reproductive isolation, whereas PSC only evaluates complete diagnosability of the two taxa for at least one character.

The BSC is the species concept traditionally advocated in the majority of ornithological research, but its practical use is quite difficult, in particular in the case of allopatric taxa. The criterion of reproductive isolation is directly applicable only in contexts where taxa are in contact (in sympatry or parapatry), and cannot be directly applied to allopatric populations (Mayr 1969; Cracraft 1983). This issue has led ornithologists (which theoretically adhered to the BSC) to delimit species on the basis of different criteria: most taxonomical revisions in the period 1950-2010 were in fact based upon (morphological or genetic) diagnosability or reciprocal monophily, instead of reproductive isolation (Sangster 2014).

Diagnosability, on the other hand, can be applied in every situation (simpatry, parapatry and allopatry) and it was regularly used as a practical species delimitation criterion well before the formalization of the PSC (Sangster 2009, 2014). As noted by deQueiroz (1998, 1999), both BSC and PSC identify species with segments of independent population lineages; however, they use different operational criteria to delimit species and, whereas BSC formally requires a bet on the future evolutionary isolation of these populations, PSC limits its inquiry to their past separation of evolutionary paths proved by their actual diagnosability.

In spite of the traditional conflict of the two criteria, however, a new theoretical and operational framework is emerging, which mixes the two approaches. Ornithologists are in fact gradually (perhaps even unconsciously) adopting a general concept of species (defined “Evolutionary Species Concept” by Mayden 1997 and “General Lineage Concept” by deQueiroz 1999) which simply

defines them as segments of population lineages. This definition leaves open the practical delimitation of species to multiple complementary criteria (morphology, vocalizations, genetic divergence and reciprocal monophily), each one corroborating the taxonomical hypothesis proposed (Schlick-Steiner et al. 2010, Sangster 2014).

Recent tendencies in taxonomic research further highlight this need for multiple independent confirmation of taxonomic hypothesis (integrative taxonomy, Padial et al. 2010). We adhere to this theoretical and operational framework and, although our data come only from genetics (but from at least two independent loci), our conclusions will always rely on the integration of our molecular results with all available data regarding geographical distribution, behaviour and morphology of the analysed taxa. Our taxonomic hypotheses will be expressed in terms of the taxonomic "entities" recently defined by Galimberti et al. (2012), a series of taxonomic "levels" distributed along the diversity continuum between individuals and species, each characterized by a greater taxonomic information content:

- Molecular Operational Taxonomic Units (MOTUs), defined as "groups of unidentified organisms sharing similar sequences";

- Unconfirmed Candidate Species (UCS), defined as "groups of organisms within a species that are distinct at the molecular level from other members of the species";

- Integrated Operational Taxonomic Units (IOTUs), defined as molecular lineages that are supported by at least one more taxonomic characteristic;

- Confirmed Candidate Species (CCS), previously defined by Padial et al. (2010) as "deep genealogical lineages that can be considered good species following standards of divergence for the group under study but that have not yet been formally described and named".

OUTLINE OF THE THESIS

Chapter 1 presents a general overview of the topic of the thesis and a description of the model species, which lists the main characteristics of their biology and reviews their past and currents taxonomical treatments at species and subspecies levels. It also includes a brief review of the theoretical and operational criteria for the definition of species, which will guide the taxonomical suggestions we report in our results. Our main conclusions are also described in the last part of the chapter.

Chapter 2 to Chapter 4 detail the results of the present research. Each chapter corresponds to a manuscript (in preparation) regarding a specific research topic.

Chapter 2 deals with a phylogeographic analysis of the molecular variation shown by Marmora's Warbler (*Sylvia sarda*) in one mitochondrial and one nuclear locus. In particular, we will focus on the taxonomic status of the Balearic Islands populations, traditionally considered a subspecies, but recently proposed as an allospecies without proper supporting data.

Chapter 3 deals with a phylogeographic analysis of the Subalpine Warbler (*Sylvia* [*cantillans*]) species complex. Following the recent identification of a morphologically cryptic species (*Sylvia moltonii*), we in depth analyse the genetic divergence of other populations of the complex in two mitochondrial and two nuclear loci.

Chapter 4 describes the results of molecular identifications of migrating and wintering individuals of the Subalpine Warbler (*Sylvia* [*cantillans*]) species complex and provides the first details on the wintering areas of each single taxa.

Notwithstanding the relative merit of each single manuscript, they are best considered as parts of our more general framework, dealing with divergence processes of songbirds within the Mediterranean basin, with particular emphasis on the lack of congruence between morphology and other biological attributes of species.

MODEL SYSTEM: THE GENUS *Sylvia*

The most representative songbird genus of the Mediterranean basin is the genus *Sylvia*. *Sylvia* is the nominate genus of a large family of insectivorous passerines, Sylviidae, including all the Old World warblers. *Sylvia* is however more strictly related to babblers (with *Chamaea fasciata* being the sister species of *Sylvia*) rather than to other typical Sylviinae (such as *Phylloscopus* and *Acrocephalus*) and this led Shirihai et al. (2001) to propose to put babblers and *Sylvia* warblers in a new subfamily of Sylviidae, Timaliinae.

Recent research about the intrageneric phylogeny of the genus *Sylvia* led to the definition of six subgenuses: *Sylvia*, *Epilais*, *Parisoma*, *Adophoneus*, *Curruca* and *Melizophilus* (Shirihai et al. 2001).
Melizophilus is the largest subgenus and includes a desertic (super)species (Desert Warbler, *Sylvia* [*nana*]), the pan-Eurasian distributed Common Whitethroat (*Sylvia communis*) and a monophyletic clade containing all species with a strictly Mediterranean distribution: Spectacled Warbler, *Sylvia conspicillata*; Dartford Warbler, *Sylvia undata*; Marmora's Warbler, *Sylvia sarda*; Tristram's Warbler, *Sylvia deserticola*; Subalpine Warbler, *Sylvia cantillans*; Sardinian Warbler, *Sylvia melanocephala*; Rüppell's Warbler, *Sylvia rueppelli*; Ménétries' Warbler, *Sylvia mystacea*; and Cyprus Warbler, *Sylvia melanothorax*.

Unfortunately, no fossils of *Sylvia* warblers exist, which prevents certain geological dating of the main splits occurred within the genus. The tentative molecular datings proposed in Blondel et al. (1996) and Shirihai et al. (2001) are based on the hypotheses of absence of rate heterogeneity between members of the group, which unfortunately cannot be taken for granted (Nguyen & Ho 2016).
Notwithstanding the lack of precise dating, however, we can readily recognise the action of geologically recent processes (perhaps due to Pleistocene ice ages) on the interspecific divergence of some *Sylvia* species. Shirihai et al. (2001), in fact, highlighted the existence of large degrees of divergence in several phenotypic and genotypic characters between populations of morphologically conservative *Sylvia* species of the subgenus *Melizophilus*.
This uncoupling of differentiation processes in terms of morphology and DNA/vocalizations may suggest the presence of cryptic taxa within the Mediterranean basin, previously not recognized due to the traditional taxonomic analysis conducted by means of anatomical and morphological analyses.

Although Shirihai et al. (2001) proposed a few taxonomical revisions of some *Melizophilus* taxa on their own, their conclusions are largely based on observational data (slight morphological differences and sonograms of a few vocalizations); conclusive supporting data (analysis of mitochondrial DNA structuration and playback experiments), which they refer to as "submitted manuscripts", unfortunately never came to light. Their conclusions therefore remain largely confined to the realm of (fairly robust) hypotheses.

With this work, we aim to thoroughly analyse several aspects of intra- and inter-specific divergence within two representative Mediterranean *Sylvia* species (Marmora's Warbler, *Sylvia sarda*; and Subalpine Warbler, *Sylvia cantillans*), in order to evaluate their relative phylogeographic structure and the reliability of the recently proposed taxonomical treatments.

MARMORA'S WARBLER (*Sylvia sarda*) BIOLOGY

Marmora's warbler (*Sylvia sarda*, Temminck, 1820) is a typical *Sylvia* warbler, close in size to Dartford Warbler (*Sylvia undata*) but with slightly shorter tail.
Its general aspect is of a small warbler, with pointed bill, short wings and long tail. Body length amounts to 12 cm, with a wingspan of 13-17.5 cm and a tail 4-5 cm long. It shows a certain degree of sexual dimorphism, constant throughout the year: whole plumage is blue-grey in males, while females are browner, with less pure grey on head and breast, more pink-brown on flanks and whitish belly. Eyes are ochre to red, with orange-red eye-ring. Juveniles differ from both adult sexes: they are browner than both adult sexes, essentially dusky-brown above and grey-brown below, with a dull yellowish eye and orange eye-ring (Cramp 1992).

Taxonomic treatment

Marmora's Warbler is traditionally considered a polytypic species, with two subspecies: *Sylvia sarda sarda* (Temminck, 1820), inhabiting Tyrrhenian islands; and *Sylvia sarda balearica* (von Jordans, 1913) inhabiting Balearic Islands except for Menorca. The two subspecies differ mainly in size and adult male plumage (see Morphology and Plumage). Whereas a difference in vocalizations between the two forms was noted by von Jordans (1914), he proposed just a subspecies rank for *balearica*. The taxonomic treatment of the two taxa has not been questioned until a few years ago.

Shirihai et al. (2001), in their monumental research on the *Sylvia* genus, recently proposed to elevate *S. s. sarda* and *S. s. balearica* to the rank of allospecies, within the superspecies *S.* [*sarda*]. They based their taxonomical hypothesis upon descriptive analyses of vocal and morphometric divergence and, secondarily, on plumage characters (not useful in all cases, in particular for adult females) and bare parts colour. They intended to accompany their proposal with an analysis of the phylogeny and phylogeography of all the species of *Sylvia* genus based upon cytochrome b gene sequences and playback response analysis between the two taxa, but the hypothesised papers never came to light, leaving some doubts about the reliability of the proposed taxonomic treatment. Whereas AERC TAC (Crochet et al. 2010) embraced the taxonomic treatment proposed by Shirihai et al. 2001 (however without unanimity), the lack of supporting data led other institutions (e.g. BirdLife International) to reject this taxonomical revision. The species treatment by BirdLife International was justified as follows: "*Sylvia balearica* has been split from *S. sarda* on the basis of size, shape (structure), coloration, song, calls, migratory behaviour and mitochondrial DNA […].

However, the size and structural differences are negligible, the colour differences are minor, the vocal differences are not particularly strong (and not properly assessed or presented), the migratory behaviour is difficult to evaluate taxonomically, and the molecular evidence remains unpublished after at least six years. Therefore this split is not accepted until better evidence is provided" (BirdLife International 2016).

Whereas this discordance might appear just as a technical controversy on an academic issue, this decision by BirdLife International directly impacts on the evaluation of the conservation status of these populations by the IUCN Red List. All *Sylvia sarda* (*sensu* Cramp 1992) populations are in fact considered as a single taxon, which does not meet any of the "concern criteria" because of a large breeding range and large, demographically stable populations: it is therefore listed as Least Concern.

However, if *Sylvia balearica* would be treated as a species *per se*, its population size and restricted geographical distribution (endemic in only one European country, with a restricted breeding range) could require a less secure treatment, which in turn could favour planning and implementing dedicated conservation efforts, which are not suggested as necessary under the current taxonomic treatment.

Geographic distribution

The species breeds strictly within the Mediterranean basin, in areas with average July temperatures up to 24–26°C and relatively frost-free winters (Cramp 1992). Its distribution is exclusively insular, with no nesting reported from mainland Europe. The species breeds on Corsica, Sardinia, Balearic Islands (except for Menorca) and several islands off Western Italy.

During non-breeding periods the species migrates to N Africa (Algeria, Tunisia and Libya) and Sicily; vagrants have been observed in England, Scotland, mainland Spain, mainland France, mainland Italy and Malta (Shirihai et al. 2001).

The two subspecies are completely allopatric, with disjointed breeding ranges. Their distributions overlap only during non-breeding periods, due to *S. s. sarda* movements to Balearic Islands.

Sylvia sarda sarda breeds mainly on Corsica and Sardinia and their off-lying islets; on several islands off Western Italy, e.g. Elba, Ischia, Montecristo, Capri, Giglio, Capraia and Ponza (Walter 1988); formerly it bred on Pantelleria and some small islands off SW Sicily (Iapichino & Massa 1989), where its occurrence is likely tied to the close wintering grounds, and where is currently

probably extinct (Gustin et al. 2009). Although many breeding islands are very close to mainland, no nesting had been reported from mainland Europe.

Sylvia sarda balearica breeds on all Balearic Islands (including small islets) except for Menorca (Shirihai et al. 2001). Last known breeding on Menorca was in 1974; after 1979 it was never observed on that island, probably replaced by Dartford Warbler, *Sylvia undata* (Muntaner 1980; but see Shirihai et al. 2001).

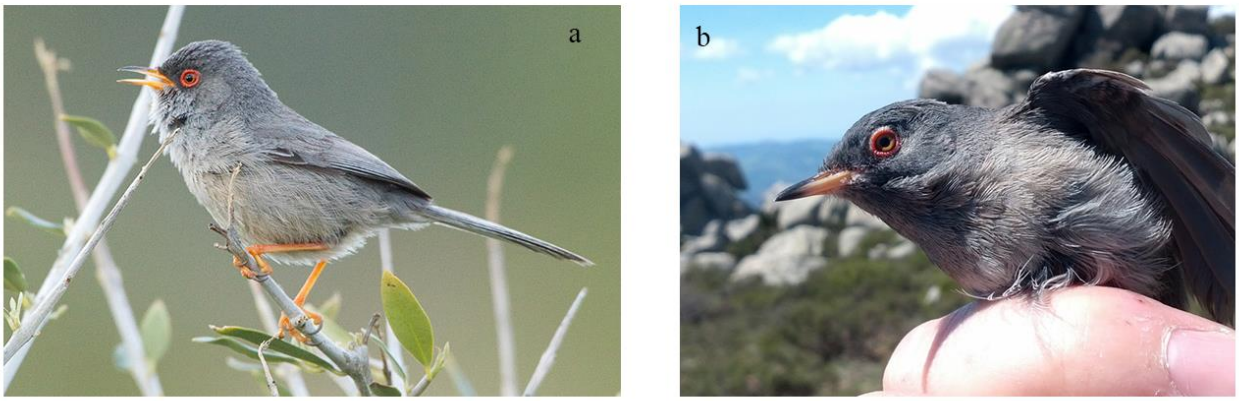
Morphology and plumage: differences between the two subspecies

Individual and seasonal variation within the two subspecies is limited, with no appreciable geographical structuration (Shirihai et al. 2001). We give here an account of the major differences between the plumages of adult males of the two subspecies (see Figure 1).

Sylvia sarda sarda: head, chest, back, and rump are uniformly slate-grey; frontal mask (lores, forehead and ear-coverts) is just only faintly dusky in autumn but blacker in spring, due to wear. Some fine whitish spots are present on chin and throat in fresh plumage (autumn) but they are uniform medium grey in spring, almost concolorous with breast and lores. Tail is black-brown, with broad dusky-white edges to external rectrices. Underparts are pale blue-grey, with just an off-white centre to belly; breast-sides, flanks and vent often show a very pale pink-buff wash. Tarsus is flesh-brown, with a more pink-straw tint in spring; however, it is consistently a bit darker and greyer than in *balearica*. Bill shows an extensive and well-marked black tip and a pale pinkish base to lower mandible.

Sylvia sarda balearica. The Balearic subspecies is about 20% smaller than nominate *sarda*, but with proportionally longer tail. Frontal mask is blacker and more contrasting with whitish and pale chin and throat (greyer in *S. s. sarda*), giving a clear pale-throated impression, even at distance. Underparts are rather uniformly coloured, with a more noticeable (than *sarda*) pink-buff wash on breast sides, flanks and vent even in spring. Tarsus is pale orange-brown. Bill base is more yellow-orange than *sarda* and the tip shows a much-reduced blackish area (Shirihai et al. 2001).

Figure 1. *Plumage differences between males of the two traditionally recognised subspecies of Marmora's Warblers: (a) Sylvia sarda balearica, Mallorca; (b) Sylvia sarda sarda, Sardinia.*



Vocalizations

Both subspecies have distinctive calls and songs. Contact call is a guttural, hard 'tak' in *S. s. sarda*, but consists of a nasal 'tsrek' or 'trt' in *S. s. balearica*. They are clearly recognizable on the field, both from each other and among all other syntopic *Sylvia* species (even *Sylvia undata*). Song is emitted only by males, mainly during the breeding season, but with a second main peak of singing activity in autumn (probably linked to territory defence outside the breeding season). *S. s. sarda*'s song is a rather short, twittering warble, usually ending with a long repetitive rolling trill; it recalls Spectacled Warbler (*Sylvia conspicillata*) in tone and tempo, but the song of the latter is sweeter and interspersed with distinctive contact calls.

S. s. balearica's song resembles more *Sylvia undata* in tone, and is clearly less liquid and clear; however, as *S. s. sarda*, it has a finale characterized by a long repetitive thrill, lacking in *S. undata* (Shirihai et al. 2001).

Shirihai et al. (2001) cited an (at that time) underway research on playback response conducted on the two subspecies, which seemed to prove the existence of a complete premating isolation mechanism mediated by vocalizations; unfortunately such a research had never been published.

Movements

The species is largely resident, with most birds remaining onto the breeding grounds all-year round (Cramp 1992). Outside breeding season only small numbers of individuals of *S. s. sarda* (both adults and first winter) disperse or migrate to N Africa (Algeria, Tunisia and Libya) and Sicily. Vagrants have been observed in England, Scotland, mainland Spain, mainland France, mainland

Italy and Malta (Shirihai et al. 2001). Recent ringing studies demonstrate that *S. s. sarda* is a very scarce, but regular, migrant to the Balearic Islands (Shirihai et al. 2001). *S. s. balearica* is essentially sedentary, with no recapture of marked adult individuals even between different Balearic Islands (Joseph Sunyer, *pers. comm.*). Just a few first-year birds disperse on east Spanish coasts (Muntaner 1997). Main movements of both taxa consist in altitudinal movements or dispersal to areas of the same island not utilized during the breeding season (Shirihai et al. 2001). In Corsica, some birds remain at high altitudes in winter, moving to lower levels only when snow persists and returning as soon as it melts (Thibault 1983).

Breeding biology

The species breeds from the second calendar year, laying one or two broods per season between April and July. Both sexes take part to nest construction, whereas male builds several "cock" nests, as typical of many *Sylvia* species. Nest is built in low bush or dense scrub, made of dry grasses, stems, and leaves, and lined with finer material including grass, roots and hair. Both sexes are involved in incubation (of 3-4 eggs, on average) and in rearing chicks, which remain in the nest for 12-13 days after hatching (Shirihai et al. 2001). The species forms monogamous pairs for most part of the year and the territory is defended all year round in sites where individuals are sedentary (Shirihai et al. 2001).

Habitat

It inhabits low, degraded and rather uniform Mediterranean maquis or garigue, with a maximum height of 2m but usually lower than 1m (thus favouring lower shrubs not so attractive to potential competitor, such as *Sylvia undata* and *Sylvia melanocephala*). Trees are avoided, unless widely scattered. It also inhabits cliff-top heaths and rocky slopes on small islands and coastal hillsides. It inhabits areas with altitude varying between 0 and about 1000m above sea level (Shirihai et al. 2001). In post-breeding season, Corsican birds may ascend up to 2000 m (Thibault 1983).

Diet

Adults feed on arthropods, including small flying insects and their larvae. Nestlings are also fed with spiders. Individuals feed mainly in low vegetation or directly on the ground, where they hop for distances of up to 200 m (Cramp 1992).

SUBALPINE WARBLER (*Sylvia cantillans*) BIOLOGY

Subalpine Warbler (*Sylvia (Motacilla) cantillans*, Pallas, 1764) is a rather small *Sylvia* warbler, close in size to Marmora's warbler (*Sylvia sarda*) but with longer wings and shorter tail. Its general aspect is of a small elegant warbler, with rather short bill. Body length amounts to 12 cm, with a wingspan of 15-19 cm and a tail 4-5 cm long. It shows plumage sexual dimorphism, mainly evident in spring: males are blue-grey above, dark pink-chestnut below, with white moustachial stripe (but see description of subspecies variability), while females are browner above and buff-white below. Eyes are gold to red, with orange-red eye-ring. Inner eye-ring is orange-red in adults and whitish in juveniles, whereas outer eye-ring is red in males, off-white in females and juveniles (Cramp 1992).

Taxonomic treatment

Subalpine Warbler has traditionally been regarded as a polytypic species, with four subspecies:
- *S. c. cantillans* (*Motacilla cantillans*, Pallas, 1764), distributed over a large part of western Mediterranean region, encompassing Portugal and continental Spain, France and Italy;

- *S. c. albistriata* (*Curruca albistriata*, Brehm, 1855), distributed over part of the Eastern Mediterranean basin, from Trieste (Italy) and Istria (Croatia) along Adriatic coastline, till Greece and Western Turkey;

- *S. c. inornata* (*Sylvia subalpina inornata*, Tschusi, 1906), breeding in Northern Africa (Morocco, Tunisia and Algeria), with a transition area to *S. c. cantillans* located in Southern Spain;

- *S. c. moltonii* (*Sylvia cantillans moltonii*, Orlando, 1937), inhabiting Balearic Islands, Corsica, Sardinia and several minor islands in Western Mediterranean (Shirihai et al. 2001).

All subspecies were therefore traditionally considered allopatric, except for the transition zone between *S. c. cantillans* and *S. c. inornata*.

The subspecies *S. c. moltonii* was not retained valid by Vaurie (1954) and even not cited by the reference manual for Western Palearctic birds (Cramp 1992). Shirihai et al. (2001), however, re-proposed the validity of the subspecies on the basis of its clear divergence from the other subspecies of *S. cantillans* in terms of vocalizations, moult pattern, colour of plumage underparts and mitochondrial DNA divergence. They even proposed to treat *moltonii* as an allospecies, within the

superspecies *Sylvia* [*cantillans*], ignoring that *moltonii* inhabited parts of continental Italy (Festari et al. 2002, Brambilla et al. 2006). More recently, Brambilla et al. (2008a) confirmed the strong divergence in mtDNA between *S.* [*c.*] *moltonii* and all other taxa of the *S.* [*cantillans*] species complex and demonstrated the existence of an apparently perfect reproductive isolation between *S.* [*c.*] *moltonii* and the locally syntopic *S.* [*c.*] *cantillans* (in Northern Italy, Brambilla et al. 2008b,c). Brambilla et al. (2008a,b) therefore suggested treating *moltonii* as a full species, *Sylvia moltonii* (syn. *Sylvia subalpina*; Baccetti et al. 2007).

Brambilla et al. (2008a) furthermore highlighted the existence of a certain degree of divergence between insular and mainland populations of *S.* [*c.*] *moltonii*, which needed an in-depth evaluation to elucidate the suitability of splitting the two forms in subspecies.
Another main result of Brambilla et al. (2008a) research was the identification of two strongly diverged clades within the traditional *S. c. cantillans* taxon: they identified a western clade ("western *cantillans*"), inhabiting the Iberian Peninsula and France, and an eastern clade ("southern *cantillans*"), embracing Italian *cantillans* populations.
Their mtDNA divergence was quite as strong as that between *S.* [*c.*] *moltonii* and all other taxa in the species complex. However these two taxa are almost indistinguishable by morphology and vocalizations, and they are completely allopatric; therefore, there is no way to directly prove their specific status. The non-existence of diagnostic character between the two taxa (apart from mtDNA) prevented Brambilla et al. (2008a) from proposing (allo)species status.

S. c. albistriata was regarded as a possible allospecies/phylogenetic species by Shirihai et al. (2001) and is in fact 1.7% differentiated in mtDNA from Italian *cantillans*. However, these two taxa form a monophyletic clade in the cytochrome-b phylogenetic tree, and some individuals (sampled during migration) proved to be intermediate between the two taxa (phenotypically *albistriata* but genetically Italian *cantillans*; Brambilla et al. 2010), suggesting potential mixed mating.

Brambilla et al. (2008a) therefore suggested this taxonomy for the *Sylvia* [*cantillans*] species complex:

- *Sylvia moltonii* (syn. *Sylvia subalpina*): monotypic species, inhabiting Balearic Islands, Corsica, Sardinia, minor islands in Western Mediterranean and parts of continental Italy (Tuscany and Emilia-Romagna).

- *Sylvia cantillans*: polytypic species, with three subspecies (left unnamed): the first subspecies, consisting of Italian *S. c. cantillans*, occurs over most continental Italy;

the second subspecies. *S. c. albistriata*, inhabits the Eastern part of Adriatic coastline, from Trieste until Greece and Turkey;

the third subspecies, inhabiting Iberian peninsula and France (western populations of the former subspecies *S. c. cantillans*).

No *S. c. inornata* individuals were sampled and analysed and this taxon could be a fourth subspecies of *S. cantillans* or could be within the range of variation of the western subspecies.

The last contributions to the taxonomical issues of this species complex came from Svensson (2013a, 2013b). In these two amateur publications on non-ISI journals, the author proposed a complete taxonomical revision, mainly based on the same data reported in Shirihai et al. (2001) and Brambilla et al. (2008a). He accepted Moltoni's Warbler, *Sylvia moltonii*, as a separate species (while designating it *Sylvia subalpina*, for priority questions; see also Baccetti et al. 2007) and split the remaining *Sylvia* [*cantillans*] taxa in two species:

- Western Subalpine Warbler, *Sylvia inornata*, with two subspecies:
    - *Sylvia inornata inornata*, breeding in North-Western Africa;
    - *Sylvia inornata iberiae*, breeding in Iberia, France and extreme north-western Italy;

- Eastern Subalpine Warbler, *Sylvia cantillans*, with two subspecies:
    - *Sylvia cantillans cantillans*, breeding in continental Italy;
    - *Sylvia cantillans albistriata*, breeding in Trieste, Balkans, Greece, Bulgaria, W-Turkey.

Svensson (2013b) even proposed a diagnostic plumage character between the two newly split species, regarding the extent and shape of white wedge on the fifth tail feather (T5, named in centrifugal order). This character, however, is of limited use in diagnosing morphologically cryptic forms of the species complex, because its efficacy is limited to adults, excluding most second-calendar-year-individuals (Svensson 2013b; and Brambilla, *pers. comm.*). Furthermore, the diagnosability of this character was not validated in association to genetic data, currently the only reliable character in distinguishing the taxa (Brambilla et al. 2008a).

Unfortunately, Svensson (2013a, 2013b) did not collect any fresh material (in particular from North African breeders) and limited his analysis to museum specimens, most of which were sampled as migrating individuals of dubious origin (Zuccon, *pers. com.*). Although we recognize that western individuals belong to a separate clade with respect to eastern ones (as stated on the basis of genetic divergence by Brambilla et al. 2008a), we believe that the attribution of the species rank to the western populations is currently poorly supported and consider the previous taxonomical treatment a much more solid working hypothesis.

Plumage differences between taxa

Morphological differences between taxa of the species complex consist in plumage colour divergence (mainly among males) and a few biometric characters (see Figure 2).

S. [*c.*] *cantillans*: underparts are brick-orange of variable intensity, with coloration extended also to flanks and vent; evident white moustachial stripe.

S. [*c.*] *albistriata*: brick chestnut-brown is largely confined to throat and breast, with sharp demarcation from wither belly; upperparts are darker and purer grey, with less brown tinge than nominate. White moustachial stripe is well developed, even more than in nominate.

S. [*c.*] *inornata*: similar to nominate, but with almost pure orange underparts, lacking most pink or chestnut hues. Birds breeding in France and Spain are somewhat intermediate between birds breeding in Italy (nominate subspecies) and birds breeding in N-Africa, with extremes approaching both.

S. moltonii: Underparts salmon-pink, without any orange hue, of variable intensity, extended to flanks and vent; white moustachial stripe scarcely developed to completely absent.

Vocalizations

Very vocal species, it emits typical contact calls (or territorial song) while moving in scrubs and bushes. Territorial song is moderately long, quite sweet and dry for a *Sylvia*. It includes high-pitched whistles, and often starts with them or an accelerated series of contact calls. Territorial song is similar to that of Sardinian Warbler, but it is slightly higher pitched and more musical, lacking rasping notes typical of Sardinian (Cramp 1992, Shirihai et al. 2001).

Vocalizations greatly vary between taxa of the species complex (Shirihai et al. 2001):

- *S.* [*c.*] *cantillans* and *S.* [*c.*] *inornata*: contact call is a “tek” similar to that of Lesser Whitethroat (*Sylvia curruca*); song is similar between the two taxa, but inornata has a slightly lower pitch;

- *S.* [*c.*] *albistriata*: contact call is a dry "tret", often repeated; song is similar to *S.* [*c.*] *cantillans*;

- *S. moltonii*: contact and alarm calls are completely different from other taxa of the complex, consisting in a "trrrrr" strictly resembling Wren (*Troglodytes troglodytes*). Song is faster, shorter, with notes uttered in fast succession and an abrupt final, lacking closing notes often present in nominate's song.

Figure 2*. Plumage differences between males of the four taxa of the Subalpine Warbler species complex described in Brambilla et al. 2008a: (a) S. moltonii, Italy (b) S. [c.] albistriata, Greece, (c) southern S. [c.] cantillans, Italy, (d) western S. [c.] cantillans, Spain.*

Habitat

The species breeds in the Mediterranean region, in areas with warm and dry summer climate, included between July isotherms 23–30°C. Breeding areas have altitudes comprised between sea-level to at least 1000-1100 m (up to *c.* 2000 m in Haut Atlas of Morocco; Cramp 1992). Overall, it occupies a broad spectrum of Mediterranean habitats, from low garigue to tall maquis, oak woodlands and mixture of these types (Shirihai et al. 2001). Its habitat preferences (Brambilla et al. 2006) differ from most other *Sylvia* warblers, in particular from Marmora's Warbler and Dartford Warbler, which normally avoid trees (Cramp 1992). Habitat requirements, however, are similar to Sardinian Warbler, which is even more arboreal; when syntopic they segregate vertically, with Sardinian Warbler using upper vegetation strata and Subalpine Warbler foraging in lower ones (Shirihai et al. 2001).

Diet

It feeds mainly upon small adult insects and their larvae, spiders and other arthropods. Outside the breeding season, it feeds also on fruits, mostly berries but also flesh of large fruits such as *Rubus*, *Ficus* or *Vitis*. It feeds in scrub, but also inside foliage of trees; only occasionally on ground (Cramp 1992; Shirihai et al. 2001). During migration, it feeds upon flower nectar in stop-over areas (*pers. obs.*).

Breeding biology

The species reproduces from second calendar year, laying 3-5 eggs per clutch between April and June. Males build a real nest in low scrub (15-150 cm on average), alongside a few "cock" nests. Both sexes incubate eggs for 11-12 days and participate in rearing chicks, which fledge 11-12 days after hatching (Cramp 1992).

The species is territorial during breeding season, with territory sizes depending on the suitability of occupied habitat. It is presumably monogamous during each breeding season. *S. moltonii* breeds about two weeks later than other taxa of the species complex (Shirihai et al. 2001).

Movements

All three European taxa in autumn cross Mediterranean Sea south-westwards on a broad front. Most N-African *S.* [*c.*] *inornata* are long-distance migrants, but populations in NW-Algeria are sedentary (Cramp 1992, Shirihai et al. 2001). Autumn migration starts in July, but peaks in mid-August and early- September (Shirihai et al. 2001).

Wintering ranges are diffused along southern edge of Sahara, from Mauritania and Senegal to Sudan (Cramp 1992). Different taxa overlap in winter quarters to a considerable but uncertain extent (Cramp 1992); an even greater confusion is expected (with respect to Cramp's considerations) due to the lumping of *S.* [*c.*] *cantillans* and *S. moltonii* in the period 1954-2001, obscuring the real pattern of each taxon. In absence of long-distance ringing recoveries, winter quarters of particular (breeding) populations remain unknown (Cramp 1992). Spring migration takes place in March-May, with *S. moltonii* arriving two weeks later, on average, on breeding grounds with respect to all other European populations (Shirihai et al. 2001). The extreme scarcity of migrants at Gibraltar in autumn compared with spring may indicate that most individuals overfly Western Mediterranean from their breeding grounds, in their route to wintering quarters (Shirihai et al. 2001). The larger numbers of migrants in spring than autumn on Cyprus and in Israel indicate (counter-clockwise) loop migration of *S.* [*c.*] *albistriata* populations (Shirihai et al. 2001).

MAIN CONCLUSIONS

Phylogeography of Marmora's Warbler (*Sylvia sarda*)

Our molecular investigation clearly revealed the existence of two deeply diverging lineages of *Sylvia sarda*: both mitochondrial and nuclear genetic markers allow complete diagnosability of the two taxa, which are also characterized by diverged vocalizations (both contact calls and male territorial song) and by unique male breeding plumage characters (Shirihai et al. 2001, Cramp & Simmons 2006). We therefore consider *Sylvia* [*sarda*] *balearica* a Confirmed Candidate Species (in an integrative taxonomic framework; Galimberti et al. 2012), awaiting for proper taxonomic description (as *Sylvia balearica*) and deposition of types. We furthermore recommend that the conservation status of *Sylvia balearica*, an insular endemic species with restricted distribution (Balearic Islands with the exception of Menorca), is correctly evaluated, in order to preserve an important element of Mediterranean biodiversity.

Phylogeography of Subalpine Warbler (*Sylvia* [*cantillans*])

Molecular variation in mitochondrial (*cytb* and *coxI*) and nuclear (g3pdh) loci, beyond confirming the complete diagnosability and deep divergence of *Sylvia moltonii* from all other taxa of the *Sylvia* [*cantillans*] species complex, highlighted a very similar situation for western populations of *S. c. cantillans*. In spite of a cryptic similarity in external morphology, this taxon is completely monophyletic and characterized by private haplotypes well diverged (the same degree of *S. moltonii*) from those of the other taxa of the complex.
The best partition of molecular variance of these three loci has been obtained by separating the populations of the species complex into three main groups ("species"): (1) *S. moltonii*, (2) western *S. c. cantillans* and (3) southern *S. c. cantillans* and *S. c. albistriata*.
Our molecular data, integrated with available information on the geographic distribution of the taxon, its partial morphological diagnosability from other members of the complex and the evolution of premating isolation mechanisms mediated by vocalizations, lead us to propose the status of Confirmed Candidate Species for western *S. c. cantillans*.
The complete failure of one nuclear locus (bFib5) in showing at least a minor degree of structuration in molecular variation in spite of the deep divergence accumulated by the taxon in several independent biological attributes confirms the recent doubts expressed about the usefulness of nuclear loci in settling taxonomic issues at the boundary of species level.

Wintering patterns of *Sylvia* [*cantillans*]

Molecular identification of wintering individuals yielded the first certain data upon wintering localities of *Sylvia moltonii*, recently elevated to species rank (Brambilla et al. 2008a), as well as of individuals of *Sylvia* [*cantillans*] *albistriata* and Western *Sylvia* [*cantillans*] *cantillans* (*sensu* Brambilla et al. 2008a). Previous data (Cramp & Simmons 2006), derived by observational or traditional ringing activities, were confounded by improper taxonomy and cryptic morphology and should be considered as generically referred to the species complex *Sylvia* [*cantillans*] (*sensu* Cramp 1992) and not precisely to a particular species/subspecies.
More importantly our data indicate that the reconstruction of the yearly patterns of the single taxa of the *Sylvia moltonii-Sylvia* [*cantillans*] complex cannot be accomplished without the institution of a more organized and widespread network of ringing stations in Northern and Central Africa, associated to molecular identification of captured individuals. We feel, indeed, that a more capillary African ringing network and widespread collaboration will benefit many other ornithological research projects, which could be conducted on other morphologically cryptic Mediterranean songbirds.

General conclusions

Our results (along with, e.g., Brambilla et al. 2008b, Pons et al. 2015a) further highlight the importance of the Mediterranean basin as a hotspot of biodiversity and we hope they will encourage future research on taxa with conservative morphology but geographically structured divergence in, e.g., vocalizations. We believe that the resolution of similar taxonomic issues lies in the domain of multi-criteria species delimitation: data on genetic structuration, while convincing *per se*, may be further accepted if supported by independent characters, such as divergence in vocalizations (Schlick-Steiner et al. 2010, Sangster 2014).

CHAPTER 2

Phylogeography of Marmora's Warbler (*Sylvia sarda*) populations

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INTRODUCTION

Mediterranean region is one of world's best explored areas in terms of ornithological research and it is considered a main biodiversity hotspot for the Western Palearctic (Myers et al. 2000), especially because it played a fundamental role in the divergence and speciation processes for many taxa, due to the dynamics linked mainly to glaciations and salinity crises (Hewitt 1996, Taberlet et al. 1998). Most taxonomical researches on Mediterranean bird species have been based on the traditional analysis of anatomical and morphological differentiation of populations. However, such kind of approach might have led to underestimating the real degree of divergence occurring in some populations in the region. This divergence could often be best evaluated in terms of genetic divergence and development of reproductive isolation mechanisms, which in oscine birds are usually mediated by vocalizations.

Recent researches on several taxa traditionally considered a single biological species, have in fact revealed the existence of morphologically indistinguishable (or nearly so) populations which are so greatly diverged in terms of genetics and vocalizations to definitely belong to separate entities, such as different allospecies (Shirihai et al. 2001) or even different 'full' species on their own even according to stringent criteria (Brambilla et al. 2008a). Those cryptic species may well remain hidden in plain sight, and the application of more modern and integrated analytical methods is urgently needed to shed light on some species complexes.

Marmora's Warbler (*Sylvia sarda*, Aves: Passeriformes: Sylviidae) is a Mediterranean endemism with an insular distribution: it inhabits Sardinia, Corsica, Balearic Islands excluding Menorca and some smaller islands alongside W Italy in the Tyrrhenian Sea. According to the 'traditional' taxonomy, it has been treated as a polytypic species with two allopatric subspecies, slightly different in terms of plumage and bare parts coloration: *S. s. balearica* (von Jordans, 1913), with distribution limited to the Balearic Islands, and the nominate *S. s. sarda* (Temminck, 1820), covering the rest of the breeding range. Whereas a difference in vocalizations between the two subspecies was noted by von Jordans (1914), the taxonomic rank of the two taxa had not been questioned until recently.

Shirihai et al. (2001), in their monumental research on the *Sylvia* genus, recently proposed to elevate *S. s. sarda* and *S. s. balearica* to the rank of allospecies, within the superspecies *S.* [*sarda*]. They based their taxonomical hypothesis upon descriptive analyses of vocal and morphometric

divergence and, secondarily, on plumage characters (not useful in all cases, in particular for adult females) and bare parts coloration. They intended to accompany their proposal with an analysis of the phylogeny and phylogeography of all the species of *Sylvia* genus based upon cytochrome b gene sequences, but such a follow up of their work, definitely needed to clarify the relationship between the two taxa, never came to light, leaving some uncertainty about the proposed taxonomic treatment.

Modern research aims to an integrated taxonomy, based upon multiple criteria spanning all main aspects of species biology (ecology, morphology, vocalizations and so on); DNA analyses are currently one of the most important kind of data available to the taxonomist and, while they preferably should not be used alone, they constitute a fundamental part of the modern multi-criteria approach to species delimitation (e.g. Padial et al. 2010, Schlick-Steiner et al. 2010, Fujita et al. 2012, Galimberti et al. 2012).

Here, we try to fill this void of knowledge in order to clarify these taxonomical issues regarding *Sylvia sarda*, complementing the already available information about morphology and vocalizations with the fundamental data about genetic divergence and its geographical structuring. We used phylogenetic and phylogeographic approaches based on mitochondrial (mtDNA) and nuclear DNA (nDNA) sequence data to analyse the differentiation among the populations of *Sylvia sarda* belonging to the two putative species.

We used both mtDNA and nDNA sequences in order to verify potential discordances in their reconstructions, which may reflect incomplete lineage sorting or other kinds of processes which may not be highlighted by mtDNA alone.

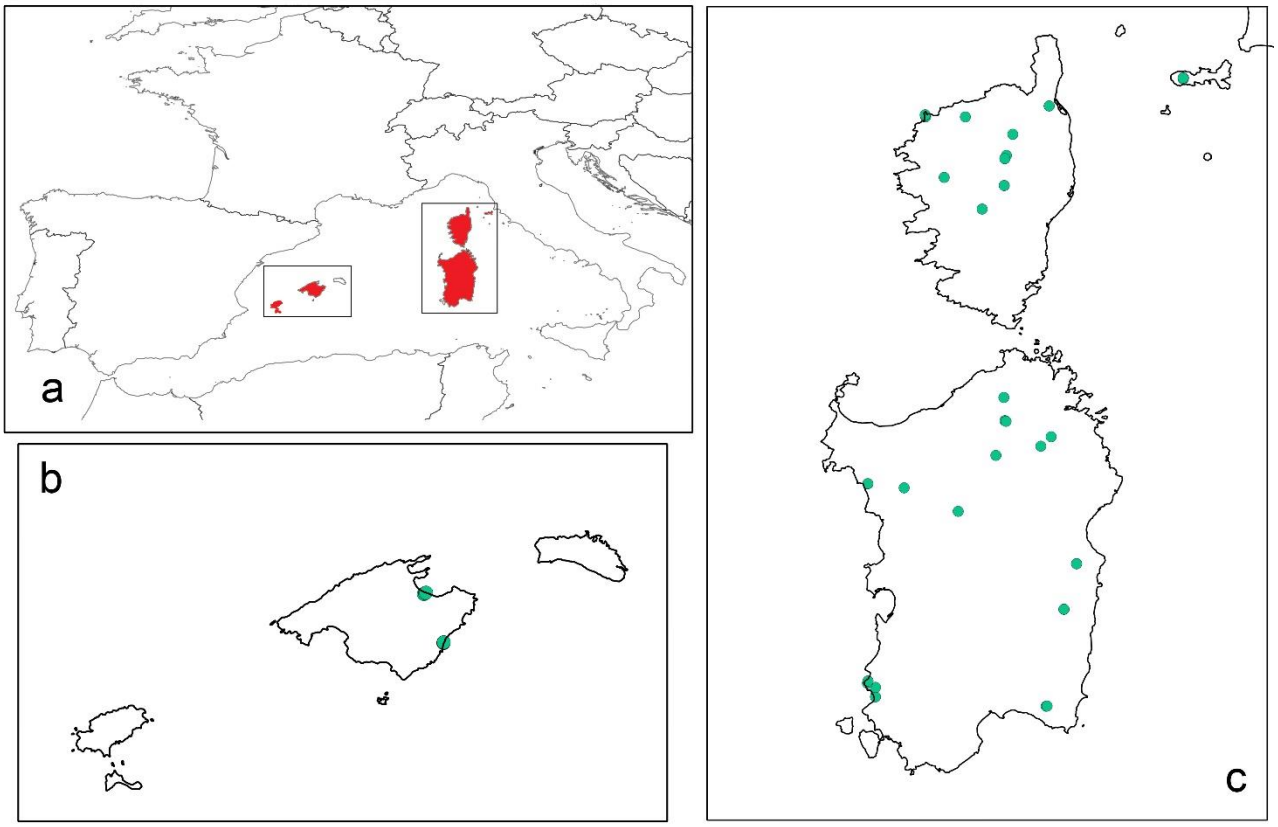
MATERIALS AND METHODS

Field methods

We sampled breeding birds between May and June in the period 2013-2015. Birds were trapped by mist-nets in different locations scattered over most of the breeding range of the species, on the islands of Mallorca (Spain), Corsica (France), Sardinia and Elba (Italy). We aimed to obtain a sampling scheme balanced both over the whole breeding range of the species and within each single island sampled, dividing each island in quadrants and sampling a balanced number of individuals in each one.

Sampled individuals are listed in Appendix and the distribution of the sampling localities is depicted in Figure 1.

Figure 1. *Geographical distribution of sampling localities: a) breeding range of Sylvia [sarda] (in red); b) sampling localities within Mallorca; c) sampling localities within Sardinia, Corsica and Elba.*



Laboratory methods

One or more feathers were collected from each bird and stored in 96% vol. ethanol at -20°C until further processing. Total DNA was extracted using Qiagen DNEasy Blood and Tissue Kit, following manufacturer's instructions, with the addition of 20µl DTT (dithiothreitol) to the initial incubation step of the extraction to accelerate tissue digestion.
The second half of the mitochondrial cytochrome b (*cytb*) gene was amplified and sequenced from each sample using PCR primers 648L and 1137H (Brambilla et al. 2008a). Amplifications were carried out in 8µl reactions using 2µl of template DNA solution, 0.80 µl 10x Taq buffer with 15mM $MgCl_2$ (5-Prime, Hilden, Deutschland), 0.80 µl of 0.2% BSA, 0.15µl of each 0.2mM primer, 0.40 µl

of 2.5mM dNTP, 0.2 U of Taq polymerase (5-Prime, Hilden, Deutschland), with 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.

Glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh) was amplified using primers G3P13b and G3P14b, and sequenced with primer G3PIntl1 and G3P14b (Irestedt et al. 2001). Amplifications were carried out following the same recipe used for *cytb* and the following thermal profile: 94°C for 2 min; 35 cycles at 94°C for 35 s, 57°C for 35 s, 72°C for 50 s; 72°C for 10 min.

In both cases, the amplicons were purified by enzymatic digestion of excess of primers with ExoSap-IT (Affymetrix) and the samples were sequenced using Big-Dye terminators (Applied Biosystems) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Software analysis methods

Sequences were manually proofread and corrected in Seqscape v2.5 (Applied Biosystems) and aligned in ClustalW (Thompson et al. 1994) with default parameters. In order to exclude the amplification of nuclear pseudogenes of mitochondrial origin (NUMTs; Sorenson & Fleischer 1996) we verified the absence of stop codons by translation of all sequences with the EMBL-EBI Emboss Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) with the subsequent options: frame=3, Codon table = "Vertebrate mitochondrial".

No g3pdh sequences contained insertions/deletions or double heterozygotes: genotype phasing was therefore straightforward and was carried out by DnaSP v5.10 (Librado and Rozas 2009) without ambiguity.

All sequences were trimmed in Bioedit (Hall 1999) and collapsed to unique haplotypes using the Dna Collapser tool in the FABOX 1.41 suite (Villesen 2007).

Estimators of molecular diversity, i.e. haplotype number, haplotype diversity (Hd), average number of nucleotide differences (k) and nucleotide diversity (π), were estimated for both loci as implemented in the program DnaSP v5.10 (for the entire dataset and for single islands). Tajima's D (Tajima 1989) and Fu and Li's D* and F* (Fu & Li 1993) tests for neutrality were conducted for each locus using DnaSP v5.10, to confirm that natural selection did not significantly influence the phylogenetic data and that the inferred phylogeny largely reflected the background rate of mutation.

We added to the *cytb*-dataset a single sequence of *Sylvia undata* (trapped on Mallorca during the ringing campaign) to be used as an outgroup for subsequent phylogenetic analyses. A phylogenetic tree was generated from *cytb* haplotypes by a Bayesian Inference approach. The identification of the best nucleotide substitution model was carried out in Jmodeltest2 (Darriba et al. 2012), among all the 203 possible models available (integrating the estimate of nucleotide frequencies, proportion of invariable sites and a gamma model with four categories), using all possible criteria of evaluation (AICc, BIC, DT). The base tree was ML optimized and the base tree search parameter was set on "best".

The Bayesian Inference tree was generated using MrBayes 3.2.6 (Ronquist et al. 2012) on CIPRES portal (Miller et al. 2010), using the single *Sylvia undata* sequence as the outgroup taxon. Four cold and one hot Metropolis-coupled Markov chain Monte Carlo chains were run for $2*10^7$ generations and sampled every 10000 generations, with default run parameters. Two runs with different starting points were carried out. The first $4*10^6$ generations were discarded as burn-in, and the posterior probability (PP) values were calculated for the remaining $1.6*10^7$ generations. We verified stationarity by checking that the potential scale reduction factor (PSRF) approached 1 (< 1.01) for all parameters and that the likelihood plot showed a pattern of "white noise" (Gelman 1996).

Haplotype networks may provide some insights into intraspecific and interspecific molecular structuring, which may easily prove to be reticulated instead of branching (Smouse 2000, Martin et al. 2013). For both *cytb* and g3pdh genes, Median Joining haplotype networks were generated by Network 5.0 (http://www.fluxus-engineering.com, Bandelt et al. 1999) with default parameters.

Genetic uncorrected p-distances (within and among groups, plus standard error calculated on 1000 bootstrap replicates) were estimated in MEGA 7 (Kumar et al. 2016).

Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) were conducted using Arlequin v3.5 (Excoffier & Lischer 2010) to examine the structure of molecular variation. AMOVA calculates Φ statistics, which are analogous to F-statistics (Wright 1951), in order to assess how much of the molecular variation is explained at different levels of structuring. Φ_{CT} values indicate the amount of variation explained among groups, Φ_{SC} values indicate the amount of variation explained among populations within groups, Φ_{ST} values indicate the amount of variation explained within populations (Martin et al. 2013). However, AMOVA cannot autonomously identify groups and populations, but the assignment of populations into *a priori* groups is needed: we decided to separate populations by the traditional subspecies definition in the two groups *S. s. sarda*

(populations of Sardinia, Corsica and Elba) and *S. s. balearica* (Mallorca).
In a first AMOVA analysis we ascribed individuals sampled within each island to a single population, for a total of four populations (i.e., Mallorca, Sardinia, Corsica and Elba). Although considering individuals of each island as members of a single panmittic population probably has more biological sense than any alternative treatments, the resulting number of populations is too low to allow the identification of significant structures at group level by means of permutation tests (Fitzpatrick 2009). The lowest possible expected p-value in the case of two groups respectively consisting of one and three populations is 0.2500.
A second AMOVA analysis was therefore performed, splitting each island in subsets, each regarded as a population: Northern and Southern Mallorca; NW-, NE-, SE- and SW-Sardinia; Western and Eastern Corsica; and Elba. We obtained two groups, respectively consisting of two and seven populations (Northern and Southern Mallorca versus all the other populations): this treatment should allow the recognition of significant structures at group levels, with a theoretical lowest expected p-value of 0.0278 (Fitzpatrick 2009). We suggest analysing only indices calculated with the first AMOVA analysis, due to a more biologically reasonable definition of populations; however the second AMOVA more appropriately evaluated the significance of group-level structure due to an adequate statistical power, and only this p-value for Φ_{CT} should be considered. Furthermore, we tested all the other possible partitions of the four populations (Mallorca, Sardinia, Corsica and Elba) in two groups, to evaluate which partition scheme was most supported by the molecular data (Fitzpatrick 2009).

RESULTS

We obtained 62 sequences of the second half of the *cytb* gene, spanning 537 bp. All the cytochrome b sequences we obtained could be functionally translated without premature terminations. Therefore, there was no evidence suggesting that the presence of NUMTs may have biased our analyses on mtDNA. Of the 537 sites, only seven were variable of which six parsimony informative. The 62 sequences were collapsed into five unique haplotypes (Genbank accession numbers: KX078574 - KX078578).

All *S. s. balearica* individuals possessed private haplotypes for *cytb*: one individual possessed haplotype *cytb*-2, all other individuals *cytb*-1. Haplotypes *cytb*-3 and *cytb*-4 were shared by individuals of *S. s. sarda* sampled in all other islands, while haplotype *cytb*-5 was present in just one Sardinian sample.

Tajima's D and Fu and Li's D* and F* tests indicated that *cytb* did not significantly depart from neutral evolution (Tajima's D = 1.10898, P>0.10; Fu & Li's D* = 0.41725, P>0.10; Fu & Li's F* = 0.76533, P>0.10). Molecular diversity indices for both loci (for the whole dataset and for single islands) are reported in Table 1.

Table 1. *Molecular diversity indices for cytb and g3pdh loci: number of individual (haploid) sequences, number of haplotypes, haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences (k).*

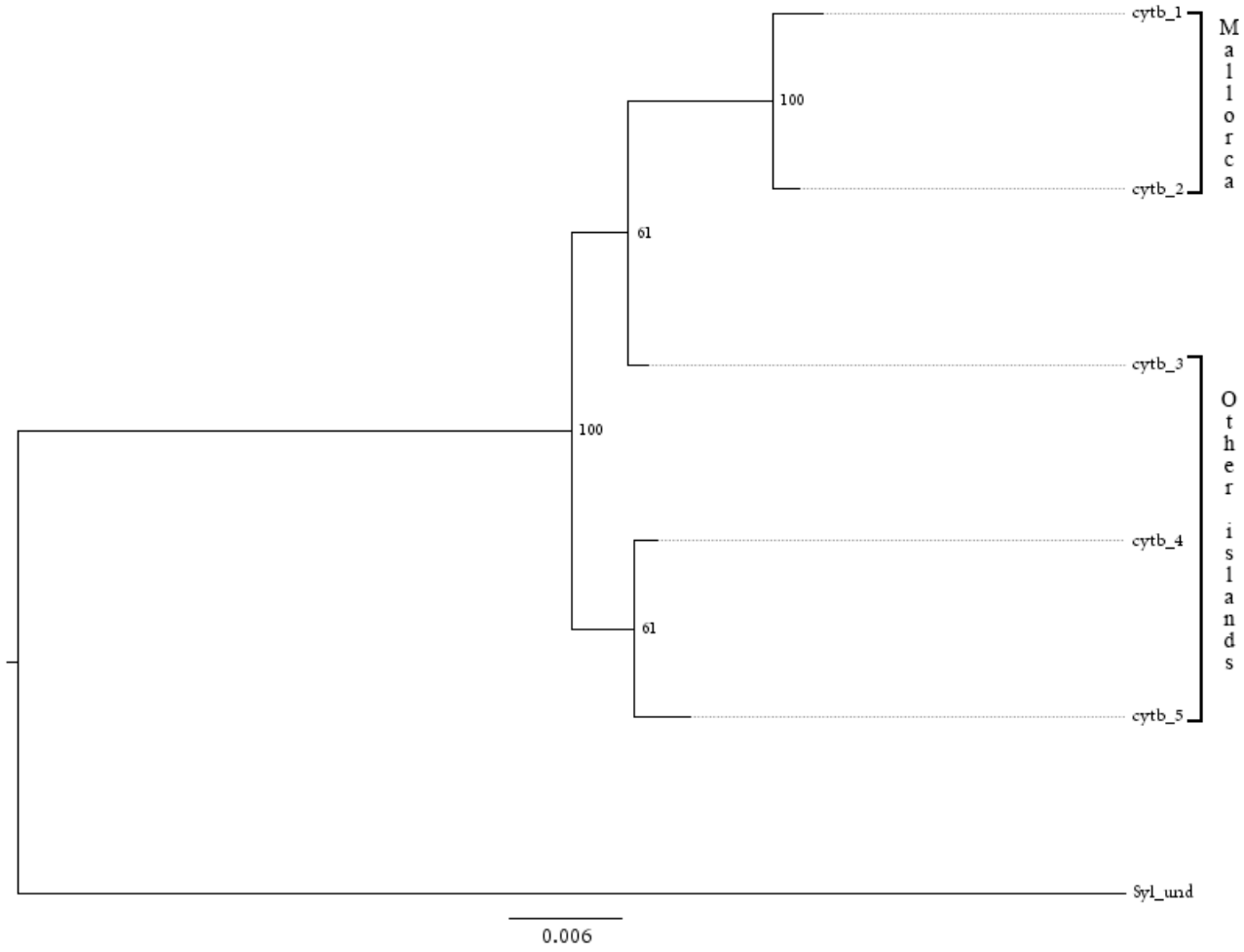
OVERALL MOLECULAR VARIABILITY					
Locus	No. Sequences	No. Haplotypes	π	Hd	k
Cytb	62	5	0.00402	0.66	2.1412
g3pdh	106	7	0.00378	0.756	1.3522
cytb DIVERSITY INDICES PER POPULATION					
Pop	No. Sequences	No. Haplotypes	π	Hd	K
Mallorca	13	2	0.00029	0.154	0.15385
Elba	4	2	0.00093	0.500	0.50000
Sardinia	31	3	0.00108	0.546	0.57634
Corsica	14	2	0.00049	0.264	0.26374
g3pdh DIVERSITY INDICES PER POPULATION					
Pop	No. Sequences	No. Haplotypes	π	Hd	K
Mallorca	24	1	0.00000	0.000	0.00000
Elba	8	2	0.00120	0.429	0.42857
Sardinia	52	6	0.00259	0.653	0.92760
Corsica	22	5	0.00306	0.758	1.09524

Unfortunately, a few samples resisted several trials of sequencing for the g3pdh intron 11, so we obtained only 53 sequences, spanning 358 bp (274 bp of intron 11 and 84 bp of flanking exons). Only four of the 358 sites were variable and parsimony informative. The 53 sequences were collapsed into seven unique haplotypes (Genbank accession numbers: KX078579-KX078585). All *S. s. balearica* samples were homozygotes for a private allele, g3pdh-1; the remaining haplotypes were shared by individuals sampled in at least two of the other islands (Sardinia, Corsica and Elba), except for g3pdh-4, occurring only in Sardinian individuals. Tajima's D and Fu and Li's D* and F* tests indicated that g3pdh did not significantly depart from neutral evolution (Tajima's D = 1.51169, P>0.10; Fu & Li's D* = 0.93570, P>0.10; Fu & Li's F* = 1.31993, P>0.10).

Phylogenetic tree Two of the three decision criteria (BIC and DT) selected the HKY model of substitution as the most appropriate for the *cytb* haplotype sequences; AICc selected TPM3uf as the best model, with HKY being selected as second-best model with a delta of 0.706. We therefore decided to use the HKY model for the subsequent analyses, also because of its availability in MrBayes v2.6.0.

Only nodes having a Bayesian posterior probability (PP) greater than 0.95 can be considered well-supported (Klicka et al. 2004). The *cytb* BI tree (Figure 2) looked therefore largely unresolved, with a single highly supported clade (PP>0.95): this clade consisted of two haplotypes (*cytb*-1 and *cytb*-2) private of individuals trapped on Mallorca, i.e. *S. s. balearica*.

***Figure 2**. 50% majority consensus rule Bayesian Inference tree inferred from 537bp cytochrome b fragment haplotypes. Bayesian Posterior Probabilities of each node are reported.*



Haplotype networks The haplotype network of *cytb* (Figure 3) better clarified the situation: *cytb*-1 and *cytb*-2, differing for a single mutation, were possessed by only Mallorca individuals; such group was separated from the main haplogroup by 4 mutations; haplotypes *cytb*-3 and *cytb*-4 were shared by individuals sampled on all other islands, while *cytb5* was possessed by Sardinian individuals only.

The haplotype network of g3pdh (Figure 4) largely gave the same information. All haplotypes were separated by a single mutation from one another. g3hpd-1 was the only haplotype found in Mallorca individuals, which were all homozygotes for this private allele. All the other haplotypes were shared by individuals sampled on at least two different islands of the remaining breeding range, except for g3pdh-4, possessed only by Sardinian individuals.

***Figure 3**. Median joining haplotype network of 537bp cytochrome b fragment. Circle sizes are proportional to the number of sequences collapsed to each haplotype (n indicated inside circles), slices reflect the geographical origin of the sequences collapsed to each haplotype. Branches between circles accounts for one mutational step; black circles were placed on the branches to represent missing intermediate steps. Legend: yellow = Mallorca, red = Sardinia, green = Corsica, blue = Elba.*

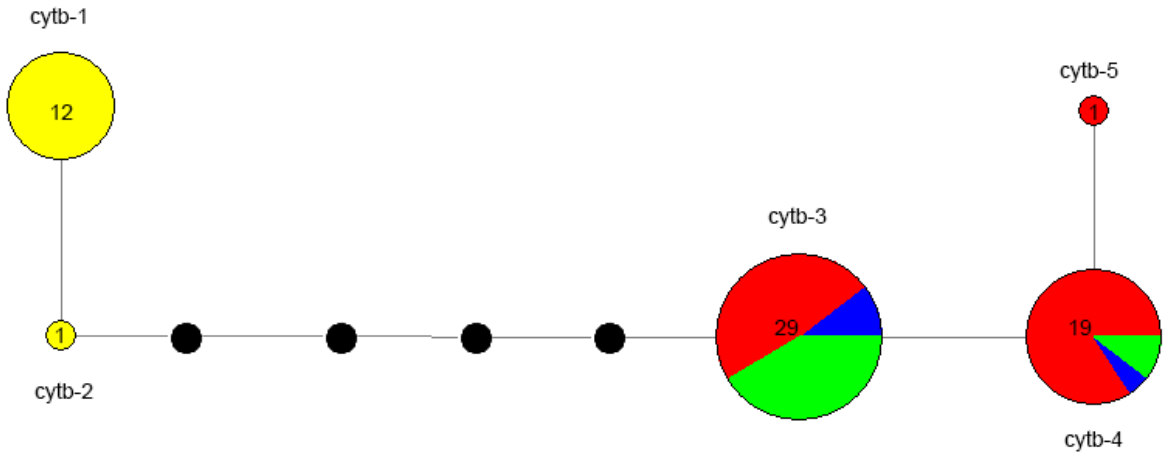
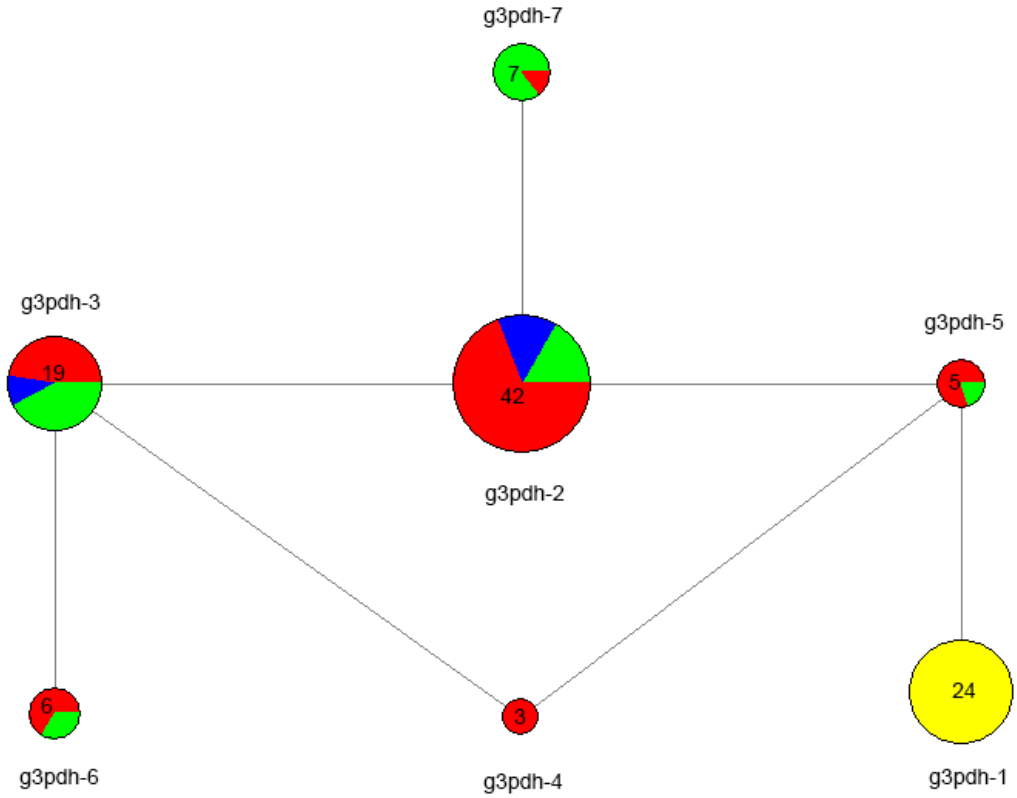


***Figure 4.** Median joining haplotype network of 358bp of glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh). Circle sizes are proportional to the number of sequences collapsed to each haplotype (n indicated inside circles), slices reflect the geographical origin of the sequences collapsed to each haplotype. Branches between circles accounts for one mutational step. Legend: yellow = Mallorca, red = Sardinia, green = Corsica, blue = Elba*



Genetic distances Genetic distances within and between different populations are reported into Table 2 for *cytb* and Table 3 for g3pdh. For *cytb* the amount of population divergence between Mallorca population and each of the others (1%) was about 10-fold greater than the divergence revealed by all other comparisons, and about 3-fold for g3pdh. The degrees of divergence between Sardinia, Corsica and Elba populations were always comparable with their respective within-island variation levels.

Table 2. *Genetic distances (uncorrected p-distances ± S.E.) within and among different island populations for 537bp of the cytochrome b. Standard errors were calculated by means of 1000 bootstrap replicates.*

	Genetic distances within island	**Genetic distances between islands**		
Island		**Sardinia**	**Corsica**	**Elba**
Mallorca	0.000 ± 0.000	0.010 ± 0.004	0.010 ± 0.004	0.010 ± 0.004
Sardinia	0.001 ± 0.001		0.001 ± 0.001	0.001 ± 0.001
Corsica	0.000 ± 0.000			0.001 ± 0.001
Elba	0.001 ± 0.001			

Table 3. *Genetic distances (uncorrected p-distances ± S.E.) within and among different island populations for 358bp of glyceraldehyde-3-phosphate dehydrogenase intron 11. Standard errors were calculated by means of 1000 bootstrap replicates.*

	Genetic distances within island	**Genetic distances between islands**		
Island		**Sardinia**	**Corsica**	**Elba**
Mallorca	0.000 ± 0.000	0.006 ± 0.004	0.007 ± 0.004	0.006 ± 0.004
Sardinia	0.003 ± 0.002		0.003 ± 0.002	0.002 ± 0.001
Corsica	0.003 ± 0.002			0.002 ± 0.001
Elba	0.001 ± 0.001			

AMOVA Results of AMOVA for *cytb* and g3pdh are reported in Table 4 and Table 5, respectively. Analyses at both loci revealed that most part of the molecular variation (90.48% and 70.78%, respectively, for *cytb* and g3pdh) was attributable to differences between groups, i.e. the traditionally recognized subspecies. The least variation occurred among different populations within subspecies (even when defined in an appropriate biological sense, i.e., AMOVA-1 for both loci); the within-populations variation was far greater than the between-populations one in both cases. All hierarchical structure levels were significant at α=0.05. In particular, group-level structuration always obtained a p-value close to the theoretical minimum p-value, both in AMOVA-1 and AMOVA-2.

Furthermore, we tested all the other possible partitions of the four populations (Mallorca, Sardinia, Corsica and Elba) in two groups, and obtained p-values much higher than both the current partition and the theoretical minimum expected p-value, in all cases (data not shown). The grouping proposed here was, therefore, the best possible treatment of geographical populations of *S. sarda* (Fitzpatrick 2009).

Table 4. *Analyses of Molecular Variance results and fixation indices for 537bp of cytochrome b. In AMOVA-1 each island was defined as a single population; in AMOVA-2 islands were subdivided in multiple populations (N-Mallorca, S-Mallorca, NW-Sardinia, NE- Sardinia, SE- Sardinia,SW-Sardinia; W- Corsica, E- Corsica, Elba).*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value	Fixation indices
AMOVA-1 (populations = Mallorca, Sardinia, Corsica, Elba)						
Among groups	1	51.567	2.46108	90.48	P=0.2522	Φ_{CT} = 0.90478
Among populations within groups	2	1.707	0.05155	1.9	P=0.0225	Φ_{SC} = 0.19904
Within populations	58	12.033	0.20746	7.63	P<0.0001	Φ_{ST} = 0.90478
AMOVA-2 (populations = N- and S-Mallorca; NW-, NE-, SE-, SW-Sardinia; W- and E-Corsica; Elba)						
Among groups	1	51.567	2.48708	91.31	P=0.02851	Φ_{CT} = 0.91313
Among populations within groups	7	2.798	0.03015	1.11	P=0.09307	Φ_{SC} = 0.12744
Within populations	53	10.942	0.20645	7.58	P<0.0001	Φ_{ST} = 0.92420

Table 5. *Analyses of Molecular Variance results and fixation indices for 358bp of glyceraldehyde-3-phosphate dehydrogenase intron 11. In AMOVA-1 each island was defined as a single population; in AMOVA-2 islands were subdivided in multiple populations (N-Mallorca, S-Mallorca, NW-Sardinia, NE-Sardinia, SE- Sardinia, SW-Sardinia; W- Corsica, E- Corsica, Elba).*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value	Fixation indices
AMOVA-1 (populations = Mallorca, Sardinia, Corsica, Elba)						
Among groups	1	33.369	0.88460	70.78	P=0.25611	Φ_{CT} = 0.70777
Among populations within groups	2	0.968	0.00589	0.47	P=0.37928	Φ_{SC} = 0.01613
Within populations	102	36.654	0.35935	28.75	P<0.00001	Φ_{ST} = 0.71248
AMOVA-2 (populations = N- and S-Mallorca; NW-, NE-, SE-, SW-Sardinia; W- and E-Corsica; Elba)						
Among groups	1	33.369	0.88699	70.95	P=0.02151	Φ_{CT} = 0.70777
Among populations within groups	7	2.940	0.00557	0.45	P=0.39003	Φ_{SC} = 0.01613
Within populations	97	34.682	0.35755	28.60	P<0.00001	Φ_{ST} = 0.71248

DISCUSSION

Our results indicated a well-defined genetic differentiation between *S. s. balearica* and *S. s. sarda*. All *S. s. balearica* individuals possessed private haplotypes for both *cytb* and g3pdh, whereas *S. s. sarda* individuals (sampled in what could have been thought of as separate populations) largely shared the remaining unique haplotypes.

Haplotypes typical of *S. s. sarda* and *S. s. balearica* differed for at least 4 mutations for *cytb* and for only one mutation for g3pdh; this difference in the degree of divergence was however expected (in case of total isolation) because of the different effective population sizes of mitochondrial (haploid and uni-parentally inherited) and nuclear (diploid and bi-parentally inherited) markers. The existence of private alleles was indicative of a prolonged isolation and a degree of differentiation which could lead to question the actual taxonomic treatment of the two taxa (Ross et al. 2010).

The AMOVAs again highlighted that most part of the molecular divergence between individuals of *S. sarda* is due to the structuration in two different blocks, coinciding with the two traditionally recognized subspecies. The divergence between individuals inhabiting different islands of the Mediterranean (Sardinia, Corsica and Elba) were very low and far lower than the normal differentiation levels realized between individuals inhabiting any single island.

The two taxa were indeed genetically well defined and clearly separated; the degree of divergence in terms of *cytb* sequence was about 1% (uncorrected proportional genetic distance). This amount of divergence is quite high for populations separated by only few hundreds of km and had been judged enough to support the proposal of elevation to species rank in Passerines (Li et al. 2006, Reddy 2008, McKay et al. 2010). However, the same amount of divergence was considered well within the range of subspecific differentiation in the Sylvioidea superfamily by Helbig et al. (1995) and not enough to propose more than a subspecific status for taxa of the *Sylvia* [*cantillans*] species complex by Brambilla et al. (2008a). Comparing the degree of divergence with known cases, however, makes sense only in light of the Biological Species Concept (BSC; Mayr 1942), in an attempt of extending its domain to allopatric geographic distributions, situations in which it cannot be applied in a direct manner. The Biological Species Concept is not useful to define the species status of allopatric taxa, because evaluating the actual degree of reproductive isolation is impossible in absence of simpatry. More direct operational criteria exist that can be easily applied to the present context.

Helbig et al. (2002), for example, considered diagnosability of genotypes and phenotypes one of the main criteria to assign species status in birds. Our research indicates that the two taxa (*S. s. sarda* and *S. s. balearica*) are clearly diagnosable by their genotypes; and Shirihai et al. (2001) demonstrated the existence of diagnosably different phenotypical characters (vocalizations, biometry and plumage) between the two forms. Diagnosability of these kinds of characters suggests long-term interruption of gene flow between populations.

Furthermore, these two conditions, diagnosability and evolutionary independence, are the only requirements for granting species status following the Phylogenetic Species Concept (Cracraft 1983). Diagnosability means that individuals of one taxon can be identified and separated with 100% certainty from individuals of the other taxa of the species complex by means of at least one fixed character (DNA mutations, morphological qualitative character, vocalization type, …) or on the basis of different ranges of variation of quantitative characters.
Our results clearly indicate the existence of two different and independent characters (genetic markers) which guarantee complete diagnosability between *S. s. sarda* and *S. s. balearica* individuals (males and females); the two taxa may therefore be considered "full" species strictly applying the original Phylogenetic Species Concept.

Recent tendencies in taxonomy, however, point to a multi-criteria delimitation of species, involving several biological attributes of the examined taxa ("integrated taxonomy"; Padial et al. 2010, Galimberti et al. 2012; for ornithological research in particular see Sangster 2014). Among these characters are vocalizations: Shirihai et al. (2001) clearly demonstrated the divergence and diagnosability of the two forms according to vocalizations (in terms of both contact call and male territorial song).

We adhere here to a recent revision of the integrated taxonomy framework (Galimberti et al. 2012), which defined a series of intermediate taxonomical grades on the road to the definition of new species, each requiring a greater content of independent data about the differentiation between the analysed taxa. They defined Molecular Operational Taxonomic Units (MOTUs) as "groups of unidentified organisms sharing similar sequences", Unconfirmed Candidate Species (UCS) as "groups of organisms within a species that are distinct at the molecular level from other members of the species" and Integrated Operational Taxonomic Units (IOTUs) as molecular lineages that are supported by at least one more taxonomic characteristic. The last step on the way to species recognition is Confirmed Candidate Species (CCS), previously defined by Padial et al. (2010) as "deep genealogical lineages that can be considered good species following standards of

divergence for the group under study but that have not yet been formally described and named. For example […] allopatric lineages with distinct morphological or bioacoustical character divergences".

The morphological diagnosability, the reciprocal monophily of individuals of the two taxa (as demonstrated here), the divergence in the vocal repertoires of males (one of the main character leading to reproductive isolation in songbirds) shown by Shirihai et al. (2001) and the correspondence between all these independent data and geography would lead to the acknowledgement of the status of IOTU (*sensu* Galimberti et al. 2012) or even CCS (Padial et al. 2010, Galimberti et al. 2012) to *S. sarda balearica*.

In the spirit of a complete integrated taxonomy approach, we believe it would be worth to explore, in the near future, other interesting biological attributes of the two species, in particular ecological needs and levels of response to playback of territorial song between the two forms. The possible divergence of the two taxa in these respects may finally lead to the wide acceptance of their "full" species status, after a formal description (Galimberti et al. 2012).

APPENDIX TO CHAPTER 2

Appendix. *Sample id; haplotypes for 537bp of cytochrome b (cytb) and 358bp of glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh); latitude, longitude and island of trapping.*

	Haplotypes					
ID	***cytb***	**g3pdh (allele 1)**	**g3pdh (allele 2)**	**Latitude**	**Longitude**	**Island**
SSA03	1	1	1	39.744286	3.170699	Mallorca
SSA04	1	1	1	39.748944	3.177025	Mallorca
SSA06	1	1	1	39.470166	3.280321	Mallorca
SSA07	1	1	1	39.470778	3.280451	Mallorca
SSA08	1	1	1	39.751638	3.182741	Mallorca
SSA09	1	1	1	39.469254	3.281154	Mallorca
SSA10	1	1	1	39.469254	3.281154	Mallorca
SSA11	1	1	1	39.471162	3.282045	Mallorca
SSA12	2	1	1	39.472109	3.281514	Mallorca
SSA13	1	1	1	39.472109	3.281514	Mallorca
SSA14	1	1	1	39.472109	3.281514	Mallorca
SSA15	1	-	-	39.472109	3.281514	Mallorca
SSA17	1	1	1	39.473225	3.282031	Mallorca
SSA18	3	2	2	42.772675	10.169999	Elba
SSA21	4	2	2	42.778167	10.171948	Elba
SSA22	3	2	2	42.77883	10.169613	Elba
SSA29	4	2	2	40.053778	9.570469	Sardinia
SSA30	3	2	2	40.053778	9.570469	Sardinia
SSA23	3	-	-	40.053778	9.570469	Sardinia
SSA24	3	2	2	40.053778	9.570469	Sardinia
SSA25	3	3	4	40.053778	9.570469	Sardinia
SSA26	3	2	2	40.053778	9.570469	Sardinia
SSA27	4	2	2	40.053778	9.570469	Sardinia
SSA31	3	3	3	42.778832	10.169613	Elba
SSA32	3	-	-	40.856249	9.167481	Sardinia
SSA33	4	2	2	40.852627	9.173701	Sardinia
SSA34	3	3	3	40.766178	9.427426	Sardinia
SSA35	3	2	2	40.713228	9.369324	Sardinia
SSA36	4	2	5	40.986329	9.161331	Sardinia
SSA37	4	2	2	40.502995	8.396651	Sardinia
SSA38	4	2	2	40.502995	8.396651	Sardinia
SSA39	4	6	6	40.347701	8.904704	Sardinia
SSA40	3	3	4	40.479583	8.600801	Sardinia
SSA41	3	2	2	40.661428	9.116621	Sardinia
SSA42	3	-	-	39.254478	9.399521	Sardinia
SSA43	4	-	-	39.255831	9.404547	Sardinia

(continues…)

(…continues)

SSA44	4	-	-	39.307357	8.439455	Sardinia
SSA45	4	3	3	39.307357	8.439455	Sardinia
SSA46	4	-	-	39.360791	8.439504	Sardinia
SSA47	3	5	5	39.384091	8.394254	Sardinia
SSA48	4	2	2	39.396183	8.397611	Sardinia
SSA49	4	2	2	39.396183	8.397611	Sardinia
SSA50	3	2	2	42.621343	9.416281	Corsica
SSA51	3	7	7	42.621343	9.416281	Corsica
SSA52	3	6	6	42.343863	9.175694	Corsica
SSA53	3	-	-	42.462384	9.211615	Corsica
SSA54	3	2	5	42.559956	8.944973	Corsica
SSA55	3	-	-	42.220507	8.826005	Corsica
SSA56	4	2	7	42.043016	9.039547	Corsica
SSA57	3	3	3	42.175148	9.163639	Corsica
SSA58	3	3	3	42.175148	9.163639	Corsica
SSA61	3	3	3	42.56073	8.721843	Corsica
SSA62	3	3	3	42.569054	8.719259	Corsica
SSA63	3	2	2	42.571837	8.719137	Corsica
SSA64	3	7	7	42.325901	9.166712	Corsica
SSA65	4	2	7	42.325901	9.166712	Corsica
SSA66	4	2	7	39.798407	9.499367	Sardinia
SSA67	5	2	2	39.798407	9.499367	Sardinia
SSA68	3	3	3	39.798407	9.499367	Sardinia
SSA69	4	2	5	39.798407	9.499367	Sardinia
SSA71	3	3	4	39.798407	9.499367	Sardinia
SSA88	4	6	6	39.798407	9.499367	Sardinia

CHAPTER 3

Phylogeography of the Subalpine Warbler (*Sylvia* [*cantillans*]) complex

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INTRODUCTION

Subalpine Warbler, *Sylvia* [*cantillans*] (Aves: Sylviidae), an Old World warbler with distribution restricted to the Mediterranean basin, has traditionally been regarded as a single polytypic species, with four subspecies (Shirihai et al. 2001):

- *S. c. cantillans*, breeding in continental Spain, France and Italy;

- *S. c. moltonii*, breeding on Mediterranean islands (Corsica, Sardinia, Balearic Islands and several minor islands) and on part of continental Italy (Brambilla et al. 2008b);

- *S. c. albistriata*, breeding from Trieste, through Balkans, till Greece and Turkey;

- *S. c. inornata*, breeding in Northern Africa and, probably, Southern Spain.

They differ from each other mainly for slight differences in breeding male plumage. Their largely conservative external morphology, however, has been proven to conceal major differences in genetics and bioacoustics. Recent analyses of molecular, bioacoustical and ecological data (Shirihai et al. 2001, Brambilla et al. 2007, 2008a, 2008c) has in fact revealed a much more intricate intraspecific pattern of divergence, leading to the elevation of *S.* [*c.*] *moltonii* to species status (*Sylvia moltonii*, syn. *Sylvia subalpina*), currently undisputed.
Whereas the taxonomic status of *S.* [*c.*] *moltonii* had been questioned before the availability of genetic analyses, on the basis of an atypical moult pattern and vocalizations different from all other taxa of the species complex, only molecular data could have revealed a more subtle pattern of divergence within a single traditional subspecies. Cytochrome b sequences gathered by Brambilla et al. (2008a) in fact revealed that western (French and Spanish) populations of the nominate subspecies (*S.* [*c.*] *cantillans*) were as much as diverged as *S. moltonii* from all other taxa of the species complex, including Italian populations of the same nominate subspecies.
This kind of divergence was absolutely unexpected, completely concealed by a cryptic similarity in morphology and by very similar vocalizations.

However, taxonomic information based on a single marker was not deemed enough to elevate the taxon to species status by Brambilla et al. (2008a). Later amateur contributions (Svensson 2013a, 2013b) tried to shed light on the issue, proposing a morphological character (extent and shape of white wedge on the fifth rectrix of adult individuals) capable of partially diagnose the two taxa. Based on this character Svensson (2013a) proposed a tentative revised taxonomy of the species

complex *Sylvia* [*cantillans*] with deposition of types for the species he defined. Unfortunately, such analyses were only based on museum specimens, most of which were sampled as migrating individuals of dubious origin (Zuccon, *pers. com.*). Furthermore, the hypothetical diagnostic character was not thoroughly evaluated in association with genetic data, currently the only certain way to assign individuals to the two lineages (Brambilla et al. 2010).

In this new reappraisal, we sought to shed some light on the taxonomic status of the *Sylvia* [*cantillans*] species complex by complementing cytochrome b information (here updated and re-analysed based on new samples) with sequences from another mitochondrial and two nuclear loci. Cytochrome c oxidase subunit 1 (*coxI*) had been regularly used to spot morphologically cryptic species and a threshold of molecular divergence has been proposed to help in the task with birds (Hebert et al. 2004, Kerr et al. 2009). However, mitochondrial DNA (mtDNA) as well as any single locus, has a specific history that can differ from the true history of the species; this problem may be particularly important when analysing lineages diverged fairly recently, due to the possible presence of incomplete lineage sorting (Ballard & Withlock 2004). We therefore analysed two nuclear DNA (nDNA) loci, in order to compare mtDNA results with an independent source of information. The best nuclear DNA loci for analysis of divergence at the species boundary are introns of protein-coding genes: whereas they coalesce at one-fourth the rate of mtDNA (due to diploidy and biparental heredity), their rate of variation is faster than the flanking exons (Pons et al. 2004) and therefore may provide more parsimony informative sites than exons.
We therefore analysed glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh) and beta-fibrinogen intron 5 (bFib5).

After a phylogeographic analysis of the molecular divergence in these four markers, we propose a taxonomic hypothesis based on an integrated approach (Padial et al. 2010, Galimberti et al. 2012), cumulating other lines of evidence coming from geography, bioacoustics and morphology.

MATERIALS AND METHODS

We analysed 50 feather samples previously deposited in the private collections of I.S.P.RA. (Institute for Environmental Protection and Research, Ozzano Emilia, Italy) and M.C.C.I. (Natural History Musem, Carmagnola, Italy). Almost all feather samples came from breeding birds, trapped by mist-nets in different locations scattered over most of the breeding range of the species, including Spain, France, Italy and Croatia. Only two samples (MCCI2981 and MCCI3122) came from migrants, but morphological identification of these individuals was straightforward. Sampled individuals and capture localities are listed in Appendix.
Birds were attributed to taxon level on the basis of sampling locality. Only two taxa occur in simpatry (*S. moltonii* and southern *S. c. cantillans*), but they were easily identified during ringing activities by means of plumage characters and contact call when released.

Samples consisted of one or more feathers stored in 96% vol. ethanol at -20°C. Total DNA was extracted using Qiagen DNEasy Blood and Tissue Kit, following manufacturer's instructions, with the addition of 20µl DTT (dithiothreitol) to the initial incubation step of the extraction to accelerate tissue digestion.
All 50 individuals were sequenced for two mitochondrial protein-coding genes: cytochrome b (*cytb*) and cytochrome oxidase subunit 1 (*coxI*). 25 individuals were also sequenced for two nuclear introns: glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh) and beta-fibrinogen intron 5 (bFib5).

cytb gene was amplified and sequenced from each sample using PCR primers 3L, 662H, 648L and 1137H (Brambilla *et al.* 2008a). Amplifications were carried out in 8µl reactions using 2µl of template DNA solution, 0.80 µl 10x Taq buffer with 15mM $MgCl_2$ (5-Prime, Hilden, Deutschland), 0.80 µl of 0.2% BSA, 0.15µl of each 0.2mM primer, 0.40 µl of 2.5mM dNTP, 0.2 U of Taq polymerase (5-Prime, Hilden, Deutschland), with 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.

coxI gene was partially (648bp) amplified and sequenced from each sample using PCR primers BirdF1 and BirdR1 (Hebert et al. 2004). Amplifications were carried out in 8µl reactions (same recipe used for *cytb*), with the following thermal profile: 94°C for 2 min; 45 cycles at 94°C for 30 s, 55°C for 30 s, 72° for 45 s; 72°C for 10 min.

g3pdh was amplified using primers G3P13b and G3P14b, and sequenced with primers G3PIntl1 and G3P14b (Irestedt et al. 2001). Amplifications were carried out following the same recipe used for *cytb* and the following thermal profile: 94°C for 2 min; 35 cycles at 94°C for 35 s, 57°C for 35 s, 72°C for 50 s; 72°C for 10 min.

bFib5 was amplified and sequenced using primers Fib5 and Fib6 (Fuchs et al. 2004), with the same recipe used for *cytb* and the following thermal profile: 94°C for 5 min; 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 45 s; 72°C for 10 min.

Amplicons were purified by enzymatic digestion of excess of primers with ExoSap-IT (Affymetrix) and the samples were sequenced using Big-Dye terminators (Applied Biosystems) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Sequences were manually proofread and corrected in Seqscape v2.5 (Applied Biosystems) and aligned in ClustalW (Thompson et al. 1994) with default parameters, as implemented in Bioedit 7.1.11 (Hall 1999). Alignment was straightforward for *cytb*, *coxI* and g3pdh, which showed no indels. A single individual showed a 2bp-deletion in Fib5 sequence. We considered it as a single informative mutational event. Sequence file was modified to take into account this form of genetic variation by replacing the missing value with a nucleotide that would induce a mutation (Pons et al. 2015b).

In order to exclude the amplification of nuclear pseudogenes of mitochondrial origin (NUMTs; Sorenson & Fleischer 1996) instead of the two mitochondrial genes, we verified the absence of stop codons by translation of all sequences with the EMBL-EBI Emboss Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) with the option: Codon table = "Vertebrate mitochondrial".

All sequences were trimmed in Bioedit (Hall 1999) and collapsed to unique haplotypes using the Dna Collapser tool in the FABOX 1.41 suite (Villesen 2007).

Estimators of molecular diversity, i.e. haplotype number, haplotype diversity (Hd), average number of nucleotide differences (k) and nucleotide diversity (π), were calculated for all loci as implemented in the program DnaSP v5.10 (Librado & Rozas 2009). Tajima's D (Tajima 1989) and Fu and Li's D* and F* (Fu & Li 1993) tests for neutrality were conducted for all loci using DnaSP v5.10, to confirm that natural selection did not significantly influence the phylogenetic data and that the inferred phylogeny largely reflected the background rate of mutation.

We added to the *cytb* and *coxI* dataset a single sequence of *Sylvia crassirostris* (retrieved in Genbank: Accession Number NC_010229.1) to be used as an outgroup for subsequent phylogenetic analyses.

Bayesian Inference phylogenetic trees were generated from *cytb* and *coxI* haplotypes. The identification of the best nucleotide substitution models was carried out in Jmodeltest2 (Darriba et al. 2012), among all the 203 possible models available (integrating the estimate of nucleotide frequencies, proportion of invariable sites and a gamma model with four categories), using all possible criteria of evaluation (AICc, BIC, DT). The base tree was ML optimized and the base tree search parameter was set on "best".

Bayesian Inference trees were generated using MrBayes 3.2.6 (Ronquist et al. 2012) on CIPRES portal (Miller et al. 2010), using the single *Sylvia crassirostris* sequence as the outgroup taxon. Four cold and one hot Metropolis-coupled Markov chain Monte Carlo chains were run for $2*10^7$ generations and sampled every 10000 generations, with default run parameters. Two runs with different starting points were carried out. The first $4*10^6$ generations were discarded as burn-in, and the posterior probability (PP) values were calculated for the remaining $1.6*10^7$ generations. We verified stationarity by checking that the potential scale reduction factor (PSRF) approached 1 (< 1.01) for all parameters and that the likelihood plot showed a pattern of "white noise" (Gelman 1996).

We generated Median Joining haplotype networks for all loci using Network 5.0 (http://www.fluxus-engineering.com, Bandelt et al. 1999) with default parameters, in order to better clarify genetic structuration in our dataset.

Genetic uncorrected p-distances (within and among groups, plus standard error calculated on 1000 bootstrap replicates) were estimated in MEGA 7 (Kumar et al. 2016). For *coxI* we also calculated Kimura 2-parameter-corrected distances, in order to compare *coxI* distances to reference thresholds (Hebert et al. 2004, Kerr et al. 2009).

Several Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) were conducted using Arlequin v3.5 (Excoffier & Lischer 2010) to examine the structure of molecular variation. Our main interest was in the main hierarchical level structures (defined "groups" in Arlequin). We already knew *S. moltonii* is a species per se, therefore *S. moltonii* individuals could have been assigned to one separate group; anyway, we evaluated all possible schemes of partitioning the four taxa in two and three groups (see below). We investigated here if western *S. c. cantillans* is best

treated as an independent species. We therefore tested whether taxa are best subdivided in two or three groups.

Because of the reduced number of "populations", our analyses *a priori* lacked the statistical power needed to highlight existing statistical significance of groupings at α=0.05. The best partitioning scheme should therefore had to be evaluated by comparison of the p-value of all possible partitioning schemes among themselves and with respect to the theoretical lowest possible p-value achievable in such a situation (Fitzpatrick 2009). We tested all possible permutations of our four taxa in two and three groups for all four loci.

RESULTS

We obtained 50 sequences for *cytb* gene, spanning 1090bp. Of the 1090 sites, 90 were variable and 76 parsimony informative. The 50 sequences were collapsed into 25 unique haplotypes (Genbank accession numbers: KX224399 - KX224423). No haplotypes were shared among taxa, each being private to a subspecies/species of the complex.

We obtained 48 sequences for *coxI* gene, spanning 648bp. 60 sites were variable and 50 parsimony informative. The two missing sequences did not compromise the sampling balance, in particular regarding the putative species (western *S. c. cantillans*). The 48 sequences were collapsed into 19 unique haplotypes (Genbank accession numbers: KX224424 - KX224442). No haplotypes were shared among taxa, each being private to a subspecies/species of the complex.

We obtained 25 diploid sequences (50 allelic sequences) for g3pdh, spanning 275bp. 13 sites were variable and 10 parsimony informative. The 50 sequences were collapsed into 17 unique haplotypes (Genbank accession numbers: KX224443 - KX224459). Haplotypes g3pdh-2, g3pdh-3, g3pdh-4 and g3pdh-7 were private to *S. moltonii*; g3pdh-5 and g3pdh-6 were private to western *S. c. cantillans*; g3pdh-9, g3pdh-14, g3pdh-15, g3pdh-16 and g3pdh-17 were private to southern *S. c. cantillans*; g3pdh-11, g3pdh-12 and g3pdh-13 were private to *S. c. albistriata*. g3pdh-1 and g3pdh-10 were shared by southern *S. c. cantillans* and *S. c. albistriata*; g3pdh-8 was shared by western *S. c. cantillans* and *S. moltonii*.

We obtained 25 diploid sequences (50 allelic sequences) for bFib5 intron region, spanning 547bp. 20 sites were variable and 12 parsimony informative. The 50 sequences were collapsed into 18 unique haplotypes (Genbank accession numbers: KX224460 - KX224477). Haplotype bFib5-2 was the most common and was shared by all the four taxa, and two other haplotypes (bFib5-1 and

bFib5-9) were shared by southern *S. c. cantillans* and *S. c. albistriata*. Four haplotypes were private to western *S. c. cantillans*, four to southern *S. c. cantillans*, four to *S. c. albistriata* and three to *S. moltonii*: however, no clear structure emerges even from private alleles, most of which are rare and separated from each other by only one mutation.

Sampling localities, sample identification numbers and haplotype attributions are reported in Appendix. Molecular diversity indices and results of neutrality tests for all loci are reported in Table 1 for the total of samples; molecular diversity indices for each locus separated by taxon are reported in Table 2. Tajima's D and Fu and Li's D* and F* tests indicated that no locus significantly depart from neutral evolution at α=0.05.

Table 1. *Molecular diversity indices and results of neutrality tests for cytb, coxI, g3pdh and bFib5 loci: number of individual (haploid) sequences (n), number of haplotypes (h), nucleotide diversity (π), haplotype diversity (Hd), average number of nucleotide differences (k); Tajima's D, Fu & Li's D* and F* values and their associated statistical significance (at α=0.05).*

Locus	n	h	π	Hd	k	Tajima's D	Fu & Li's D*	Fu & Li's F*
Cytb	50	25	0.0283	0.937	30.831	1.41918 (n.s.)	0.66535 (n.s.)	1.34063 (n.s.)
COI	48	19	0.02980	0.926	19.3076	1.89339 (n.s.)	0.57813 (n.s.)	1.05507 (n.s.)
g3pdh	50	17	0.0099	0.924	2.70939	-0.40404 (n.s.)	0.09159 (n.s.)	-0.08923 (n.s.)
FIB	50	18	0.0053	0.906	2.92327	-1.20767 (n.s.)	-1.14617 (n.s.)	-1.38531 (n.s.)

Table 2. *Molecular diversity indices for cytb, coxI, g3pdh and bFib5 loci calculated within each single taxon: number of individual (haploid) sequences (n), number of haplotypes (h), nucleotide diversity (π), haplotype diversity (Hd).*

cytb					
Taxon	**n**	**h**	**π**	**Hd**	**k**
Southern S. c. cantillans	16	8	0.00162	0.825	1.76667
S. moltonii	15	9	0.00538	0.914	5.86667
Western S. c. cantillans	12	3	0.00031	0.318	0.33333
S. c. albistriata	7	5	0.00253	0.905	2.76190

coxI					
Taxon	**n**	**h**	**π**	**Hd**	**k**
Southern S. c. cantillans	15	6	0.00356	0.762	2.30476
S. moltonii	15	6	0.00617	0.743	4.00000
Western S. c. cantillans	11	5	0.00135	0.709	0.87273
S. c. albistriata	7	2	0.00176	0.286	1.14286

g3pdh					
Taxon	**n**	**h**	**π**	**Hd**	**k**
Southern S. c. cantillans	14	7	0.00583	0.813	1.60440
S. moltonii	14	5	0.00707	0.802	1.94505
Western S. c. cantillans	10	3	0.00541	0.689	1.48889
S. c. albistriata	12	5	0.00242	0.576	0.66667

bFib5					
Taxon	**n**	**h**	**π**	**Hd**	**k**
Southern S. c. cantillans	14	7	0.00406	0.890	2.21978
S. moltonii	14	4	0.00143	0.626	0.78022
Western S. c. cantillans	10	5	0.00557	0.756	3.04444
S. c. albistriata	12	7	0.00496	0.924	2.71212

Genetic distances Genetic distances within and between different taxa are reported in Table 3.

Table 3. *Genetic distances (uncorrected p-distances ± S.E.) within and among different taxa for cytb, coxI, g3pdh and bFib5 loci. Standard errors were calculated by means of 1000 bootstrap replicates. K2P-corrected distances are also reported for coxI.*

Cytb	**Distances within population**	**Distances between populations**		
Taxon		***Southern S. c. cantillans***	***S. c. albistriata***	***Western S. c. cantillans***
S. moltonii	0.005 ± 0.001	0.046±0.006	0.040 ± 0.006	0.039 ± 0.006
Southern S. c. cantillans	0.002 ± 0.001		0.016 ± 0.004	0.036 ± 0.006
S. c. albistriata	0.003 ± 0.001			0.033 ± 0.005
Western S. c. cantillans	0.000 ± 0.000			

coxI	**Distances within population**	**Distances between populations**		
Taxon		***Southern S. c. cantillans***	***S. c. albistriata***	***Western S. c. cantillans***
S. moltonii	0.010 ± 0.002	0.034±0.006	0.041 ± 0.006	0.045 ± 0.006
Southern S. c. cantillans	0.004 ± 0.001		0.014 ± 0.004	0.044 ± 0.008
S. c. albistriata	0.000 ± 0.000			0.053 ± 0.008
Western S. c. cantillans	0.001 ± 0.001			

coxI (K2P)	**Distances within population**	**Distances between populations**		
Taxon		***Southern S. c. cantillans***	***S. c. albistriata***	***Western S. c. cantillans***
S. moltonii	0.010 ± 0.002	0.035±0.006	0.042 ± 0.007	0.047 ± 0.008
Southern S. c. cantillans	0.004 ± 0.001		0.015 ± 0.004	0.046 ± 0.008
S. c. albistriata	0.000 ± 0.000			0.055 ± 0.010
Western S. c. cantillans	0.001 ± 0.001			

g3pdh	**Distances within population**	**Distances between populations**		
Taxon		***Southern S. c. cantillans***	***S. c. albistriata***	***Western S. c. cantillans***
S. moltonii	0.007 ± 0.003	0.012 ± 0.004	0.012 ± 0.005	0.017 ± 0.006
Southern S. c. cantillans	0.006 ± 0.003		0.006 ± 0.003	0.012 ± 0.005
S. c. albistriata	0.002 ± 0.001			0.009 ± 0.004
Western S. c. cantillans	0.005 ± 0.003			

bFib5	**Distances within population**	**Distances between populations**		
Taxon		***Southern S. c. cantillans***	***S. c. albistriata***	***Western S. c. cantillans***
S. c. moltonii	0.001 ± 0.001	0.004 ± 0.001	0.008 ± 0.003	0.005 ± 0.002
Southern S. c. cantillans	0.004 ± 0.001		0.007 ± 0.002	0.005 ± 0.002
S. c. albistriata	0.005 ± 0.002			0.007 ± 0.002
Western S. c. cantillans	0.006 ± 0.002			

Cytb showed deep divergences between taxa: *S. moltonii* differed from all other taxa for about 4% (3.9% to 4.6%); furthermore, western *S. c. cantillans* differed for 3.3-3.6% from *S. c. albistriata* and southern *S. c. cantillans*, respectively. Southern *S. c. cantillans* and *S. c. albistriata* differed from each other, on average, for 1.6% in *cytb* sequences.

The same pattern of divergence was shown by *coxI*: *S. moltonii* differed (in terms of uncorrected p-distances) from all other for about 4% (3.4% to 4.5%) and western *S. c. cantillans* differed from all other taxa for 4.4 to 5.3%. *S. c. albistriata* and southern *S. c. cantillans* differed from each other for only 1.4%, on average. K2P-corrected distances between *S. moltonii* and all other taxa amounted to 3.5 to 4.7%; western *S. c. cantillans* differed from all other taxa for 4.6 to 5.5%.

Divergences between taxa for nuclear introns were much lower (about one fourth with respect to mitochondrial loci), with g3pdh showing major divergences between *S. moltonii* and western *S. c. cantillans* and all the other taxa, and southern *S. c. cantillans* and *S. c. albistriata* being the less diverged taxa.

bFib5, in addition to show smaller divergences than any other loci, did not highlight particular patterns of divergences between any pair of taxa, all separated by approximately the same percentage of sequence divergence.

Phylogenetic trees

DT and BIC decision criteria selected the HKY+I model of substitution as the most appropriate for *cytb* haplotype sequences; AICc selected a sub-partition of the GTR model (012010+I+F) as the most appropriate, with HKY+I showing a ΔAICc of only 2.89. We therefore selected the HKY+I model of substitution for *cytb* analyses. All three decision criteria selected the HKY+I model of substitution as the most appropriate for the *coxI* haplotype sequences.

Both the *cytb* and the *coxI* BI tree (Figure 1 and Figure 2, respectively) appeared well resolved at the taxon level, with high support. All main nodes had 100% Bayesian posterior probabilities in both trees, with two exceptions in *coxI* tree: 97% support for the western *S. c cantillans* clade and 82% support for the split between *S. moltonii* and southern *S. c. cantillans* - *S. c. albistriata*. The main structure of the trees was the same, with three major groups corresponding to *S. moltonii*, western *S. c. cantillans* and southern *S. c. cantillans* - *S. c. albistriata*. These groups were clearly reciprocally monophyletic.

A slight difference between the two trees occurred inside the clade western *S. c. cantillans* - *S. albistriata*: whereas *cytb* identified a complete lower-level separation between the two taxa, *coxI* seemed not to contain enough information to unambiguously resolve their reciprocal relationships.

Figure 1. *50% majority consensus rule Bayesian Inference tree inferred from 1090bp cytochrome b (cytb) fragment haplotypes. Bayesian Posterior Probabilities of each node are reported.*

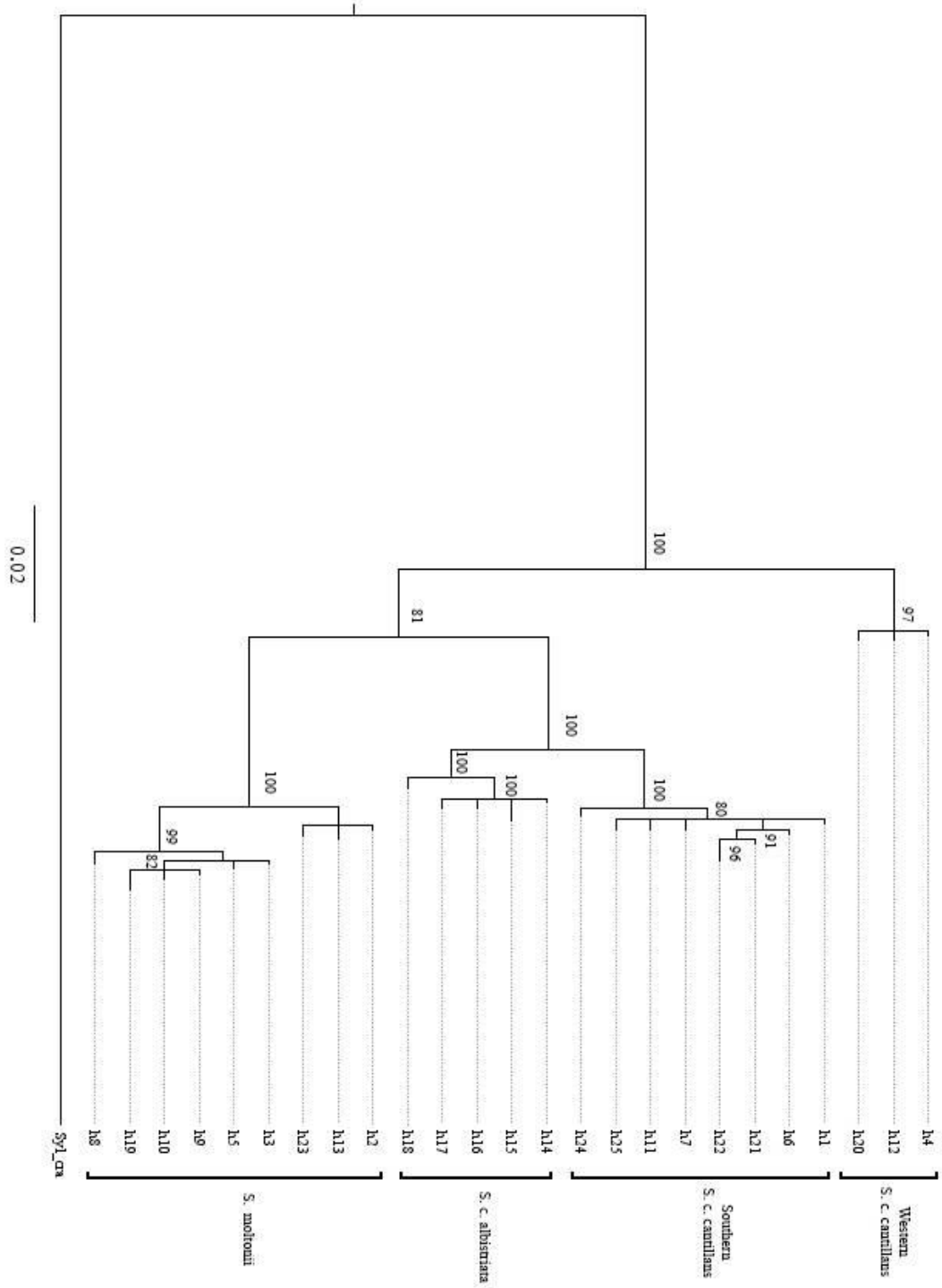
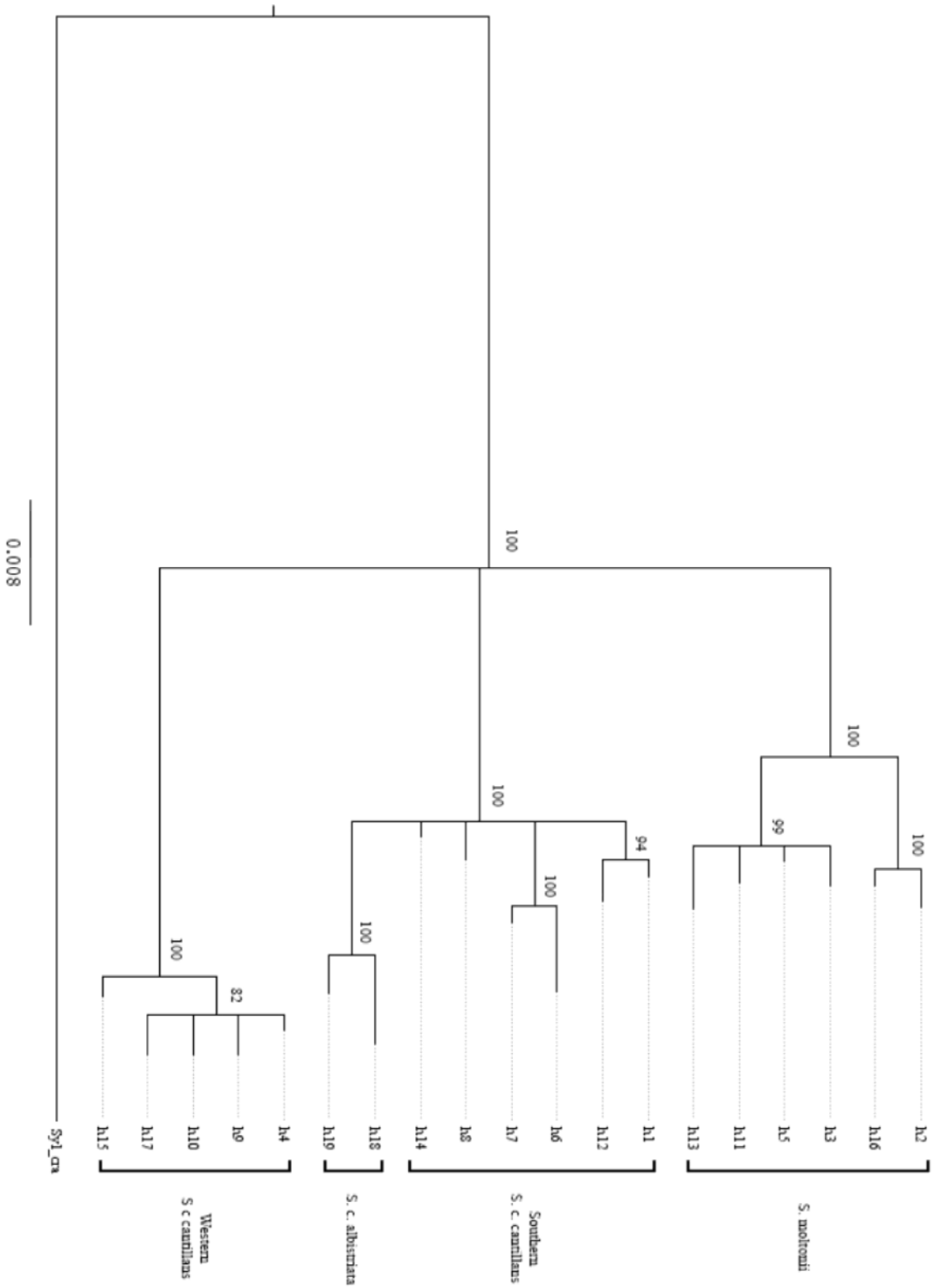
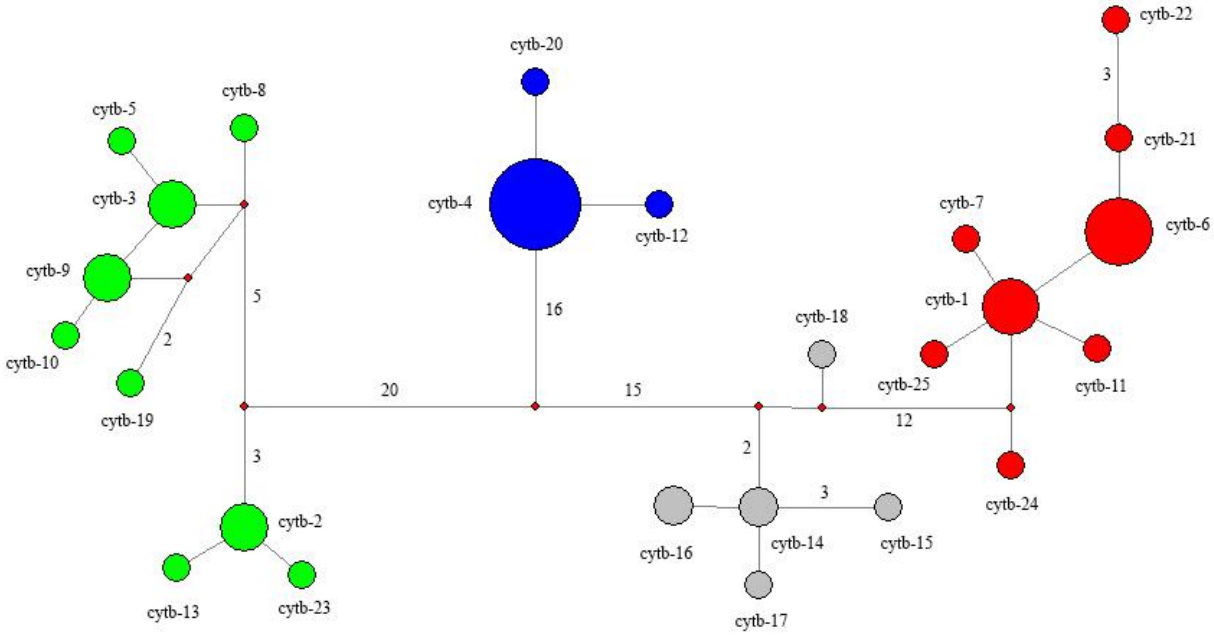


Figure 2. *50% majority consensus rule Bayesian Inference tree inferred from 648bp cytochrome c oxidase subunit I (coxI) fragment haplotypes. Bayesian Posterior Probabilities values larger than 70% are reported.*



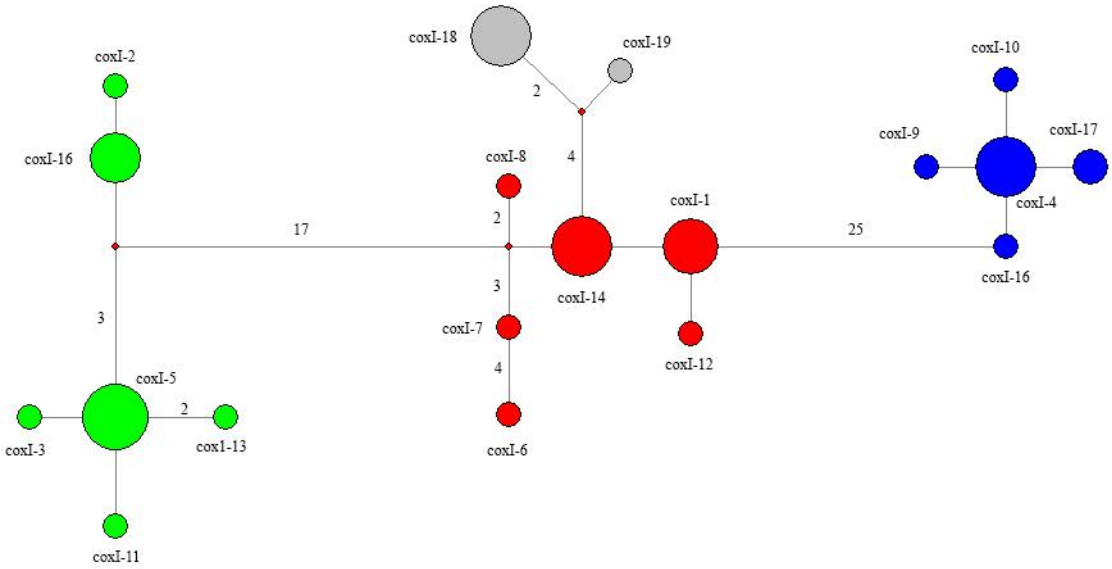
Haplotype networks The *cytb* haplotype network (Figure 3) showed four well separated clusters of haplotypes, each consisting of sequences observed in only one taxon (private haplotypes). Inter-haplogroup distance was at least 12 mutations.

Figure 3. *Haplotype network of 1090bp cytochrome b (cytb) fragment. Circle sizes are proportional to the number of sequences collapsed to each haplotype; slices reflect the taxon of the individuals bearing that haplotype. Unlabelled branches between circles account for one mutational step; labels indicate numbers of mutations larger than one. Legend: green = S. moltonii, red = southern S. c. cantillans, grey = S. c. albistriata, blue = western S. c. cantillans.*



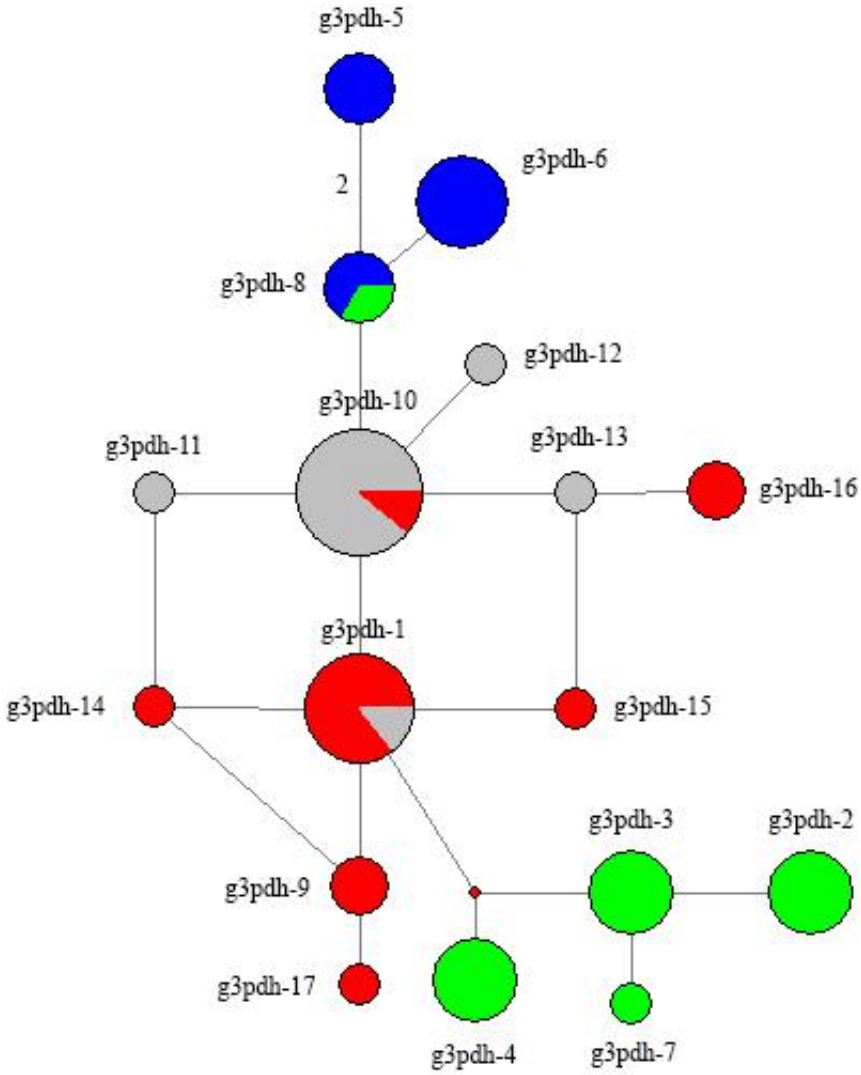
The *coxI* haplotype network (Figure 4) showed three well separated haplogroups, corresponding to *S. moltonii*, western *S. c. cantillans* and southern *S. c. cantillans* - *S. c. albistriata*. Whereas *S. c. albistriata* was characterized by private haplotypes, its cluster was separated by the southern *S. c. cantillans* cluster by only four mutations; *S. moltonii* and western *S. c. cantillans* haplogroups, instead, differed from southern *S. c. cantillans* for 17 and 25 mutations, respectively.

Figure 4. *Haplotype network of 648bp cytochrome c oxidase subunit I (coxI) fragment. Circle sizes are proportional to the number of sequences collapsed to each haplotype; slices reflect the taxon of the individuals bearing that haplotype. Unlabelled branches between circles account for one mutational step; labels indicate numbers of mutations larger than one. Legend: green = S. moltonii, red = southern S. c. cantillans, grey = S. c. albistriata, blue = western S. c. cantillans.*



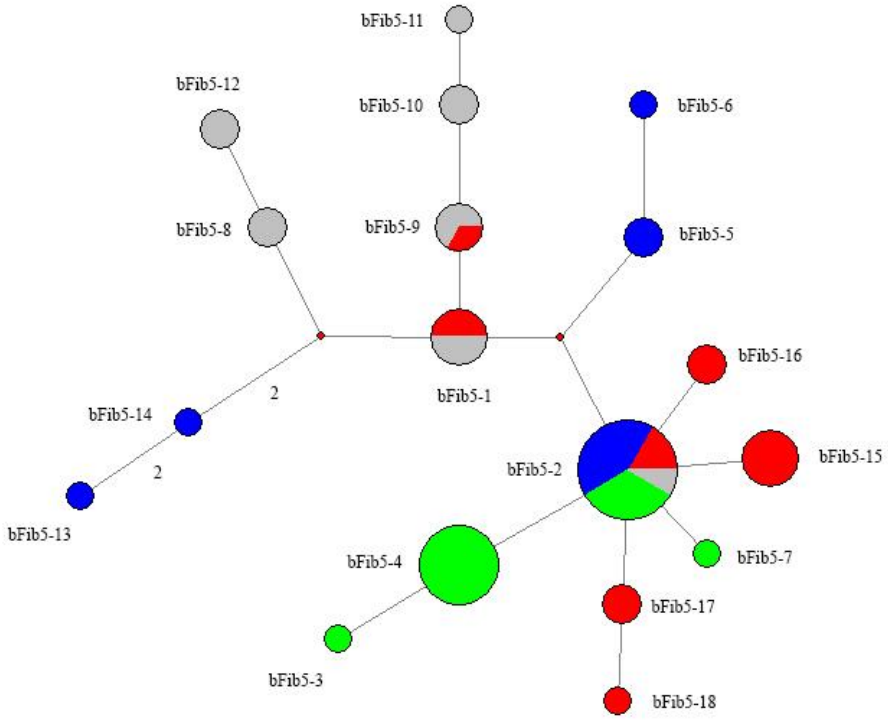
The g3pdh haplotype network (Figure 5) did not show any clearly separated haplogroup: most haplotypes diverged for just a single mutation from each other. Whereas *S. moltonii* and western *S. cantillans* were mainly characterized by private haplotypes clustering separately (apart from a single haplotype, g3pdh-8, shared by these two taxa), southern *S. c. cantillans* and *S. c. albistriata* shared two common haplotypes (g3pdh-1 and g3pdh-10) and even their private haplotypes were organized in a reticular way, which prevented the delimitation of two separated clusters.

Figure 5. *Haplotype network of 275bp glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh) fragment. Circle sizes are proportional to the number of sequences collapsed to each haplotype; slices reflect the taxon of the individuals bearing that haplotype. Unlabelled branches between circles account for one mutational step; labels indicate numbers of mutations larger than one. Legend: green = S. moltonii, red = southern S. c. cantillans, grey = S. c. albistriata, blue = western S. c. cantillans.*



The bFib5 haplotype network (Figure 6), despite showing some private haplotypes peculiar of each taxa, did not show any kind of structuration of genetic divergence.

Figure 6. *Haplotype network of 547bp beta-fibrinogen intron 5 (bFib5) fragment. Circle sizes are proportional to the number of sequences collapsed to each haplotype; slices reflect the taxon of the individuals bearing that haplotype. Unlabelled branches between circles account for one mutational step; labels indicate numbers of mutations larger than one. Legend: green = S. moltonii, red = southern S. c. cantillans, grey = S. c. albistriata, blue = western S. c. cantillans.*



AMOVA We reported in Table 4 only the results referring to two particularly informative partitions dealing with our aims: two groups, with *S. moltonii* (group 1) versus all other taxa (group 2); and three groups, *S. moltonii* (group 1), western *S. c. cantillans* (group 2) and southern *S. c. cantillans* - *S. c. albistriata* (group 3).

The *S. moltonii vs.* all other taxa was the best among all possible partitions of four taxa in two groups, both in terms of percentage of molecular variation explained at group level and p-value.

Cytb, *coxI* and g3pdh gave the same main results: among all the six possible partitions of four taxa ("populations" in Arlequin) in three groups (for each locus) the partition *S. moltonii vs.* western *S. c. cantillans vs.* southern *S. c. cantillans* - *S. c. albistriata* was always the best both in terms of percentage of molecular variation explained at group level and p-value. In all cases p-value for this partition was far lower than that of all other possible partitions and strictly close to the minimum theoretically possible p-value (p=0.16667).
For these three loci, the three-group treatment described above always explained a much higher percentage of molecular variation and/or was associated to lower p-values (in turn closer to the minimum expected p-value) than the two-group treatment mirroring the current taxonomy of the species complex.

bFib5 was the only locus for which AMOVA did not clearly indicate one of the tri-partitions as the best nor clarified if a two-groups or three-groups treatment should have been preferred (data not shown).

Table 4. *Results of AMOVA for cytb, coxI and g3pdh loci. For each locus percentage of variation explained among groups, its p-value (± s.e.) and theoretically expected minimum p-value are reported, for both the best two-groups and three-groups classification of the four taxa (M= S. moltonii, W = western S. c. cantillans, S = southern S. c. cantillans, A = S. c. albistriata).*

Locus	Treatment	Percentage of variation explained among groups	p-value among groups (± s. e.)	Expected minimum p-value
cytb	M *vs* W-S-A	26.37	0.25318 ± 0.01511	0.25000
cytb	M *vs* W vs S-A	58.48	0.17400 ± 0.00843	0.16667
coxI	M *vs* W-S-A	4.33	0.47898 ± 0.02009	0.25000
coxI	M *vs* W vs S-A	64.81	0.17791 ± 0.01011	0.16667
g3pdh	M *vs* W-S-A	26.77	0.24536 ± 0.01496	0.25000
g3pdh	M *vs* W vs S-A	42.67	0.16618 ± 0.01114	0.16667

DISCUSSION

Our *cytb* sequences confirmed the deep molecular divergence between *S. moltonii* and all other taxa of the species complex already described by Brambilla et al. (2008a), as well as the large divergence (3.3-3.6%) between the western populations of the nominate subspecies *S. c. cantillans* and southern *S. c. cantillans* - *S. c. albistriata*. The absence of shared haplotypes (which were all private to a specific taxon), the reciprocally monophyletic clades formed by all taxa in the Bayesian phylogenetic tree and the well distinct haplogroups formed by all taxa in the median joining network depicted a clear picture of prolonged isolation within the species complex, in particular for *S. moltonii* and western *S. c. cantillans*.

CoxI analyses described a largely analogous situation, with a (slightly lower but still remarkably) strong divergence between *S. moltonii* and all other taxa. Western *S. c. cantillans* diverged from all other taxa even more strongly in *coxI* than in *cytb*, with a difference of 4.4 to 5.3% (4.6 to 5.5% Kimura 2-parameter distances). Southern *S. c. cantillans* and *S. c. albistriata* showed the least divergence from each other, among the species complex.
A *coxI* Kimura 2-parameter divergence of 2.7% was the threshold indicated by Hebert et al. (2004) for the identification of morphologically unrecognized species of birds by means of DNA barcoding. Kerr et al. (2009) subsequently lowered this threshold value to 1.6%. The divergence between western *S. c. cantillans* and all other taxa in the species complex is well above these thresholds.
Whereas the automatic application of this criterion for species definition is no more accepted on its own, it should be noted that the degree of divergence between western *S. c. cantillans* and all other taxa of the species complex *S.* [*cantillans*] was even higher than the divergences between all taxa and *S. moltonii*, which is undoubtedly recognised as a "full" biological species.
S. moltonii and western *S. c. cantillans*, furthermore, formed monophyletic clades on their own in the BI tree; southern *S. c. cantillans* and *S. c. albistriata*, instead, were mixed in a single clade. The *coxI* haplotype network gave the same information: whereas *S. moltonii* and western *S. c. cantillans* formed two distinct haplogroups on their own, *S. c. albistriata* cluster was strictly connected to southern *S. c. cantillans* and seemed to be part of the same radiation.

Genetic distances between the taxa were far lower in nuclear sequences than in mitochondrial ones. However, such results were expected, due to the different ploidy and effective population sizes of the two types of markers (Pons et al. 2004).

In spite of the lower divergences, g3pdh showed a genetic structuration similar to *cytb* and *coxI*: again, *S. moltonii* and western *S. c. cantillans* were the most divergent lineages (1.2 to 1.8% divergence with respect to all other taxa), with only a minor distance between southern *S. c. cantillans* and *S. c. albistriata*. However, g3pdh haplotype network did not show well separated haplogroups, with most distances between haplotypes amounting to only one mutation. *S. c. moltonii* and western *S. c. cantillans* seemed to form two exclusive clusters, with only haplotype g3pdh-8 shared between the two taxa: however g3pdh-8, which seemed characteristic of western *S. c. cantillans*, was only found in a single *S. moltonii* individual, leaving open the door to the possibility of a rare episode of hybridization. This hypothesis was reinforced by the fact that the individual unusually emitted both kinds of contact calls (respectively typical of *S. moltonii* and western *S. c. cantillans;* Boano, *pers. comm.*) as well as by the sampling location, comprised within a narrow contact zone recently discovered between the two taxa. Southern *S. c. cantillans* and *S. c. albistriata* shared two common haplotypes and their "cluster" showed some sign of reticulations, again suggesting these two taxa are not deeply differentiated from one another.

bFib5 showed the least amount of divergence: inter- and intra- taxa differentiation largely overlapped, with no signs of genetic structuration within the species complex. All taxa shared a common haplotype (bFib5-2) and each possessed some more rare private haplotypes, derived from singleton mutations and perhaps of more recent origin. However, no structuration existed even among these private haplotypes, with private haplotypes of each taxon scattered over much of the network, not forming any cluster.

These results highlighted the complete molecular diagnosability of western *S. c. cantillans* from all other members of the *S.* [*cantillans*] species complex and the prolonged history of independent evolution experienced by this taxon.

AMOVA analyses corroborated this picture of the situation. The best possible two-group (i.e., two-species) treatment of the complex was the separation of *S. moltonii* in one group (species) and western *S. c. cantillans*, southern *S. c. cantillans*, *S. c. albistriata* in the other one. The p-value for the among-group variance component obtained by this treatment was the lowest among all possible alternative partitions of four taxa in two groups and was very close to the theoretical lowest possible p-value for such a configuration (for all loci except bFib5). However, the percentage of total molecular variation explained among groups amounted to 4.3-26.77%. The best possible three-group (three-species) treatment of the complex was, by far: (1) *S. moltonii*,

(2) western *S. c. cantillans*, (3) southern *S. c. cantillans* - *S. c. albistriata*. The p-value of among-groups structuration level was strictly close to the theoretical minimum for the classification of four taxa in three groups (for all loci except for bFib5). Furthermore, the best three-group treatment explained 42.67 to 64.81% of molecular variation at the among-groups hierarchical level. We therefore retain that a three-group treatment best describes the structuration of molecular variation within the species complex *S.* [*cantillans*].

Our molecular results, therefore, strongly indicate the need to consider western *S. c. cantillans* as a Molecular Operational Taxonomic Unit (Galimberti et al. 2012).

The strict association between this molecular degree of divergence and the allopatric geographical distribution of this taxon with respect to all other populations of *S. cantillans* (*sensu* Brambilla et al. 2008a) qualifies western *S. c. cantillans* as an Integrated Operational Taxonomic Unit (*sensu* Galimberti et al. 2012).

Partial morphological diagnosability (Svensson 2013a, 2013b) and a premating mechanism of reproductive isolation based on differential responses to vocalizations (Nespoli 2011) further require considering western *S. c. cantillans* as a Confirmed Candidate Species (Padial et al. 2010, Galimberti et al. 2012), waiting formal description with deposition of proper type specimens.

Our results also confirm that mitochondrial DNA alone is capable of discovering real independent evolutionary units, whereas nuclear markers may fail to show appreciable structure in molecular differentiation even in cases of prolonged history of isolation and speciation (Drovetski et al. 2014).

APPENDIX TO CHAPTER 3

Appendix. *Sample id; sampling locality; taxon ; haplotypes for 1090bp of cytochrome b (cytb), 648bp of cytochrome c oxidase subunit I (coxI), 275bp of glyceraldehyde-3-phosphate dehydrogenase intron 11 (g3pdh), and 547bp of beta-fibrinogen intron 5 (bFib5).*

ID	SAMPLING LOCALITY	TAXON	Haplotypes					
			cytb	*coxI*	g3pdh (allele 1)	g3pdh (allele 2)	bFib5 (allele 1)	bFib5 (allele 2)
SCA001	Italy (Lazio)	*Southern S. c. cantillans*	1	1	1	9	2	1
SCA017	Italy (Lazio)	*Southern S. c. cantillans*	6	6	.	.	.	.
SCA021	Italy (Lazio)	*Southern S. c. cantillans*	7	7	.	.	.	.
SCA030	Italy (Abruzzo)	*Southern S. c. cantillans*	6	8	.	.	.	.
SCA044	Spain	*Western S. c. cantillans*	4	9	8	6	2	2
SCA049	Spain	*Western S. c. cantillans*	4	10	.	.	.	.
SCA050	Spain	*Western S. c. cantillans*	4	.	8	6	6	2
SCA060	Italy (Tuscany)	*S. moltonii*	8	5	2	3	4	7
SCA069	Italy (Emilia-Romagna)	*S. moltonii*	3	11	2	2	4	4
SCA072	Italy (Emilia-Romagna)	*S. moltonii*	9	5	3	3	4	2
SCA079	Italy (Tuscany)	*Southern S. c. cantillans*	1	12	.	.	.	.
SCA081	Italy (Tuscany)	*S. moltonii*	3	13	.	.	.	.
SCA084	Italy (Tuscany)	*S. moltonii*	10	5	.	.	.	.
SCA089	Italy (Abruzzo)	*Southern S. c. cantillans*	6	14	.	.	.	.
SCA093	Italy (Umbria)	*Southern S. c. cantillans*	1	1	.	.	.	.
SCA107	Italy (Emilia-Romagna)	*S. moltonii*	9	5	4	4	2	2
SCA112	Italy (Emilia-Romagna)	*Southern S. c. cantillans*	11	14	.	.	.	.
SCA116	France	*Western S. c. cantillans*	12	4	.	.	.	.
SCA118	France	*Western S. c. cantillans*	4	15	.	.	.	.
SCA119	Corsica	*S. moltonii*	13	16	.	.	.	.
SCA126	Spain	*Western S. c. cantillans*	4	17	.	.	.	.
SCA133	Spain	*Western S. c. cantillans*	4	4	.	.	.	.
SCA135	Greece	*S. c. albistriata*	14	18	10	11	8	8
SCA136	Greece	*S. c. albistriata*	15	18	10	10	9	10
SCA137	Greece	*S. c. albistriata*	16	18	10	10	10	11
SCA139	Greece	*S. c. albistriata*	16	18	10	10	1	1
SCA141	Greece	*S. c. albistriata*	17	18	12	10	12	12
SCA142	Greece	*S. c. albistriata*	14	18	1	13	9	2
SCA143	Greece	*S. c. albistriata*	18	19	.	.	.	.
SCA144	Italy (Emilia-Romagna)	*S. moltonii*	19	5	.	.	.	.
SCA156	France	*Western S. c. cantillans*	4	4	.	.	.	.
SCA161	France	*Western S. c. cantillans*	4	17	6	6	13	14
SCA162	France	*Western S. c. cantillans*	20	4	.	.	.	.
SCA164	France	*Western S. c. cantillans*	4	4	5	5	2	5

(continues…)

(…continues)

SCA259	Italy (Sicily)	*Southern S. c. cantillans*	6	14	.	.	.	.
SCA264	Italy (Sicily)	*Southern S. c. cantillans*	21	.	1	1	15	15
SCA265	Italy (Sicily)	*Southern S. c. cantillans*	22	14	.	.	.	.
SCA271	Italy (Tuscany)	*S. moltonii*	2	16	.	.	.	.
SCA289	Italy (Tuscany)	*S. moltonii*	9	5	.	.	.	.
SCA290	Mallorca	*S. moltonii*	2	16	.	.	.	.
SCA291	Mallorca	*S. moltonii*	23	16	.	.	.	.
SCA410	Italy (Puglia)	*Southern S. c. cantillans*	24	1	14	15	16	16
SCA411	Italy (Campania)	*Southern S. c. cantillans*	25	1	1	10	15	17
SCA412	Italy (Abruzzo)	*Southern S. c. cantillans*	6	14	16	17	15	9
SCA413	Italy (Abruzzo)	*Southern S. c. cantillans*	6	14	16	9	18	17
MCCI2981	Italy (Sicily)	*Southern S. c. cantillans*	1	1	1	1	1	2
MCCI3666	Italy (Piedmont)	*Western S. c. cantillans*	4	4	5	6	2	5
MCCI3122	Italy (Sicily)	*S. moltonii*	2	2	2	3	2	3
MCCI3483	Italy (Piedmont)	*S. moltonii*	3	3	4	4	4	4
MCCI3876	Italy (Piedmont)	*S. moltonii*	5	5	7	8	4	4

CHAPTER 4

Genetic analyses reveal temporal turnover of species of the *S.* [*cantillans*] complex at stop-over and wintering sites

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Manuscript

INTRODUCTION

Recent research shed new light on the taxonomy and the phylogeographic patterns of the Subalpine Warbler species complex (*Sylvia* [*cantillans*]; Shirihai et al. 2001, Brambilla et al. 2008a, 2010). *Sylvia* [*cantillans*] have been demonstrated to consist of a complex of genetically distinct but morphologically cryptic taxa, only hardly distinguishable by male breeding plumage and vocal cues. These traits, however, are available only during the breeding season (Shirihai et al. 2001, Brambilla et al. 2008a, 2010).

Information on the migratory and wintering dynamics of these long-distance migrants is scarce (Cramp 1992), amounting to sparse observations carried out in a period of taxonomic confusion: in particular *S. moltonii*, a biological species on its own (Brambilla et al. 2008a), was not even considered a subspecies and all information regarding this taxon has been completely mixed with those from other taxa of the species complex. *S. c. albistriata*, one of the most identifiable taxon (in spring/summer male plumage), is the only one for which a hypothesis of annual trajectory has been advanced: it is supposed to accomplish an anti-clockwise loop migration starting from breeding areas (Balkans, Greece and Turkey), pointing straight down overflying Mediterranean Sea till wintering quarters and then coming back eastwards, through Israel/Cyprus (Cramp 1992). Capture-recapture data, the traditional method for the study of bird annual movements, are scarcely available due to the lack of specific studies conducted on these elusive taxa on the breeding areas and, even more so, to the reduced amount of ringing activities in northern and central Africa, where the taxa of the *Sylvia* [*cantillans*] complex spend winter and migration periods. Even an increased ringing effort, however, would not be enough to investigate these patterns at the taxon level. Molecular identification of the captured individuals is mandatory to identify individuals to the taxon level, in particular during migration and wintering (Brambilla et al. 2010).
The species complex consists of three main clades: *Sylvia moltonii* (syn. *S. subalpina*), currently considered to be a separate species, inhabiting Corsica, Sardinia, the Balearic islands and parts of continental Italy; the phylogroup constituted by *S. c. albistriata*-southern *S. c. cantillans (sensu* Brambilla et al. 2008a), distributed in Italy and along the Balkan peninsula, till Turkey; and western *S. c. cantillans* (*sensu* Brambilla et al. 2008a), distributed in France, the Iberian peninsula and, probably, northern Africa (Brambilla et al. 2008a).

Our study aims to determine the patterns of migration and wintering of the different taxa within the *S.* [*cantillans*] complex using molecular identification of several individuals opportunistically

trapped during migration or wintering in several locations of Northern and Central Africa.

MATERIALS AND METHODS

Birds were trapped with mist-nets at various sites in Northern and Central Africa in the course of different ringing projects: Morocco (Yasmina, 31.213°N, 3.988°W), Niger (Tibiri, 13.583°N, 7.033°E; Aoulikiss, 15.35°N, 5.35°E), Mauritania (Bellar, 17.033°N, 11.983°W), Nigeria (Dagona, 12.818°N, 10.745°E; Malamfatori; 13.617°N, 13.383°E).

A total of 44 birds were included in the present analyses: 11 from Yasmina, 1 from Tibiri, 1 from Aoulikiss, 1 from Bellar, 11 from Dagona, 19 from Malamfatori.

Capture sessions took place in different seasons (spring, autumn and winter) in the period 2000-2011 (see Table 1 for details).

One or more feathers were collected from each bird and stored in 96% vol. ethanol at -20°C until further processing. Total DNA was extracted by Qiagen DNEasy Blood and Tissue Kit, following manufacturer's guidelines. A 544 bp fragment of the cytochrome b (*cytb*) gene was amplified from each individual using PCR primers 3L and 662H (see Brambilla et al. 2010 for primer definition and cycling conditions), purified with ExoSap-IT (Affymetrix) and sequenced using Big-Dye terminators (Applied Biosystems) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Sequences were manually corrected in FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and aligned in ClustalW (Thompson et al. 1994) with default parameters. In order to exclude the possibility of amplification of nuclear pseudogenes of mitochondrial origin (NUMTs; Sorenson and Fleischer 1996) we verified the absence of stop codons by translation of all sequences with the EMBL-EBI Emboss Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/).

We added a single sequence of *Sylvia melanocephala* to the dataset (retrieved by Genbank: accession no. EU760694.1) to be used as an outgroup for subsequent analyses and sequences from 157 breeding birds analysed in Brambilla et al. (2008a) as a reference for phylogenetic analyses (see later).

All sequences were trimmed in Bioedit (Hall 1999) and collapsed to unique haplotypes by the Dna Collapser tool in the FABOX 1.41 suite (Villesen 2007). Genbank accession numbers for the 51 resulting haplotype sequences are KU521803-KU521853.

We attributed each migrating individual to one of the four taxa of the *Sylvia [cantillans]* complex considered in Brambilla et al. 2008a (*S. moltonii*, *S. c. albistriata*, southern *S. c. cantillans*, western *S. c. cantillans*) by a blastn search (Altschul *et al.* 1990) with default values.

In order to ensure that 544 bp of the first half of *cyt-b* gene contained sufficient information to identify the phylogenetic relationships among, and separate the four taxonomic groups of the *Sylvia* [*cantillans*] complex, we generated phylogenetic trees using the haplotypes of all individuals in the dataset (migrating, wintering and breeding birds).

Phylogenetic trees were generated by Maximum Likelihood and Bayesian Inference methods. The identification of the best nucleotide substitution model was carried out in Jmodeltest2 (Darriba et al. 2012), among all the 203 possible models available (integrating the estimate of nucleotide frequencies, proportion of invariable sites and a gamma model with four categories), using all possible criteria of evaluation (AICc, BIC, DT). The base tree was ML optimized and the base tree search parameter was set on "best".

Maximum Likelihood tree was generated by GARLI 2.1 (GARLI web service hosted at http://www.molecularevolution.org; Zwickl 2006, Bazinet *et al.* 2014), with 8 different starting trees and 2000 bootstrap replicates.

Bayesian Inference (BI) tree was generated by MrBayes 3.2.6 (Ronquist et al. 2012) on CIPRES portal (Miller *et al.* 2010). Four cold and one hot Metropolis-coupled Markov chain Monte Carlo chains were run for 10^7 generations and sampled every 1000 generations, with default run parameters. Two runs with different starting points were carried out. The first $2*10^6$ generations were discarded as burn-in, and the posterior probability (PP) values were calculated for the remaining $8*10^6$ generations. We verified stationarity by checking that the potential scale reduction factor (PSRF) approached 1 (< 1.0001) for all parameters and that the likelihood plot showed a pattern of "white noise" (Gelman 1995).

Either analyses used the single *Sylvia melanocephala* sequence as outgroup taxon.

RESULTS

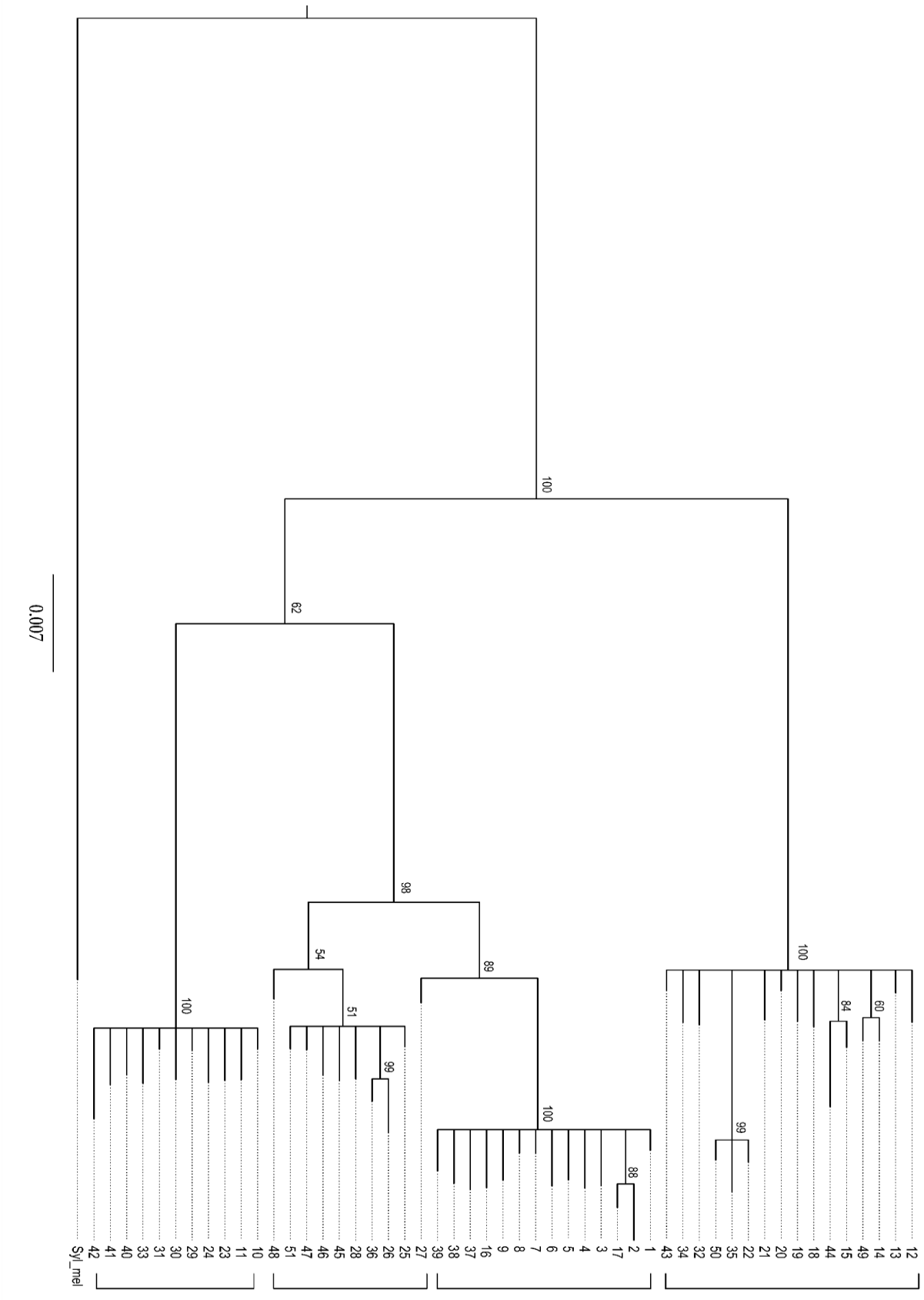
All decision criteria selected the HKY+I model of substitution as the most appropriate for the haplotype sequences.

Both phylogenetic trees (BI and ML) showed the same structure, with the Bayesian tree being slightly more resolved (see Figure 1).

The same phylogeographic patterns of Brambilla et al. 2008a were retrieved, with only a minor shift of one haplotype from the *S. c. albistriata* group to a position intermediate between *S. c. albistriata* and eastern *S. c. cantillans*. This minor difference, probably caused by a smaller information content in 544 bp versus the original 1147 bp (Brambilla et al. 2008a), is negligible for the aims of the current study.

All molecular identifications by blastn searches matched a Genbank reference sequence with at least 99% confidence, with E- values approaching 0 and total scores equal to (or larger than) 994, without any ambiguity. The molecular identifications revealed the presence of *S. moltonii*, *S. c. albistriata* and western *S. c. cantillans* individuals among our samples; we did not sample any migrating or wintering southern *S. c. cantillans* individuals. For details on the identification and scoring refer to Appendix.

Figure 1. *50%-majority rule Bayesian consensus tree of cytb haplotypes. Bayesian Posterior Probabilities for each node are reported.*



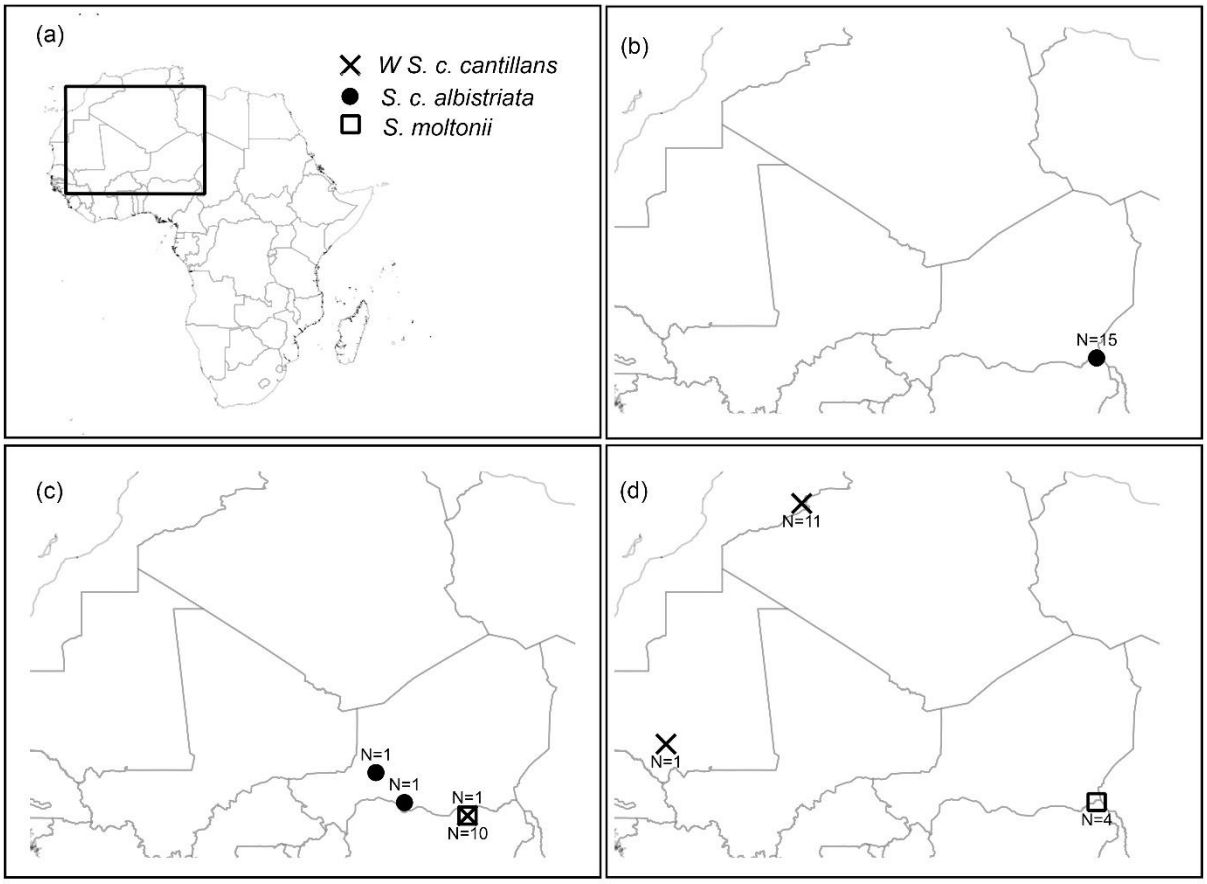
All our autumn samples came from only one locality, Malamfatori (Northern Nigeria). All the 15 individuals were molecularly identified as *S. c. albistriata*.

We trapped and analysed a total of 13 individuals wintering (December-February) in 3 localities: of the 11 individuals trapped in Dagona (Northern Nigeria) 10 were identified as *S. moltonii* and one as western *S. c. cantillans*; two Nigerien samples (one from Tibiri and one from Aoulikiss) were molecularly identified as *S. c. albistriata*.

We trapped and analysed 16 individuals during spring (March) in three localities: all the 4 individuals from Malamfatori (Nigeria) were identified as *S. moltonii*; 1 Mauritanian and 11 Moroccan individuals as western *S. c. cantillans*.

For a graphical representation of these results refer to Figure 2.

Figure 2*. Study area (a) and numbers of individuals per taxon trapped in autumn (b), winter (c) and spring (d) in Central and Northern Africa: S. moltonii (square), western S. c. cantillans (cross), S. c. albistriata (circle). The number of individuals of a taxon sampled in a single location is shown near the corresponding symbol ('N=10' in Figure 2(c) refers to S. moltonii).*



DISCUSSION

Although the small sample size (in terms of both sampled individuals and sampling localities), our results indicate that the wintering/migration patterns of the taxa ascribed to the *S.* [*cantillans*] complex are highly dynamic: different taxa visit the same areas in different seasons, while other ones occasionally occur in the same locality or localities nearby in some periods. In particular, only *S. c. albistriata* seems to be present in autumn in a site in northern Nigeria (Figure 2a), while during spring in the same site only *S. moltonii* were recorded (Figure 2c). However, during winter, all three taxa seems to be present in (Western) central Africa, in a belt extending over southern Niger and northern Nigeria (Figure 2b). Western *S. c. cantillans* was recorded only once in winter (in northern Nigeria; Figure 2b), but a larger number of individuals were sampled in NW-Africa during spring (Mauritania and Morocco).

Whereas our results do not allow the resolution of the yearly patterns of the single taxa of this species complex, they strongly suggest the importance to avoid generalizations based on a few ringing data or field observations. Sparse data cannot be reliable given the complex patterns our results suggested, and in both cases of ringed birds and visual observations, additional challenges derive from the fact that only-visual-identification of individuals at the taxon level would be sometimes difficult or even impossible.

Clinching the matter will require in-depth analyses, based on an extended network of ringing stations scattered over large parts of Northern and Central Africa, associated with molecular analyses and/or capture programs with tagging of molecularly identified breeding individuals.

APPENDIX TO CHAPTER 4

Appendix. *Capture data and molecular identifications of sampled birds based on 544 bp of cyt-b gene: capture date, country, locality, blastn taxon matching and goodness-of-fit values (percent identity, E-value and total score).*

DATE	COUNTRY	LOCALITY	TAXON	IDENTITY	E-VALUE	TOTAL SCORE
2000/03/07	Nigeria	Malamfatori	*S. moltonii*	100%	0.0	1005
000/03/07	Nigeria	Malamfatori	*S. moltonii*	100%	0.0	1003
2000/03/09	Nigeria	Malamfatori	*S. moltonii*	100%	0.0	1003
2000/03/16	Nigeria	Malamfatori	*S. moltonii*	100%	0.0	1005
2000/08/26	Nigeria	Malamfatori	*S. c. albistriata*	99%	0.0	1000
2000/08/26	Nigeria	Malamfatori	*S. c. albistriata*	99%	0.0	1000
2000/08/28	Nigeria	Malamfatori	*S. c. albistriata*	99%	0.0	998
2000/08/28	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1003
2000/08/30	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1003
2000/08/31	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1003
2000/09/01	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1003
2000/09/02	Nigeria	Malamfatori	*S. c. albistriata*	99%	0.0	998
2000/09/02	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2000/09/03	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2000/09/04	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2000/09/04	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2000/09/06	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2000/09/08	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2000/09/09	Nigeria	Malamfatori	*S. c. albistriata*	100%	0.0	1005
2007/01/22	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/23	Nigeria	Dagona	*W S. c. cantillans*	99%	0.0	994

(continues…)

(…continues)

2007/01/24	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/24	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/25	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/26	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/27	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/28	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/29	Nigeria	Dagona	*S. moltonii*	99%	0.0	1000
2007/01/30	Nigeria	Dagona	*S. moltonii*	100%	0.0	1005
2007/01/30	Nigeria	Dagona	*S. moltonii*	99%	0.0	1002
2007/02/12	Niger	Tibiri	*S. c. albistriata*	100%	0.0	1005
2007/02/19	Niger	Aoulikiss	*S. c. albistriata*	99%	0.0	1000
2007/03/11	Mauritania	Bellar	*W S. c. cantillans*	100%	0.0	1005
2011/03/25	Morocco	Yasmina	*W S. c. cantillans*	99%	0.0	1000
2011/03/27	Morocco	Yasmina	*W S. c. cantillans*	99%	0.0	994
2011/03/29	Morocco	Yasmina	*W S. c. cantillans*	99%	0.0	994
2011/03/22	Morocco	Yasmina	*W S. c. cantillans*	99%	0.0	989
2011/03/06	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005
2011/03/12	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005
2011/03/13	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005
2011/03/19	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005
2011/03/19	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005
2011/03/22	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005
2011/03/27	Morocco	Yasmina	*W S. c. cantillans*	100%	0.0	1005

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ABSTRACT

Mediterranean region is one of world's best explored areas in terms of ornithological research and it is considered a main biodiversity hotspot for the Western Palearctic (Myers et al. 2000), especially because it played a fundamental role in the divergence and speciation processes for many taxa, due to dynamics linked mainly to glaciations and salinity crises (Hewitt 1996; Taberlet et al. 1998). Most taxonomical research in ornithology carried on within this region has been based on anatomical and morphological differentiation of populations; this could have led to underestimating the real degree of divergence occurring in some morphologically cryptic populations in the region. We here analyse genetic divergence in two *Sylvia* (Aves: Sylviidae) species, formerly suspected of hiding complexes of cryptic sister species.

We demonstrate the existence of complete molecular diagnosability and strong patterns of structuration of molecular variability in mitochondrial and nuclear markers for two taxa, which we propose to elevate to the rank of Confirmed Candidate Species (Galimberti et al. 2012): *Sylvia* [*sarda*] *balearica* and western *Sylvia* [*cantillans*] *cantillans*.

We simultaneously urge the need to properly re-evaluate conservation status and policies for at least *Sylvia* [*sarda*] *balearica*, endemic to only a small archipelago.

Molecular identification of migrating and wintering individuals of *Sylvia* [*cantillans*] allowed us to gather the first certain data about the wintering areas of single taxon of the species complex and to highlight a pattern of great dynamism in their yearly trajectories. We suggest expanding our preliminary research by coupling a more capillary distribution of ringing efforts in Northern and Central Africa with molecular identifications of trapped individuals.

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