SHORT COMMUNICATION

Running head: *Migration pattern in the* Sylvia cantillans *complex*

An unexpected pattern of migration revealed in the Subalpine Warbler *Sylvia cantillans* complex by mitochondrial DNA analyses

MATTIA BRAMBILLA,[1,2]* SEVERINO VITULANO,[1,3] ANDREA FERRI,[4] FERNANDO SPINA,[4] ELENA FABBRI[4] & ETTORE RANDI[4]

[1]*Fondazione Lombardia per l'Ambiente, Settore Biodiversità e Aree protette, Piazza Diaz 7, I-20123 Milan, Italy*

[2]*Museo Tridentino di Scienze Naturali, Sezione Zoologia dei Vertebrati, Via Calepina 14, I-38122 Trento, Italy*

[3]*Associazione FaunaViva, Viale Sarca 78, I-20125 Milano, Italy*

[4]*Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Sede amministrativa ex-INFS, via Ca' Fornacetta 9, 40064 Ozzano Emilia, BO, Italy*

*Corresponding author.

E-mail: brambilla.mattia@gmail.com

Genetic markers are often used to trace the geographical origin of migrating birds. Such an approach has been used to attribute individuals of a given species to a given population, but it could also be applied to cryptic species, which are not fully diagnosable on the basis of their morphological appearance alone, despite often being genetically distinct. We sampled migrants of the *Sylvia cantillans* complex, which include cryptic taxa not readily diagnosable on the basis of their morphological appearance, at stopover sites in the central Mediterranean. We identified these individuals taxonomically using mitochondrial DNA sequence data. Molecular analyses enabled us to establish that four different taxa/clades of the *S. cantillans* complex (two *S. c. cantillans* clades, *S. c. albistriata* and *S. subalpina*) migrate through the central-western Mediterranean from Africa.

Genetic markers are often used to investigate the phylogenetic origin of species or populations (e.g. Sibley & Ahlquist 1990), or the historic origin of populations colonizing new geographical areas through dispersal (e.g. Cann *et al.* 1987, Avise & Nelson 1989, Quinn *et al.* 1991). In recent years, genetic markers have been increasingly used to trace the geographical origin of migrating birds. The distribution of birds with different genetic traits (e.g. different haplotypes) in their breeding areas can be used to estimate the breeding origin of migrating individuals, allowing researchers to 'follow' different populations throughout their annual cycle which often encompasses long annual migrations between breeding and wintering areas (Wennerberg 2001, Wink 2006). Such an approach makes use of genetic markers to identify the geographical origin of sampled individuals, but potentially could also be applied to identifying cryptic species, which are not fully diagnosable on the basis of their morphological appearance, yet are often genetically distinct. This is; however, a rather unexplored context, probably due to the lack of detailed knowledge of genetic variation

within many migratory species complexes, coupled with scarce data about individual migrant flyways.

The Subalpine Warbler *Sylvia cantillans* is a complex of taxa, all breeding within the Mediterranean basin, from Spain and Morocco to Greece and western Turkey, and winters almost entirely south of the Sahara (Cramp 1992). The *Sylvia cantillans* complex includes at least four taxa, traditionally ranked as subspecies (Shirihai *et al.* 2001): (1) the nominate race *cantillans*, which breeds in France, Spain and mainland Italy; (2) the more eastern race *albistriata*, distributed from Trieste (northeast Italy) to the Balkans and western Turkey; (3) *inornata*, inhabiting northern Africa (Morocco, Algeria and Tunisia) and southern Iberia; (4) *subalpina* (syn. *moltonii*), endemic to a restricted area in the central Mediterranean, encompassing Sardinia, Corsica, the Balearics and a few regions in mainland Italy (Brambilla *et al.* 2006). The latter taxon is now regarded as a full species (*Sylvia subalpina* synonymous *Sylvia moltonii*; see e.g. Fracasso *et al.* 2009, Sangster *et al.* 2009), on the basis of genetic divergence (Brambilla *et al.* 2008a), reaction to playback song (Brambilla *et al.* 2008b), distribution (Brambilla *et al.* 2008c), and diagnosibility of male plumage (Brambilla *et al.* 2010). On the basis of spatial structure of genetic variation (Brambilla *et al.* 2006), *cantillans* breeding in central and southern Italy are clearly different from the *cantillans* populations of France and Spain, being closer to *albistriata* (from which they are not always fully distinguishable based on visual appearance, Brambilla *et al.* 2010) rather than to the French-Iberian populations (Brambilla *et al.* 2008a).

The migration routes of these taxa are largely unknown and further study is complicated by difficult field identification: species and subspecies of the *S. cantillans* complex are cryptic with females being especially difficult to distinguish (Shirihai *et al.* 2001), and males are often also difficult to diagnose on morphological grounds (Brambilla *et al.* 2010). The main identification character among these taxa is vocal repertoire (Shirihai *et al.* 2001, Brambilla & Guidali 2005, Brambilla *et*

al. 2008a), but migrants are rarely vocal and trapped birds are almost invariably silent. Therefore, for a large proportion of migrating birds at stopover sites it is often extremely difficult to identify individuals at the (sub)specific level. In addition, ringing data from breeding grounds are scarce because birds belonging to the *S. cantillans* complex are primarily trapped during migration, and thus the very few ringing recoveries cannot be used to safely link breeding populations to migration routes and stopover sites.

The eastern race *albistriata* has been reported to display a loop migration, being more common during spring than autumn in the eastern Mediterranean basin (Shirihai *et al.* 2001 and references therein), while western populations seem to cross the Strait of Gibraltar in large numbers in spring, but not in autumn (Cramp 1992 and references therein). However, birds belonging to the *S. cantillans* complex are abundant during spring migration in many areas in the central Mediterranean. In summary, information about migration strategies of members of the complex is scant and, somewhat conflicting.

We studied the occurrence of taxa during spring migration at several stopover sites in the central Mediterranean in order to: (1) demonstrate the potential use of genetic markers to identify cryptic taxa in species complexes, and more specifically, (2) to establish the distribution of taxa belonging to the *S. cantillans* complex in the central Mediterranean basin via the use of molecular methods, as a means to avoid the difficulties of field identification using morphology. For molecular markers to prove effective in delineating lineages at stopover sites, detailed knowledge of the genetic differences among cryptic taxa belonging to the complex is required, and there should be limited gene flow among them. A previous study using the mitochondrial cytochrome-*b* gene (Brambilla *et al.* 2008a) suggests that both conditions are met in the *S. cantillans* complex.

METHODS

Birds were trapped through mist-netting at three island or coastal stopover sites in the central Mediterranean (Fig. 1): Camargue (coastal; France; 43°21', 4°47'), Palmaria Island (Liguria, Italy; 44°03', 9°51') and Ventotene Island (Lazio, Italy; 40°48', 13°26'). We also included in our analyses a bird caught at Palude Brabbia (45°47', 8°43) in April 2006, near Varese in (mainland) northwest Italy, where the Subalpine Warbler has not been recorded before. Overall, 79 passage migrants were included in our analyses (14 from Camargue, one from Brabbia, four from Palmaria, 60 from Ventotene). GenBank accession numbers for haplotype sequences are EU760644-EU760693, GQ433965-GQ433979, JN048669- JN048682 (see Supporting information for details).

For each bird, one or more feathers were collected and stored in ethanol. Laboratory procedures were the same as those adopted by Brambilla *et al.* (2008a, 2010). A fragment of 598 base pairs was obtained from each individual from the cytochrome-*b* gene using PCR primers 4L and 662H (Brambilla *et al.* 2010).

Individuals were assigned to a subspecies/group using a phylogenetic approach by building trees with reference sequences (e.g. Wennerberg 2001, Brambilla *et al.* 2010) obtained from breeding birds of each of the subspecies (see Fig. 2), an approach comparable to DNA barcoding (e.g. Kerr *et al.* 2009, Casiraghi *et al.* 2010). A Spanish sequence downloaded from GenBank (accession no. AJ534543) was also added. Given that the four groups are distinct, and that breeding individuals of the different subspecies/diverged populations do not share any haplotype (Brambilla *et al.* 2008a), we expected the assignment of all of our samples to their respective taxa/groups to be unambiguous. We carried out sample assignment using Neighbour Joining (performed in MEGA5, Tamura *et al.* 2011), Maximum Parsimony (performed in PAUP*4.08, Swofford 2001), Maximum Likelihood (performed in MEGA5) and Bayesian inference (with the selection of a specific model of molecular evolution, HKY+G, following Brambilla *et al.* 2008a, 2010). Posterior probabilities were calculated in the software programme MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). Four Metropolis-

coupled Markov chain Monte Carlo chains were run for $1.5*10^6$ generations and sampled every 100 generations. The temperature was set to 0.1 to improve the mixing of the chains, given that it was found to be poor at the default temperature 0.2. The remaining settings were set at default values. The first $5*10^5$ generations, before the chain reached apparent stationarity were discarded (burn-in), and the posterior probability values were calculated for the remaining $1*10^6$ generations. Stationarity was confirmed by checking that the potential scale reduction factors approached 0.001 and by the plot looking like 'white noise'. The Sardinian Warbler *S. melanocephala* (GenBank accession no. AJ534544) was used as the outgroup taxon.

RESULTS

Of the 60 individuals caught in Ventotene, 46 were southern *cantillans*, six were *albistriata* and eight *moltonii* (Fig. 2). The four birds trapped in Palmaria were three *moltonii* and one southern *cantillans*. The French migrants comprised 10 western *cantillans*, one southern *cantillans* and three *moltonii*. The female trapped at Palude Brabbia proved to be *albistriata*. Therefore, all four primary groups identified to date on the basis of mitochondrial DNA haplotypes were found within this rather narrow geographical belt across the central Mediterranean.

DISCUSSION

Despite our relatively small and rather unbalanced sampling, which prevents us from deriving any conclusion about numerical relationships among the different taxa during migration at stopover sites, the use of genetic markers facilitated interesting and somewhat unexpected insights into the migratory patterns of the taxa within the *S. cantillans* complex.

Roughly two thirds of the total number of individuals were members of the southern *cantillans* group, which was represented at all stopover sites and was by far the most abundant at Ventotene Island. The taxon *moltonii*, also occurred at all sampled stopover sites. In contrast western

6

cantillans was sampled exclusively at the French site. The easternmost known populations of this taxon inhabit southeastern France (and possibly also northwestern Italy), and therefore probably follow a western route towards their breeding grounds (Cramp 1992).

Despite being the rarest taxon, *albistriata* accounted for almost 10% of the total samples; this subspecies occurred at Ventotene Island and was also sampled in northwestern Italy. This suggests that some individuals of this taxon migrate through the central Mediterranean in spring, including western Italy, despite the supposed eastern spring flyway followed by the subspecies during its loop migration (Shirihai *et al*. 2001). This result is in agreement with the recent observations of *albistriata*-like birds during spring migration in eastern France (Duquet & Flitti 2007). The occurrence of these genetically determined *albistriata* birds confirms the regular occurrence of this taxon in the western Mediterranean during spring migration, whose field identification is somewhat complicated by imperfect diagnosability with respect to *cantillans* (Brambilla *et al.* 2010).

The occurrence of *moltonii* in Camargue is also interesting. Camargue is northwest of the primary range of the species, and individuals passing through Camargue may be either Corsican breeders, as it appears from their similarity with the Corsican and Balearic reference haplotypes (see Fig. 2), or, but less likely, northwestern Italian breeders that have taken a western route to their breeding grounds (cf. distribution map in Brambilla *et al.* 2006). *Moltonii* is relatively common also at Ventotene Island, situated to the east of the main breeding range of this taxon. Taken together, these observations suggest that *moltonii* does not follow a single return migratory route towards the breeding areas, but probably follows a western flyway or alternatively a more direct route across the central Mediterranean (Tyrrhenian). All individuals belonging to this form staging in Camargue and half of those passing Ventotene belong to the 'insular' group (the clade comprising the Corsican and Balearic haplotypes), thus suggesting that even island breeders (Corsica and Sardinia) may have different routes to their breeding grounds. As expected, the three *moltonii* sampled in Palmaria

(further north with respect to the insular portion of the species' range) had a 'mainland' haplotype, suggesting northern Italy as a possible breeding area.

The southern *cantillans* individual sampled in France may be a bird breeding in northern or central Italy (scattered populations of this race occur within the *moltonii* range, see Brambilla *et al.* 2006, 2008c) undertaking a western migratory route, or alternatively may be a migrant, which moved too far. However, there is very little recent data, and no genetic data on the populations breeding between western Liguria and eastern France (see comments in Brambilla *et al.* 2006); therefore, the possibility that southern *cantillans* or even *moltonii* may breed in the region between Italy and France cannot be rejected, and our results further highlight the need for targeted surveys in this area.

Molecular analyses allowed for easy identification of taxa, which would be impossible based only on field observations. In the present study, our limited sample size prevented quantitative conclusions with respect to the prevalence of taxa to be drawn, but allowed us to determine that all four taxa migrate through the central-western Mediterranean, despite previous studies suggesting a different pattern (Cramp 1992, Shirihai *et al.* 2001).

Our study confirmed that molecular methods may provide a powerful tool for fine scale diagnosis and may enable the identification of migratory routes in cryptic taxa belonging to species complexes, within which morphological identification can be challenging.

Fuchs, R. Bowie and three anonymous reviewers for helpful comments on previous drafts of the ms. Results from ''Progetto Piccole Isole'' (ISPRA): paper n. 51.

REFERENCES

Avise, J.C. & Nelson, W.S. 1989. Molecular genetic relationships of the extinct Dusky Seaside Sparrow. *Science* **243:** 646–648.

Brambilla, M. & Guidali, F. 2005. Quando la voce è tutto: l'identificazione delle sottospecie di sterpazzolina *Sylvia cantillans*. *Avocetta* **29:** 154.

Brambilla, M., Janni, O., Guidali, F. & Sorace, A. 2008b. Song perception among incipient species as a mechanism for reproductive isolation. *J. Evol. Biol.* **21:** 651-657.

Brambilla, M., Tellini Florenzano, G., Sorace, A. & Guidali, F. 2006. Geographical distribution of Subalpine Warbler *Sylvia cantillans* subspecies in mainland Italy. *Ibis* **148:** 568-571.

Brambilla M., Vitulano, S., Spina, F., Baccetti, N., Gargallo, G., Fabbri, E., Guidali F. & Randi, E. 2008a. A molecular phylogeny of the *Sylvia cantillans* complex: Cryptic species within the Mediterranean basin. *Mol. Phylogenet. Evol.* **48:** 461-472.

Brambilla, M., Quaglierini, A., Reginato, F., Vitulano, S. & Guidali, F. 2008c. Syntopic taxa in the *Sylvia cantillans* species complex. *Acta Ornithol.* **43:** 217-220.

Brambilla, M., Vitulano, S. Ferri, A., Spina, F., Fabbri, E. & Randi, E. 2010. What are we dealing with? An explicit test reveals different levels of taxonomical diagnosability in the *Sylvia cantillans* species complex. *J. Ornithol.* **151:** 309-315.

Cann, R.L., Stoneking, M. & Wilson, A.C. 1987. Mitochondrial DNA and human evolution. *Nature* **325:** 31–36.

Casiraghi, M., Labra, M., Ferri, E., Galimberti, A. & De Mattia, F. 2010. DNA barcoding: a six-question tour to improve users' awareness about the method. *Brie. Bioinform.* **11:** 440-453.

Cramp, S. 1992. *The Birds of the Western Palearctic, Vol. VI.* Oxford: Oxford University Press.

Duquet, M. & Flitti, A. 2007. Éléments d'identification de la Fauvette passerinette orientale *Sylvia cantillans albistriata*. *Ornithos* **12:** 135-139 (2005).

Fracasso, G., Baccetti, N. & Serra, L. 2009. La lista CISO-COI degli Uccelli italiani – Parte prima: liste A, B e C. *Avocetta* **33:** 5-24.

Huelsenbeck, J.P. & Ronquist, F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17:** 754–755.

Kerr, K.C., Birks, S.M., Kalyakin, M.V., Red'kin, Y.A., Koblik, E.A. & Hebert, P.D. 2009. Filling the gap - COI barcode resolution in eastern Palearctic birds. *Front. Zool.* **6:** 29.

Quinn T.W., Shields, G.F. & Wilson, A.C. 1991. Affinities of the Hawaiian goose based on two types of mitochondrial DNA data. *Auk* **108:** 585–593.

Sangster, G., van den Berg, A.B., van Loon, A.J. & Roselaar, C.S. 2009. Dutch avifaunal list: taxonomic changes in 2004–2008. *Ardea* **97:** 373–381.

Shirihai, H., Gargallo, G. & Helbig, A.J. 2001. Sylvia *warblers*. London: Helm.

Sibley C.G. & Ahlquist, BL. 1990. *Phylogeny and Classification of Birds: a Study in Molecular Evolution*. New Haven: Yale University Press.

Swofford, D.L. 2001. *PAUP*: phylogenetic analysis using parsimony (and other methods) version 408b*. Sunderland: Sinauer Assoc.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol* **28:** 2731-2739.

Wennerberg, L. 2001. Breeding origin and migration pattern of dunlin (*Calidris alpina*) revealed by mitochondrial DNA analysis. *Mol. Ecol.* **10:** 1111–1120

Wink, M. 2006. Use of DNA markers to study bird migration. *J. Ornithol.* **147:** 234–244.

Supporting material: Supplementary Table S1. Accession numbers of haplotype sequences used for this study.

Figure 1. Map showing location of stopover sites in the central Mediterranean; vertical lines: *cantillans* (both western and southern populations); grey area: *moltonii*; barred area: *albistriata*; dotted area: *inornata*. See text for further details.

Figure 2. Phylogram showing consensus values (only if ≥ 70) for NJ, MP, ML and Bayesian inference (shown as posterior probability*100), respectively. For reference haplotypes (breeding individuals, marked with black dots), the country where the individual was sampled follows the taxon designation. Open dot and open square represent sequences downloaded from GenBank.