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What are we dealing with? An explicit test reveals different levels of taxonomical diagnosability in the *Sylvia cantillans* species complex

Mattia Brambilla, Severino Vitulano, Andrea Ferri, Fernando Spina, Elena Fabbri, Ettore Randi

M. Brambilla

Fondazione Lombardia per l'Ambiente

Settore Biodiversità e Aree protette

Piazza Diaz 7, I-20123 Milano, Italy

S. Vitulano, A. Ferri, F. Spina, E. Fabbri, E. Randi

Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA)

Sede amministrativa ex-INFS

via Ca' Fornacetta 9, I-40064 Ozzano Emilia (BO), Italy

Correspondence:

M. Brambilla

e-mail address: brambilla.mattia@gmail.com

Phone number: +390280616140; Fax number: +390280616180

Running head: *Diagnosability in the Sylvia cantillans complex*

Abstract

Diagnosability is our ability in discriminating between similar taxa, including sibling or cryptic ones. We made an explicit test of diagnosability using the *Sylvia cantillans* species complex as a model, comparing phenotypic with genetic identifications. Individual warblers sampled during migration in Central Italy were (sub)specifically identified using putatively diagnostic plumage traits. Nucleotide sequences of a fragment (598 bp) of the mtDNA cytochrome *b* were then used to assign each individual to distinct phylogenetic clades, as determined by reference haplotypes sequenced in breeding individuals, defining well-distinct clades corresponding to known taxa of the complex. The new haplotypes were assigned to one of the previously identified groups (corresponding to three different taxa); no sample was assigned outside them. In contrast, when using plumage traits 11 out of 58 birds were classified as ‘uncertain/intermediate’ among two taxa, while 5 were differently classified under the two methods. Only for one taxon (*Sylvia subalpina*, syn. *S. moltonii*) there was a perfect concordance between the two methods. For the other two taxa of the complex, diagnosability is not guaranteed and their field and hand identification should be carefully addressed. Our work provides an example for an explicit test of taxa diagnosability, where the use of two or more ‘markers’ for diagnosis may point out discordant identification and thus miss unambiguous diagnosability. Our results outline the importance of considering different features for taxa diagnosis and point out the weakness of visual appearance-based identification (currently widely used for taxa determination) in our study complex.

Keywords appearance - identification - mtDNA - plumage - subspecies- variability

Introduction

Evolution promotes speciation, which means increasing diversity through divergence among taxa that become progressively distinguishable to us in genotype and phenotype (de Queiroz 1999). Diagnosability (see Helbig et al. 2002; Watson 2005) is our ability to discriminate between similar taxa based on characters or character state. It is an obvious requirement for taxon distinction, and a necessary condition for assigning species rank (Helbig et al. 2002, and references therein). A taxon is ‘diagnosable’ when individuals of at least one age/sex class can be distinguished from individuals of the same class(es) of all other taxa by at least one qualitative difference, or at least one class is separated by a complete discontinuity in at least one continuously varying character from the same class(es) of other taxa (Helbig et al. 2002). These diagnostic traits may be of various origins, as e.g. external and internal morphology or morphometrics, molecular data and karyotypes, distribution range and habitat preferences, behavioural and reproduction traits (Watson 2005). Suitable diagnostic traits in taxonomy should have strong genetic and low environmental components of variation (Helbig et al. 2002).

Diagnosability has often been claimed for assigning taxonomic rank (particularly in birds), and is considered essential to all species concepts at the operational level (Li et al. 2006). However, tests of taxa diagnosability are scarce. In this study we aim to show the potential importance of such a test with a simple worked example. We carried out an explicit test of diagnosability in a complex of closely related taxa and evaluated the reliability of current field diagnosis among the taxa of the complex.

Our model species complex, the subalpine warbler [*Sylvia cantillans*], is a Mediterranean passerine bird. It has formerly been considered a polytypic species (Cramp 1992), but actually it is better regarded as a complex of different taxa (traditionally ranked as subspecies, but

including at least two different species; Brambilla et al. 2008a), closely related to each other but with distinctive characteristics. The main differences among the taxa of the complex rely on contact/alarm calls (Brambilla and Guidali 2005) and on plumage, especially underpart colours in males (Shirihai et al. 2001). Song perception has been shown to be the most likely underlying mechanism of reproductive isolation between two taxa of the complex which occur in sympatry and parapatry but do not hybridise (Brambilla et al. 2008a; Brambilla et al. 2008b). Call differences are rather diagnostic and are handily being used to identify three differentiated taxa (Shirihai et al. 2001; Brambilla and Guidali 2005), of which one (the nominate subspecies *S. c. cantillans*) has been shown to be polyphyletic, including two genetically well-diverged clades (Brambilla et al. 2008a). In contrast, morphometrics are of rather limited use, with only *S. c. albistriata* being on average larger than other taxa (Shirihai et al. 2001). Most ringed birds belonging to the *S. cantillans* group are trapped during migration, when they are rarely vocal, and are currently identified mainly on the basis of their visual appearance. According to literature, males (especially individuals at least two years old) are often easily identified at the (sub)specific level based on their plumage colours, while juveniles and females are often indistinguishable, with rather few exceptions (see Shirihai et al. 2001).

We aimed to assess the reliability of plumage diagnosability in males of the subalpine warbler species complex. Plumage-based recognition has a major role in bird identification, including (sub)specific attribution of taxa belonging to the *S. cantillans* complex, and thus the implications of our experiments could be useful for field observers and ringers. Moreover, plumage-based identification is particularly interesting for explicitly testing diagnosability, because of its wide use in bird diagnosis. In our study, plumage identification is compared with genetic classification, obtained using independent data (mtDNA haplotypes).

We decided to work on the cytochrome *b*, one of the most used genes for phylogeny and phylogeography of passerine birds (Wink 2006), as it was known to show diagnostic variations within our study species complex (Brambilla et al. 2008a). We considered a taxon ‘diagnosable’ if all the individuals classified on the basis of plumage features belong to the relevant mtDNA clade, and vice-versa (all individuals of a mtDNA clade showed the plumage characters of such clade). Otherwise, taxa were considered to be not diagnosable (i.e. when some individuals resulted in discordant identifications).

Methods

Study area and samples collection

All samples used in this study were collected during spring migration at Ventotene island (40.48°N, 13.25°E), a ringing station in the central Tyrrhenian Sea. Samples were collected from 58 trapped individuals, caught through mist netting during the “Progetto Piccole Isole”, between 31st March and 28th May 2006.

Preliminary data indicate that trapping birds at this stopover site should likely lead to capture the following recognized taxa of the complex: the nominate race *Sylvia cantillans cantillans*, which breeds in the western Mediterranean to south Italy (Shirihai et al. 2001; Brambilla et al. 2006; now regarded as including two distinct groups: see below and Brambilla et al. 2008a); *S. c. albistriata*, an eastern subspecies, which breeds from Trieste (NE Italy) to Greece and western Turkey; Moltoni’s warbler *S. subalpina* (synonym *S. moltonii*) endemic to a restricted area in the central Mediterranean (Sardinia, Corsica, Balearics, and few regions in CN Italy, Brambilla et al. 2006; Brambilla et al. 2007; now considered as a full species, Brambilla et al. 2008a, b). Virtually, we could miss only *S. c. inornata*, the subspecies inhabiting northern Africa (Shirihai et al., 2001), and the western (Spain, France) populations formerly belonging to *S. c. cantillans*, which are genetically rather diverged from southern (Italian) *cantillans*, despite morphological and vocal similarity (Brambilla et al. 2008a), and probably perform a more western route towards the breeding grounds, crossing Gibraltar (see Cramp 1992, and references therein; Shirihai et al. 2001).

Studying migrants rather than breeding birds, we cannot *a priori* presume what (sub)species are we dealing with (with the exception of the contact zone between *cantillans* and *subalpina*, all taxa of the complex are mainly allopatric, and thus breeding birds in many cases could be

assigned to a given taxon simply based on their geographical origin), hence the (sub)specific identification should not be biased by known distribution data.

Plumage-based (sub)specific attribution of trapped birds

A minimum of four pictures were taken for each trapped bird. All pictures were carefully examined and subspecies assigned by judging and comparing the photographs. Birds were (sub)specifically determined based on the features of breast, belly, submoustachial stripe and head (table 1). It should be noted that Italian *cantillans* breeders are generally similar to western *cantillans*, but they often have wide white moustache, a rather large white belly patch, sometimes quite dark lores and also slightly washed-out tinge on flanks (Brambilla 2007), thus being in these traits a bit more reminiscent of *albistriata* (although not reaching its typical appearance) with respect to western *cantillans*; notably, descriptions of *cantillans* in classical handbook usually refer to the latter populations. Anyway, southern *cantillans* birds typically have a definitely orange-red tinge on underparts, rather different from the brick-red or vinaceous colour of *albistriata*, and the red colour on breast is not sharply demarcated against the white belly (Brambilla 2007). However, some birds were classified as uncertain/intermediate, as they appeared somewhat between *cantillans* and *albistriata*. In most cases, they had a pronounced submoustachial stripe, a separation between red breast and white belly but just approaching the characteristic neat demarcation of typical *albistriata*, a deep red (but not vinaceous or brick-red) colouration on throat and breast, and flank colour slightly paler than breast and belly, not almost uniform as in typical *cantillans* neither clearly different as in typical *albistriata*.

Genetic identification

DNA was extracted from feathers stored in ethanol, following laboratory procedures described by Randi et al. (2003) and Brambilla et al. (2008a). PCR primers (named 4L and 662H) used to amplify the first half of the mtDNA cytochrome *b* gene were the same of Brambilla et al. (2008a). A potential problem with phylogenetic analyses based on mtDNA data is the occurrence and amplification of mitochondrial DNA homologues from the nuclear genome (numts) (e.g. Sorenson and Fleischer 1996, Kidd and Friesen 1998). All the cyt *b* sequences we obtained could be functionally translated without premature terminations and there was no evidence suggesting that the presence of numts may bias our analyses on mtDNA.

A contiguous fragment of 598 bp was obtained from each sample. Sequences of migrant haplotypes have been deposited in GenBank under Accession Nos. XXXXXXXX-XXXXXXXXX. Individuals were assigned to a subspecies/group using a phylogenetic approach. A mtDNA cytochrome *b* phylogenetic tree (Brambilla et al. 2008a) revealed the existence of large phylogenetic distinctions among *albistriata*, *subalpina* and two (western and southern) *cantillans* mtDNA clades. These clades showed genetic distances D comprised between 1.7% and 5.0%. We used a subset of reference haplotypes determined in breeding individuals caught in the range of the four clades, and thus representing *cantillans* (southern and western clades; see above), *subalpina* (both mainland and insular populations) and *albistriata* (both from the western and eastern portions of its range). For each group, we selected the four commonest haplotypes (as found in Brambilla et al. 2008a); for *subalpina*, we selected the two commonest mainland haplotypes and two insular haplotypes, the latter representing Corsican and Balearic populations. The tree resulting from the reference haplotypes showed a clear distinction among the three subspecies and strong statistical support for each group (bootstrap values for each group $\geq 99\%$), consistent with the wider analysis of breeding birds (Brambilla et al. 2008a). Together with the reference haplotypes we

158 then included all migrants, classified on the basis of their plumage features (see above) and
159 re-built the phylogenetic tree, recording the placement in the tree of the newly included
160 (migrant) samples (see Fig. 1). On the basis of the assignment (no sample was placed outside
161 the previously identified groups; see Results and Fig. 1) we determined the (sub)specific
162 attribution based on mtDNA. One sequence of *Sylvia atricapilla* (GenBank accession no.
163 Z73494.2) was used as outgroup. Phylogenetic relationships were inferred using: 1)
164 neighbour-joining (NJ) method, based on number of differences among sequences (using
165 MEGA 3.1; Kumar et al. 2004); bootstrap tests with 1000 replicates were performed to assess
166 the robustness of the clades (i.e., bootstrap support); 2) heuristic search strategy, carried out in
167 PAUP* 4.08b (Swofford 2001), with exclusion of all uninformative nucleotide positions,
168 unordered and equally weighted characters, starting trees obtained via random stepwise
169 addition, random haplotype additions with 10 replicates, multiple minimal trees swapped by
170 TBR (tree-bisection-reconnection) branch swapping, collapsed zero length branches;
171 MulTrees option in effect; Steepest descent option not in effect, gaps treated as missing data,
172 300 replicates for assessing bootstrap values (10^6 rearrangements tried); 3) Maximum
173 Likelihood (ML) method, carried out in PAUP* 4.08b (Swofford 2001) and bootstrap values
174 assessed by means of 100 replicates on 100 equiprobable trees; 4) Bayesian inference using
175 MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; www.mrbayes.net). The choice of model
176 (HKY+G) mirrored the results of the previous phylogenetic study (Brambilla et al. 2008a).
177 Posterior probabilities were calculated in MrBayes for cytochrome *b* under a Hasegawa-
178 Kishino-Yano with gamma-shaped variation across sites (HKY+G) model (see above and
179 Brambilla et al. 2008a). Four Metropolis-coupled MCMC chains were run for 10^6 generations
180 and sampled every 100 generations. The temperature was set to 0.1 to improve the mixing of
181 the chains, given that it was found to be poor at the default temperature 0.2 (cf. Olsson et al.
182 2005). Other settings were kept as default values. The first 200,000 generations, before the

183 chain reached apparent stationarity (burn-in), were discarded and the posterior probability
184 estimated for the remaining 800,000 generations. Stationarity was confirmed by potential
185 scale reduction factors (all the parameters comprised between 1.000 and 1.013) and by the
186 plot looking like ‘white noise’, with no tendency of increase or decrease over time.
187 In all cases, trees were collapsed to obtain strict and 50% majority-rule consensus trees and
188 the results obtained through the three different procedures were compared.
189

Results

The reference haplotypes lead to a well-resolved phylogenetic tree, according to all methods: the breeding birds of the four clades were grouped into four different and clearly separated groups.

Of the 58 photographed and examined birds, 29 were classified as *cantillans*, 10 as *albistriata*, 8 as *subalpina* based on plumage traits, while 11 were classified as ‘uncertain/intermediate’ *cantillans/albistriata*, the latter showing intermediate features between the typical appearance of the two taxa, not allowing us to unequivocally assign them to one of the two subspecies. Moreover, some *cantillans* birds showed a paler colour on flanks and a rather wide submustachial stripe, yet possessing all the other typical *cantillans* traits and were assigned to this group. The latter all resulted as belonging to this group according to the *cyt b*.

Genetic analysis revealed that all birds belonged to three distinct clades perfectly associated with the ‘reference’ haplotypes. As expected, no western *cantillans* was sampled (see above). Bootstrap values of the three methods confirmed the validity of the tree, as all the groups had a support equal to or greater than 87% for bootstrap procedure in the neighbour joining tree, equal to or greater than 78% according to the maximum parsimony tree, equal to or greater than 74% according to the maximum likelihood tree, and had a posterior probability equal to or larger than 0.88 in the Bayesian analysis (see Fig. 1). Therefore, each group received a good statistical support from bootstrap and posterior probability values.

However, the position in the phylogenetic tree imperfectly matched identification based on photographs, and thus genotypic identification in some cases differ from phenotypic attribution. In fact, out of ten birds determined as *albistriata*, only five showed an *albistriata*-like *cyt b* sequence, while the remaining five were grouped together with *cantillans*. Three of

215 these misclassified birds showed a nearly ‘unmistakable’ *albistriata* plumage, with all typical
216 features of this subspecies (large white moustache, vinaceous-red tinge restricted to the upper
217 breast, faint red colour on flanks, dark lores).
218 Of the 11 intermediate *cantillans/albistriata* birds, one proved to have an *albistriata*-like cyt
219 *b*, while the other ten were genetically *cantillans*.
220 The 29 males classified as *cantillans* were actually all grouped in the phylogenetic tree with
221 the reference *cantillans* breeders.
222 All the eight males classified as *subalpina* were identically classified by the genetic data,
223 being grouped with the respective reference haplotypes in a unique group (but evidently
224 mutually with ‘mainland’ or ‘insular’ haplotypes, respectively), and no *subalpina* mtDNA
225 was found among differently classified birds.
226

Discussion

Diagnosability is a discussed topic in current ornithology (Watson 2005). Correct identification of taxa is required both in taxonomic research and field ornithology (Helbig et al. 2002). However, although a variety of different suites of traits are potentially diagnostic (i.e., morphometrics, external or internal morphology, behavioural or ecological traits, molecular data, etc.), most of them have unknown taxonomical value and weight (Helbig et al. 2002). Thus, explicit tests of diagnosability are needed, particularly in assemblages including sibling or cryptic taxa. In this study we test the strength of diagnoses based on plumage in a group of strictly related taxa, currently mainly identified based on male plumage, as contrasted with mtDNA-based diagnosability, by analysing trapped males. The results of this study show that different levels of diagnosability apply to the different taxa comprised within the *Sylvia cantillans* complex. The haplotypes of migrant warblers were assigned to the distinct haplogroups as defined based on the reference breeding birds: there were three well-distinct groups; no haplotype showed problematic position and all groups had a good statistical support. However, plumage-based identification was not always consistent with the classification provided by mtDNA. Intermediate birds (not safely classifiable based on their plumage) were placed in both the *albistriata* and (especially) *cantillans* groups and, more importantly, *albistriata*-like birds, as identified on the basis of plumage traits, were not all classified as belonging to this subspecies, as some of them were determined as *cantillans* according to their *cyt b*. Therefore, weakness of plumage-based identification currently complicates field and hand identification of these taxa, whose subspecific attribution requires caution. MtDNA did not allow us to check for the occurrence of birds originating from a secondary contact area with mixed mating between *cantillans* and *albistriata*; such birds would probably show intermediate or even ‘wrong’ plumages when compared with mtDNA.

Intermediate and misclassified birds could be hybrids of *cantillans* and *albistriata*, or could result from (possibly unilateral) introgression, or they could be southern *cantillans* with an outer appearance similar to *albistriata*. However, these ambiguities could be resolved only with the use of a nuclear marker and a larger sample.

On the other side, *subalpina* clearly stands out as the most differentiated taxon; all birds assigned to this subspecies by plumage were placed together with breeders of this race, and no *subalpina* haplotype was found among birds otherwise identified. Therefore, according to our sample this species appears as 100% diagnosable on the basis of males' plumage features (and of mtDNA too). Although the low sample prevents us from drawing definitive conclusions, the perfect diagnosability of *subalpina* in the Ventotene sample confirms the strong differentiation between this and the other taxa of the complex (Brambilla et al. 2008a), as demonstrated also by placement in the mtDNA tree (Figs. 1-2).

In conclusion, plumage- and mtDNA- based diagnosability of taxa belonging to the *Sylvia cantillans* complex may be considered as straightforward for *subalpina*, but unreliable for many individuals, which despite a rather typical '*albistriata*' appearance showed a *cantillans* mtDNA; other traits should be studied in order to develop a safe method for hand diagnosability of the latter taxa. Under a taxonomic point of view, the splitting of *subalpina* (cf. Shirihi et al. 2001) into another different species (*Sylvia subalpina*, or *S. moltonii*), as recently proposed on the basis of song perception (Brambilla et al. 2008b) and genetic divergence among breeding populations (Brambilla et al. 2008a), is acknowledged also by males' diagnosability, other than by strong differences in vocalizations (Brambilla and Guidali 2005) and particular distribution pattern (Brambilla et al. 2006).

At the same time, our study provides a worked example of an explicit test of taxa diagnosability. In particular, our results outline the importance of considering different features for taxa diagnosis: if we only consider mtDNA, also *cantillans* and *albistriata* may

appear fully diagnosable. The use of two or more ‘markers’ for diagnosis may point out discordant identification and thus lack of unambiguous diagnosability. Procedures based upon two or more ‘independent’ traits should be suggested for judging diagnosability among closely related taxa in taxonomic studies. With this respect, Alström et al. (2008) provided with a fine example of how the use of multiple criteria can greatly improve assessment of taxonomic status in a bird species complex, the *Bradypterus thoracicus* complex, and similar multidimensional analyses greatly enhanced taxonomic diagnosability in Asian *Phylloscopus* Warblers (Martens et al. 2008) and Canary Island Goldcrests (Päckert et al. 2006). In this species complex, mtDNA and song were the most useful traits for taxonomic assessment (Alström et al. 2008), similarly to the *Sylvia cantillans* complex, where mtDNA identified main clades (Brambilla et al. 2008a) and song seemed to represent the most important isolation mechanism (Brambilla et al. 2008b).

Genetic attribution may be a powerful tool if a clear pattern of genetic divergence among populations is known; the use of finer molecular markers, such as microsatellites or nuclear genes, may help in determining the occurrence of hybridisation and introgression among related taxa, which was not possible in our analysis based on mtDNA.

Zusammenfassung

Worum handelt es sich? Ein expliziter Test offenbart verschiedene Niveaus der Artenbestimmung im Artenkomplex *Sylvia cantillans*

„Diagnosability“ ist unsere Fähigkeit zwischen ähnlichen Taxa zu unterscheiden, Schwester- oder kryptische Taxa eingeschlossen. Wir haben die „Diagnosability“ am Modell-Artenkomplex *Sylvia cantillans* explizit getestet und die Bestimmung mittels phänotypischer mit der mittels genetischer Merkmale verglichen. Individuelle

304 Weißbartgrasmücken, gefangen während der Zugperiode in Zentralitalien, wurden
305 bis zur Unterart identifiziert mittels vermeintlich artbestimmender Merkmale des
306 Federkleides. Mittels Referenzhaplotypen, die in brütenden Individuen sequenziert
307 wurden und eindeutige Stammbäume entsprechend der bekannten Taxone dieses
308 Komplexes lieferten, wurden Nukleotidsequenzen des mtDNS Cytochrom b
309 Fragmentes (598 bp) verwandt um jedem Individuum ein eindeutiges
310 phylogenetische Taxon zuzuordnen. Diese neuen Haplotypen wurden einer der
311 vorher identifizierten Gruppen (entsprechend drei verschiedenen Taxa) zugeordnet.
312 Bei dieser molekularen Methode fiel keine Probe außerhalb besagter Taxa. Trotzdem
313 wurden bei der Bestimmung mittels Gefiedermerkmalen 11 von 58 Vögel als
314 „unsicher/intermediär“ zwischen zwei Taxa eingeordnet, und 5 Vögel wurden von
315 beiden Methoden unterschiedlich klassifiziert. Nur bei einem Taxon (*Sylvia*
316 *subalpina*, syn. *S. moltonii*) stimmten die Resultate beider Methoden perfekt überein.
317 Für die anderen zwei Taxa dieses Artenkomplexes kann die „Diagnosability“ nicht
318 garantiert werden, und die Bestimmung im Feld sollte mit Vorsicht betrachtet werden.
319 Unsere Arbeit liefert ein Beispiel für einen expliziten Test von „Diagnosability“. Wir
320 konnten zeigen, dass die Nutzung von zwei oder mehr Merkmalen zur
321 Artbestimmung kontroverse Ergebnisse liefert, und damit der Anspruch auf
322 Eindeutigkeit verfehlt wird. Unsere Ergebnisse betonen die Bedeutung der Nutzung
323 mehrerer, unterschiedlicher Merkmale zur Artbestimmung und zeigen, in dem von
324 uns studierten Artenkomplex, die Schwächen der visuellen, auf äußere Erscheinung
325 basierten Identifikation auf.

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Table 1 Characters and character states adopted for (sub)specific identification of studied birds of the *S cantillans* complex (Cramp 1992; Shirihai et al 2001; Brambilla 2007).

	<i>cantillans</i>	<i>albistriata</i>	<i>subalpina/moltonii</i>
breast and belly colouration	extending to lower breast and belly; uniform (or almost so) on breast, belly and flanks	restricted to a clear-cut breast band; paler on flanks than on breast	extending to lower breast and belly; uniform on breast, belly and flanks
breast and belly colour	orange-red	deep brick-red; white belly	salmon pink without orange hue
submoustachial stripe	well defined or even large	large	ill-defined or fine, often with rounded lower end
head		purser grey with darker lores than other taxa	

Fig. 1 Phylogenetic tree for migrant attribution with node estimates based on (in this order) neighbour-joining (based on number of differences; 1000 bootstraps), maximum parsimony (300 bootstraps), maximum likelihood (100 bootstraps) and Bayesian posterior probability estimates (see text for details). For reference haplotypes (breeding individuals), the country (or region) where the individual was sampled follows the (sub)species designation. The two alternative names *subalpine* and *moltonii* are both shown for clarity.