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Molecular analyses of flightless weevils *Chiloneus* from Sicily and adjoining islands revealed new synonymy (Coleoptera: Curculionidae)

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Abstract
The trophic ecology of *Chiloneus* weevil species from Sicily was unknown until recently when the host plant of these closely related species was discovered. The opportunity to collect many samples of these elusive species allowed us to re-evaluate the morphology and the genetic relationships among the three closest of the four species inhabiting Sicily and the adjoining islands. We analysed a fragment of the cox1 mitochondrial gene of adult specimens collected from the host plant *Drimia pancration* in Sicily, Pantelleria, Pelagie Islands and Malta to study the population structure and the relationships among the different nominal species known so far. Both morphological and genetic results suggest that the previous three species are actually two species with disjunct distribution, for which the new synonymy *C. hoffmanni* (González, 1970) (= *C. solarii* Pesarini, 1970, syn. nov.) is established. *Chiloneus meridionalis* (Boheman, 1840) inhabits the main island of Sicily and Pantelleria island, while *C. hoffmanni* is restricted to Malta and Pelagie islands. Adults of the two species are very similar in general appearance but still distinguishable by a set of well-established characters presented here in a key to their identification. The genetic approach provided evidence of a certain degree of structure of the genetic variation within the two species that would be worth addressing from a phylogeographic perspective in future work.

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Keywords: Chiloneus, Drimia, *Sicily*, *mtDNA*, *integrative taxonomy*

Introduction

Chiloneus Schoenherr, 1842 is a genus of Sciaphilini Sharp, 1891 that currently includes 41 species, 40 included in the subgenus *Chiloneus* and a single one from southern Italy, *Chiloneus* (*Mylaconeus*) *lonai* Pesarini, 1970, placed in the quite diverse subgenus *Mylaconeus* Pesarini, 1970 (Alonso-Zarazaga et al. 2017; Casalini et al. 2017). All the species, except the possibly misplaced *Chiloneus pallidus* Bajtenov, 1974 from Kazakhstan, are distributed in the warmer parts of the Mediterranean basin. The most recent, although now outdated, revision of this group was published by González (1970). Unfortunately, González confused members of *Chiloneus* with those of the morphologically similar *Desbrochersella* Reitter, 1906, which is a genus of Omiini Shuckard, 1840 (Alonso-Zarazaga & Lyal 1999). Precise distinguishing features of both genera were provided by Borovec and Perrin (2016), who revised several type specimens, described new species, and established new combinations and new synonymies.

In general, most *Chiloneus* are uncommon, and a good few of them are known only from type series and a handful of additional specimens, probably due to the complete lack of information about their biology. Four species of the nominotypical subgenus are

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currently known to be distributed in Sicily and neighbouring islands: *C. aliquoi* (Pesarini, 1975) from Sicily, *C. hoffmanni* (González, 1970) from Malta and Lampedusa islands, *C. meridionalis* (Boheman, 1840) from Sicily and *C. solarii* Pesarini, 1970 from Malta and Linosa islands (Alonso-Zarazaga et al. 2017). The first of them, *C. aliquoi* from the Madonie mountains, is already set apart from all other species dealt with in this note because of its entirely piliform elytral scales and small size (3.6–4.2 mm). The latter feature is shared with 27 other species of *C.* (*Chiloneus*), which, in turn, can be divided into at least two groups according to their body shape and vestiture. The remaining 13 larger species are rather similar to each other and badly in need of revision.

We had the opportunity to discover the habits and phenology (Casalini et al. 2017) of the three close species *C. hoffmanni*, *C. meridionalis* and *C. solarii* and to collect enough specimens to submit them to molecular analyses in order to clarify their true identity, given their extreme similarity. It is worth pointing out that these three species were separated by Pesarini (1970) on the basis of tiny morphological differences, having scarce material at hand.

Materials and methods

Sampling

Specimens of several different populations were collected by us in various sites of Sicily, mostly along the coast or just inland, between 2016 and 2018 (Figure 1). Fresh samples of two populations were collected by E. Colonnelli in 2017 in Malta. Specimens from Pantelleria were sampled by Pietro Ferrandes on the suggestion of Andrea Corso. We hand-collected more than 300 adult specimens on plants that had feeding damage, of which 133 individuals were used in the molecular screening here reported. Table S1 (see Supplementary material) lists the details of the localities and host plants. The recorded host plant was *Drimia pancration* (Steinh.) J. C. Manning and Goldblatt (Asparagaceae). Given the still controversial generic arrangement of Mediterranean bulbous Asparagaceae, in this paper the nomenclature of the genus follows Crespo et al. (2020) rather than that proposed by Bartolucci et al. (2018). Specimens were labelled by location, date and host plants and stored in American Chemical Society (ACS) grade acetone for the molecular genetic analyses. In four populations a share of the specimens were stored in ethanol 100°.

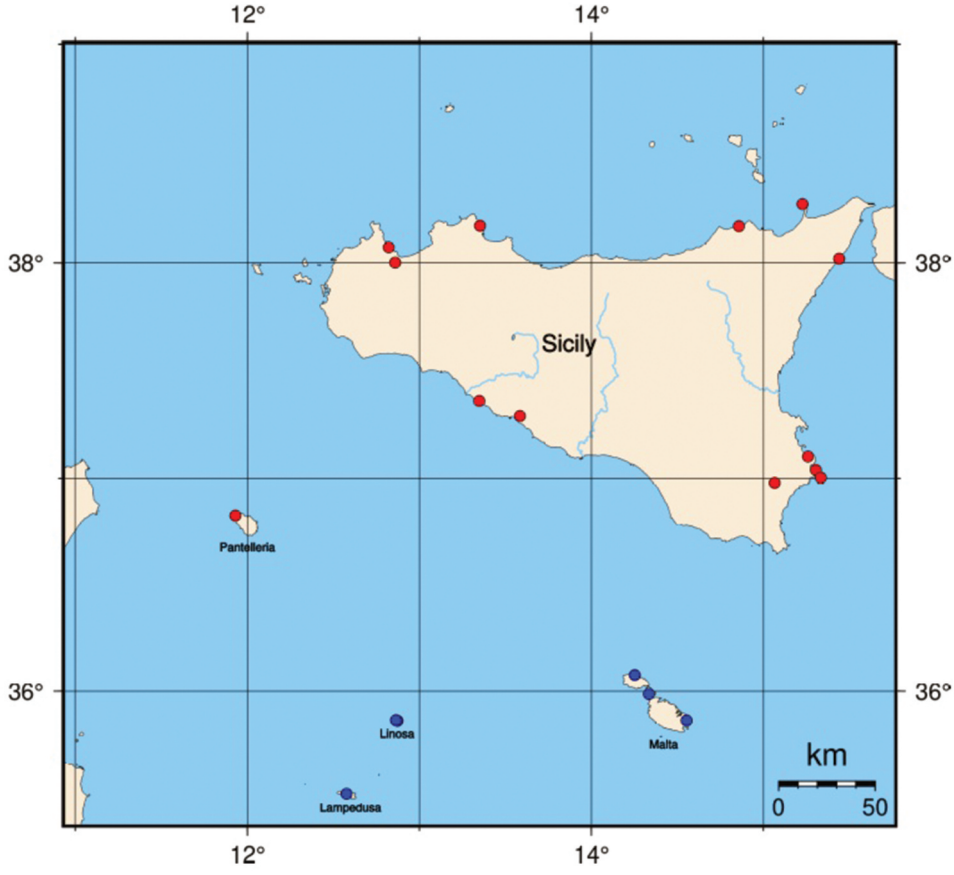

Figure 1. Area of study. Red and blue dots refer to sampling sites of *Chiloneus meridionalis* and *C. hoffmanni*, respectively.

Morphological analysis

Samples were morphologically analysed by two of us (EC, RC) using Pesarini (1970) and Casalini et al. (2017) to confirm the assignment to morphospecies and to detect possible differences useful in the discrimination of the available populations. The morphological analysis was performed using a Leica M205C and a Wild M5 binocular microscope on some 500 dried or wet samples. When necessary, specimens were dissected to permit a more in-depth analysis of internal structures such as the median lobe of aedeagus and the spermatheca. Pictures were taken by Francesco Sacco with a Nikon 810 camera provided with a Mitutoyo Plan Apo 10× objective and an f80 mm 4× tube lens. Photos were then stacked using the program Helicon Focus v. 6.1, and further processed with the program Adobe Photoshop CS5.

Mitochondrial marker sequencing

Following the procedure described in De Biase et al. (2019), the total genomic DNA was extracted and used as a template in polymerase chain reactions (PCR) to amplify a fragment of the mitochondrial genome coding for the cytochrome c oxidase subunit I (COI). Folmer's primers LC01490 and HC02198 (Folmer et al. 1994) were used to amplify the 5′ upstream region of the *cox1* gene or, when needed, the TY-J-1460 primer of Simon et al. (1994) as the forward one. We used several PCR thermal cyclers among those available at our laboratory (i.e. MWG® Biotech Primus 25, Biometra® TPersonal 48, Biometra® TProfessional 96 Gradient), with the following amplification conditions: 94°C denaturation (5 min), followed by 35 cycles of 95°C denaturation (1 min), 41°C annealing (1 min), and 72°C extension (1 min and 30 s), followed by a final 7 min elongation step at 72°. In vitro reactions were carried out in 25 µL of cocktail containing $(NH_4)_2SO_4$ 16 mM, Tris-HCl 67 mM (pH 8.8), $MgCl_2$ 3 mM, Tween-20 0.01%, 1 mM of each deoxynucleotide, 0.8 pM of each primer, and 1.25U of Taq DNA polymerase (Bioline Reagents Ltd, London, UK).

Amplified products were purified by Exo-SAP enzymatic reactions and sequenced at the Macrogen Europe genomic centres (Amsterdam, The Netherlands and Madrid, Spain), employing Applied Biosystems® 3730xl DNA Analysers and using the BigDye Terminator Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Sequencing primers were LC01490 or TY-J-1460. When needed, the DNA of a few individuals was sequenced on both strands using the same reverse primers used during the PCR amplifications. The acquired sequences were screened by a blast search of the GenBank nucleotide collection of the National Center for Biotechnology Information (NCBI) using the Mega BLAST procedure (Wheeler et al. 2007) available at its website (http://www.ncbi.nlm.nih.gov/blast). The screening procedure was aimed at checking the assignment of the specimens to high-level categories (e.g. family and subfamily). Next, the sequences were edited and aligned using the Staden Package v. 2.0.0b11 software (Staden et al. 2000). All peaks were checked for wrong base calls and noise and were cleaned when required. The alignment was visually assessed without requiring any insertion–deletion (indel) typing and was collapsed using FaBox tool (Villesen 2007) to retain the detected haplotypes only.

Data analysis

Bayesian analyses were performed on haplotypes using BEAST v. 2.6.3 (Bouckaert et al. 2019) with the bModelTest module (Bouckaert & Drummond 2017) for the evaluation of the substitution model (*BEAST Model Test; transistionTrasversionSplit*). We used the *Random Local Clock* model (Drummond & Suchard 2010) implemented in the software to take into account the variation of the substitution rate among lineages with *Clock.rate* 0.0177 according to Papadopoulou et al. (2010). The tree prior was set using the Yule model (Yule 1925). Two analyses were run adopting the adaptive Metropolis coupled MCMC strategy implemented in the *coupledMCMC* module of Beast 2.6.3 (Müller & Bouckaert 2020) using for each analysis a random starting tree and two Markov chains with length 50×10^6, resampling every 1000 iterations and logging trace and tree every 1000 iterations. Finally, the same analysis was performed sampling from priors only, to evaluate the priors that we applied to the analysis parameters. Convergence was evaluated with Tracer v. 1.7 (Rambaut et al. 2018), and the two chains were combined with Logcombiner routine of Beast 2.6.3, discarding 25% of burn-in trees each; the combined set of trees was summarised as a Maximum clade credibility tree with Beast's TreeAnnotator program. Finally, genetic divergence was estimated using the Kimura two-parameter evolutionary model K80 = K2P (Kimura 1980); distances and standard errors (1000 bootstrap replicates) were computed as pairwise values among all haplotypes and as net averages among the groups detected on the inferred Bayesian topology, by means of MEGA X v. 10.1.8

(Kumar et al. 2018). To further display the genealogical relationships among haplotypes and to indicate possible missing mutational connections, we performed a network analysis of the entire set of nucleotide sequences, using the statistical parsimony method (Templeton et al. 1992) as implemented in the software TCS v. 1.23 (Clement et al. 2000). The results were depicted as a network using the web-based program tcsBU (Múrias dos Santos et al. 2016) available at https://cibio.up.pt/software/tcsBU/ (last access January 2021).

As discussed in De Biase et al. (2016), the degree of genetic divergence has a non-linear relationship with the taxonomic rank of detected genetic groups. In the framework of the species delimitation approach, the automatic barcoding gap discovery (ABGD) approach (Puillandre et al. 2012) is a fast and simple method to discover partitions in DNA sequence data sets thus suggesting those clusters to be considered as putative distinct species on a phylogenetic tree. The ABGD method stems from the barcoding methodology, which was originally focused on the identification of biological samples using a standard nucleotide sequence (a 5′ fragment of the mitochondrial cox1 gene) compared with a reference data set of previously characterised species. The method aims at defining partitions in a set of cox1 sequences that must be considered as hypotheses of prospective distinct species to further investigate in an integrative framework. The partitions are defined by analysing the distribution of all pairwise distances between sequences to locate the most reliable "barcode gap" between the intraspecific and interspecific divergence. After the initial partitions are defined, the algorithm is performed in a recursive way until no new partitions are defined. Our analyses were carried out on the alignment of the all 133 cox1 sequences obtained for the sampled populations by using the ABGD method as available at the website https://bioinfo.mnhn.fr/abi/public/abgd/ (Puillandre et al. 2012; last access January 2021) with the following parameters: Pmin 0.001, Pmax 0.1, Steps 10, X (relative gap width) 1.5, Nb bins (for distance distribution) 20, K80 distance, and TS/TV 2.0.

We used as another approach for species delimitation the general mixed yule-coalescent (GMYC) model (Pons et al. 2006; Fontaneto et al. 2007; Monaghan et al. 2009; Fujisawa & Barraclough 2013), which helps in seeking the threshold that marks the transition between evolutionary dynamics within and among species, thus suggesting those clusters to be considered as distinct species on a phylogenetic tree. The method is aimed at modelling in a probabilistic framework both the coalescence processes that occur within species at population level, as described by topology and length of the branches of a phylogenetic gene tree, and the speciation processes occurring at a certain level of divergence and identified as a threshold above which all nodes describe speciation events as defined by the Yule speciation model (Yule 1925). This approach thus combines standard coalescent models that consider the diversification within populations (Hudson 1991; Wakeley 2008) with those models that describe the branching pattern of speciation events (Nee 1994, 2001; Nee et al. 1994). The method evaluates, by means of a likelihood test, alternative scenarios by assessing several thresholds as a boundary between intra- and inter-specific dynamics, and fitting the best one for delimiting the species encompassed by the gene tree under analysis. The analyses were performed starting from the ultrametric Bayesian tree previously inferred on the cox1 haplotypes data set by using the web service available at the URL https://species.h-its.org/gmyc/ (more information at The Exelixis Lab https://cme.h-its.org/exelixis/index.html) setting both the single and multiple threshold option of the method (Monaghan et al. 2009).

Finally, considering the results obtained with the previous analyses, we analysed in detail the alignment of the nucleotide sequences to detect all the positions that showed alternative bases shared among all individuals belonging to each of the entities highlighted through the statistical analyses described above. Subsequently, the amino acid sequences obtained from the translation of the nucleotide sequences, on the basis of the standard code for Invertebrate Mitochondrial DNA (Table S5), were analysed in the same way. This procedure was performed using the AliView v. 1.27 (Larsson 2014).

Results and discussion

Morphological analysis revealed that certain external and internal characters used by Pesarini (1970) are too variable to use to reliably differentiate some populations at the species level. In particular, we noted that the only diagnostic external features are the disposition and number of lifted elytral setae (Figure 2a, b), the shape of the protibiae (Figure 3a, b), the length of the last segments of the antennal funiculus (Figure 3c, d) and the shape of the aedeagus (Figure 3e–h). In contrast, the depth of elytral striae, density of recumbent scales, and shape of spermatheca are of little use for separating taxonomic entities.

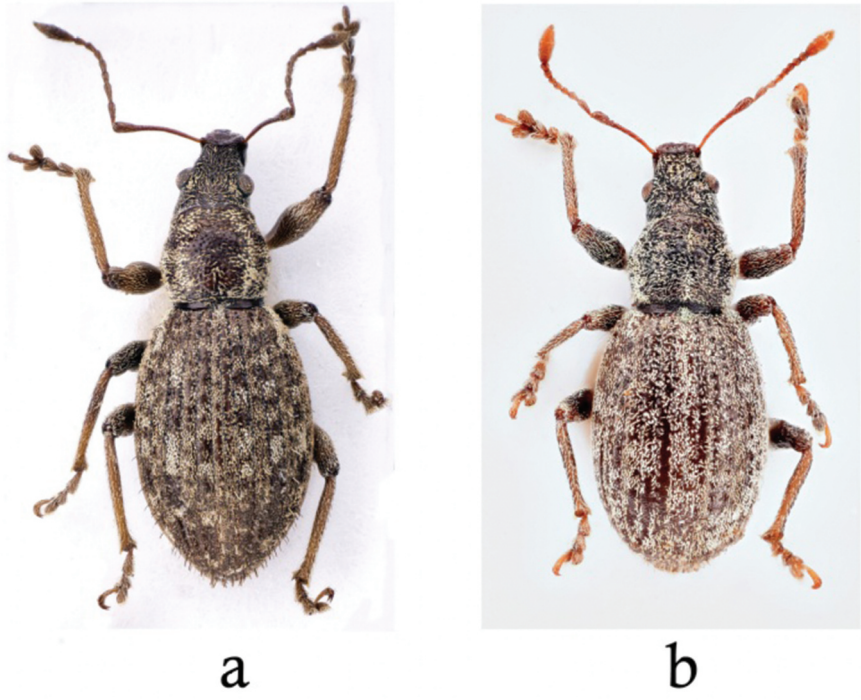

Figure 2. Habitus of (a) *Chiloneus meridionalis* from Palermo, Monte Pellegrino, and (b) *C. hoffmanni* from Malta, St. Thomas Bay.

Based on morphological evidence, only two morphospecies can be identified among the studied material, one distributed in Sicily and in the Aegadian Islands, and the other occurring in Malta and the Pelagie Islands, both showing a moderate variability of external features, often depending also on the freshness of the studied individuals. Thus, we propose here the following synonymy: *Chiloneus hoffmanni* (González, 1970) (= *Chiloneus solarii* Pesarini, 1970; syn. nov.). The species dealt with in this note, both quite variable in terms of the density of their scaling and punctuation of the pronotum, can be separated using the following key, which modifies that published by Pesarini (1970).

1 – Elytral setae erect also near base, although here they are shorter than those on elytral declivity. Anterior tibiae of both sexes quite strongly curved inwards at apical fifth. Segments 5–7 of funiculus at least a trifle longer than wide. Apex of aedeagus bisinuose at apex in lateral view, extreme apex curved downward. Sicily and Aegadian islands…….
…….*meridionalis* (Boheman, 1840)

1' – Elytral setae recumbent near base. Anterior tibiae of both sexes only feebly curved inwards at apical fifth. Segments 5–7 of funiculus not longer than wide. Apex of aedeagus slightly bisinuose at apex in lateral view, extreme apex not or imperceptibly curved downward. Malta and Pelagian islands…….*hoffmanni* (González, 1970)

For the purpose of testing whether morphological differences coincide with genetic ones, we amplified a fragment of the mitochondrial cox1 gene, of nearly 680 bp, from 133 adult samples. The alignment was cut at the shortest aligned sequence, giving a final set of sequences each 552 bp long. The collapsed alignment was composed of 28 unique haplotypes; the distribution among samples and the accession numbers of the nucleotide sequences deposited in the NCBI/EMBL/DDBJ databanks are listed in Table S2. Table S3 gives the complete matrix of pairwise K80 distance values among the scored haplotypes (range 0.002–0.029).

Bayesian analyses carried out on the haplotype data set produced a tree topology (Figure 4) highlighting two clades that were well differentiated (K80 distance = 0.194; SE = 0.021) and supported by very high posterior probability values (pp 1.00 and 1.00; Figure 4). Clade #1 (pp = 1.00) includes eight haplotypes (h1–h8; mean K80 distance within clade = 0.004, SE = 0.002) scored from individuals collected on *Drimia pancration* in Pelagie Islands (Lampedusa and Linosa) and Malta archipelago only. One haplotype (h1) is shared between Malta and Lampedusa while

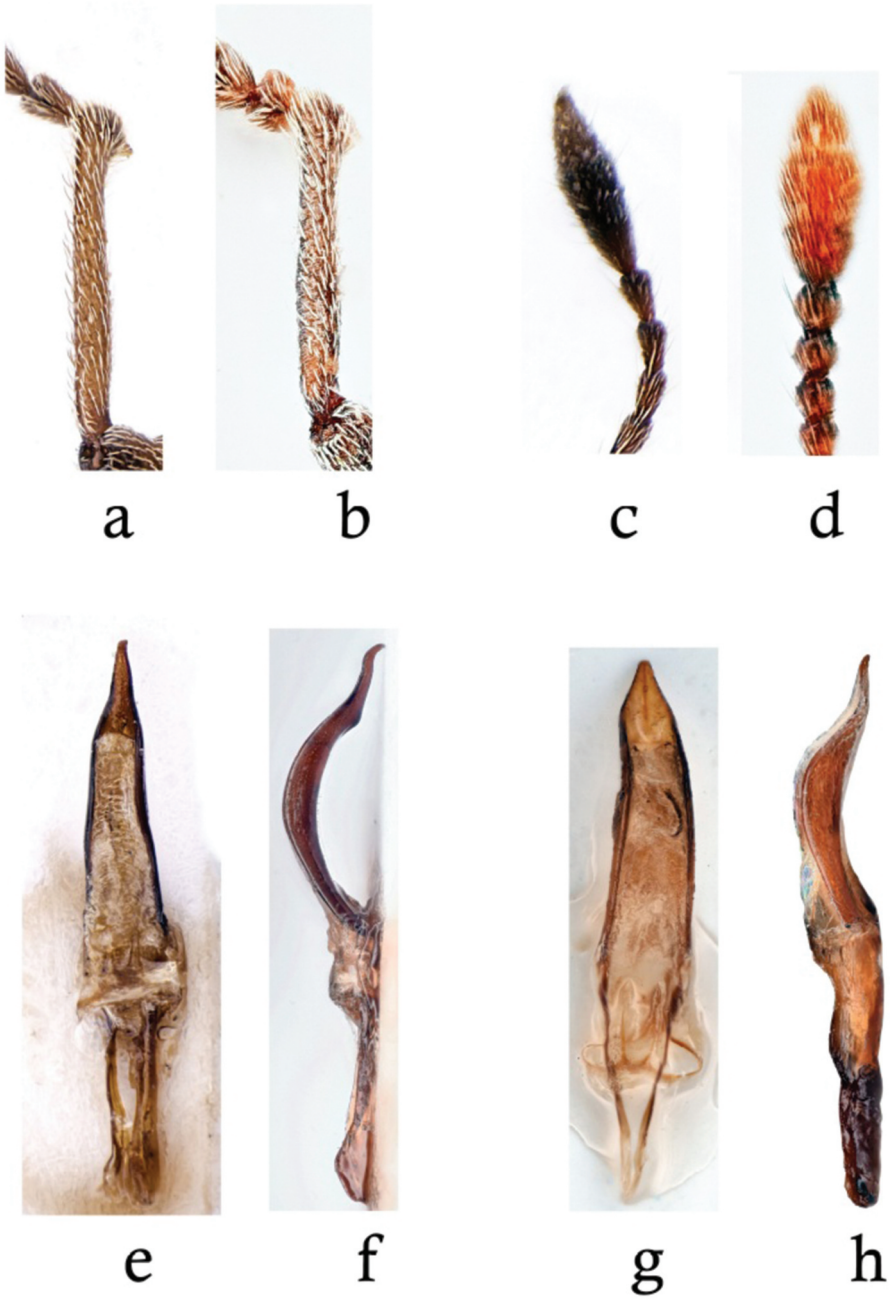

Figure 3. Left protibia of (a) *C. meridionalis* and (b) *C. hoffmanni* of the same specimens as those shown in Figure 2. Last antennomeres and club of (c) *C. meridionalis* and (d) *C. hoffmanni*. Aedeagus of *C. meridionalis* in (e) dorsal and (f) lateral views. Aedeagus of *C. hoffmanni* in (g) dorsal and (h) lateral views

Linosa reveals two unique haplotypes only (h3–h4); the remaining haplotypes (h2, h5–h8) are unique to Malta. Clade #2 (pp = 1.00) is made up of all remaining haplotypes (h9–h28) scored from samples collected in the whole of Sicily and from Pantelleria Island. It shows two clusters (2a: h9, h10, h11, h13, h15, h16, h17, h20, h21, h24, h25, h26; 2b: h12, h14, h18, h19, h22, h23, h27, h28) at a small divergence level of 0.007 (K80 distance) nonetheless suggesting a somewhat structured genetic variation for this clade.

Table S3 lists the pairwise K80 distance values among all scored haplotypes. The inferred statistical parsimony network (Figure 5) shows an overall topology reflecting that obtained by the Bayesian analysis, bringing to light two separate networks matching clades #1 and #2. The smaller network shows the

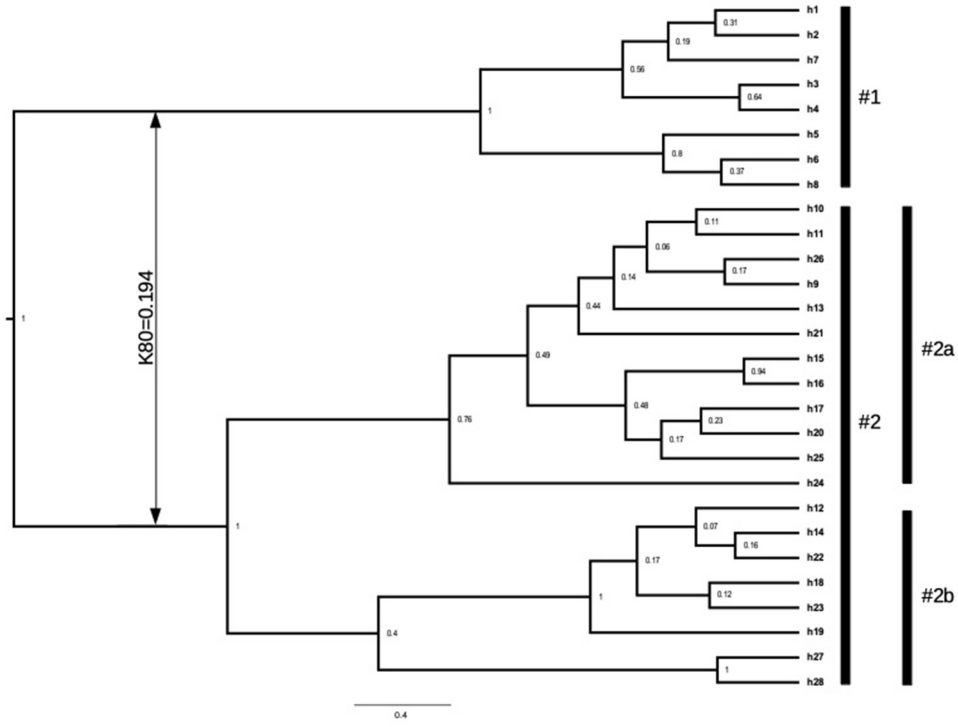

Figure 4. Bayesian consensus tree of the scored haplotypes of the cox1 gene; figures at nodes are posterior probability values; vertical black bars refer to scored groups discussed in the text; vertical double arrow shows the K80 distance between groups #1 and #2.

relationships among haplotypes h1–h9, all sampled from the Malta archipelago and Pelagian Islands as discussed. The second network mirrors clade #2 of the Bayesian topology, highlighting the more entangled relationships among haplotypes. The network topology clearly shows two haplotype groups corresponding to clusters 2a and 2b discussed for the Bayesian topology. Even if the geographic distribution of the haplotypes does not unveil a clear-cut pattern, it is noteworthy that haplotypes h14 and h18 are more frequent in south-eastern Sicily whereas h9 is more widely distributed in and frequent in the westernmost part of the island (Figure 5; Table S2). However, it must be noted that, overall, the two clusters exhibit a largely overlapping geographical distribution.

The results of the ABGD analysis (Figure 6) clearly indicate that our data set contains two partitions that coincide with the clades identified through Bayesian analysis and statistical parsimony. The list of haplotypes and individuals contained in the two clades are listed in Table S4 (including some parameters). In contrast, single-threshold (Figure S1a) and multiple-threshold (Figure S1b) GMYC analyses produce six and five putative species, clearly oversplitting our data set, probably owing to the already discussed genetic differentiation observed in clades #1 and #2 (Figures 4 and 5). Many authors have discussed the oversplitting behaviour of the GMYC model due to several different circumstances (e.g. Talavera et al. 2013), and we are convinced that the convergence of the results of all of the analyses except GMYC strongly suggests that our data set includes haplotypes of two well-distinct taxa only. Both taxa show a certain degree of intra-specific variation that, among other factors, could also be related to the flightless characteristic of these insects. The two taxa include individuals that were morphologically identified as *C. hoffmanni* (group/clade #1) and *C. meridionalis* (group/clade #2) and show a very high level of genetic divergence (K80 = 0.194), well above the 0.01 threshold level between intra- and inter-specific divergence frequently encountered in the DNA barcoding literature (e.g. Bergmann et al. 2013; Magoga et al. 2018). The K80 values computed as net divergence within groups #1 and #2 are 0.004 and 0.010, respectively, falling within the range of intraspecific distance variability frequently reported for weevil taxa (e.g. Astrin et al. 2012; De Biase et al. 2016). Overall, we are therefore inclined to believe that the K80 value scored between groups #1 and #2 suggests, as do the results of the morphological analysis and of the other adopted approaches (except GMYC), an interspecific level of differentiation.

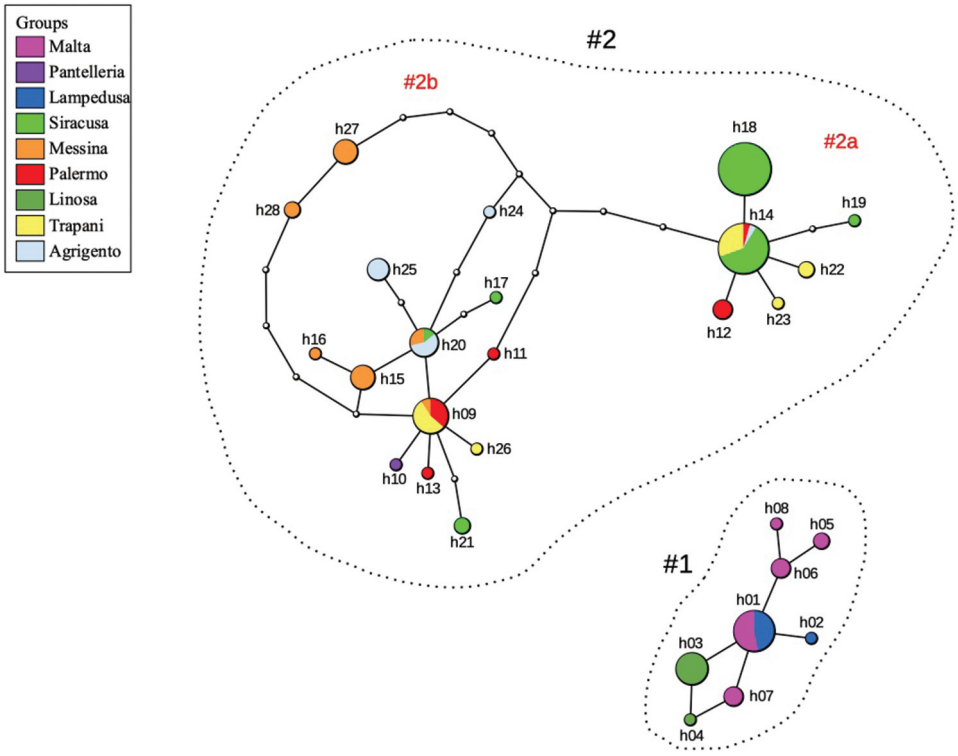

Figure 5. Statistical parsimony network of the scored cox1 haplotypes; each haplotype is represented by a circle whose size is proportional to the haplotype frequency (number of individuals found); empty circles correspond to missing or not sampled (presumed) haplotypes; numbers and abbreviations refer to groups and clades discussed in the text and illustrated in Figure 4.

Finally, Table S5 lists all the nucleotide positions that showed alternative fixed bases for *C. hoffmanni* and *C. meridionalis*. Altogether, 83 fixed alternative nucleotide positions are observed, some of which give rise to seven fixed alternative amino acid substitutions. These are therefore molecular characteristics that can be used as diagnostic characters for the two species, in addition to the morphological characters illustrated above. However, it must always be borne in mind that further analyses of the mitochondrial genetic variability of these two species could change the picture of variability of some characters now considered diagnostic. Therefore, the use not of single molecular characters but of the group of them as a whole is the best strategy to identify specimens taken in nature with a greater degree of safety.

Conclusions

The above results clearly show the existence of only two population groups that might be considered distinct species of *Chiloneus*. Group #1 is very clearly differentiated and clumps haplotypes distributed in Malta and the Pelagie Islands; this group coincides with the taxonomic entity *C. hoffmanni*. Group #2 clumps all other sampled populations and matches the taxonomic entity *C. meridionalis* that is distributed in the whole of Sicily and on Pantelleria Island, being more variable than the previous species, although the level of genetic differentiation is still within the range of intra-specific variation. The indication of *C. meridionalis* from Pantelleria is a new record for this island.

Further investigations are needed to improve our knowledge of the relationships among these species and with other members of the genus *Chiloneus*, particularly with the Greek ones very close to those dealt with in this note. Finally, we are currently running a project to unveil the phylogeographic history of these taxa, which appear to be of great interest for evaluating the expansion–contraction model of Pleistocene biogeography.

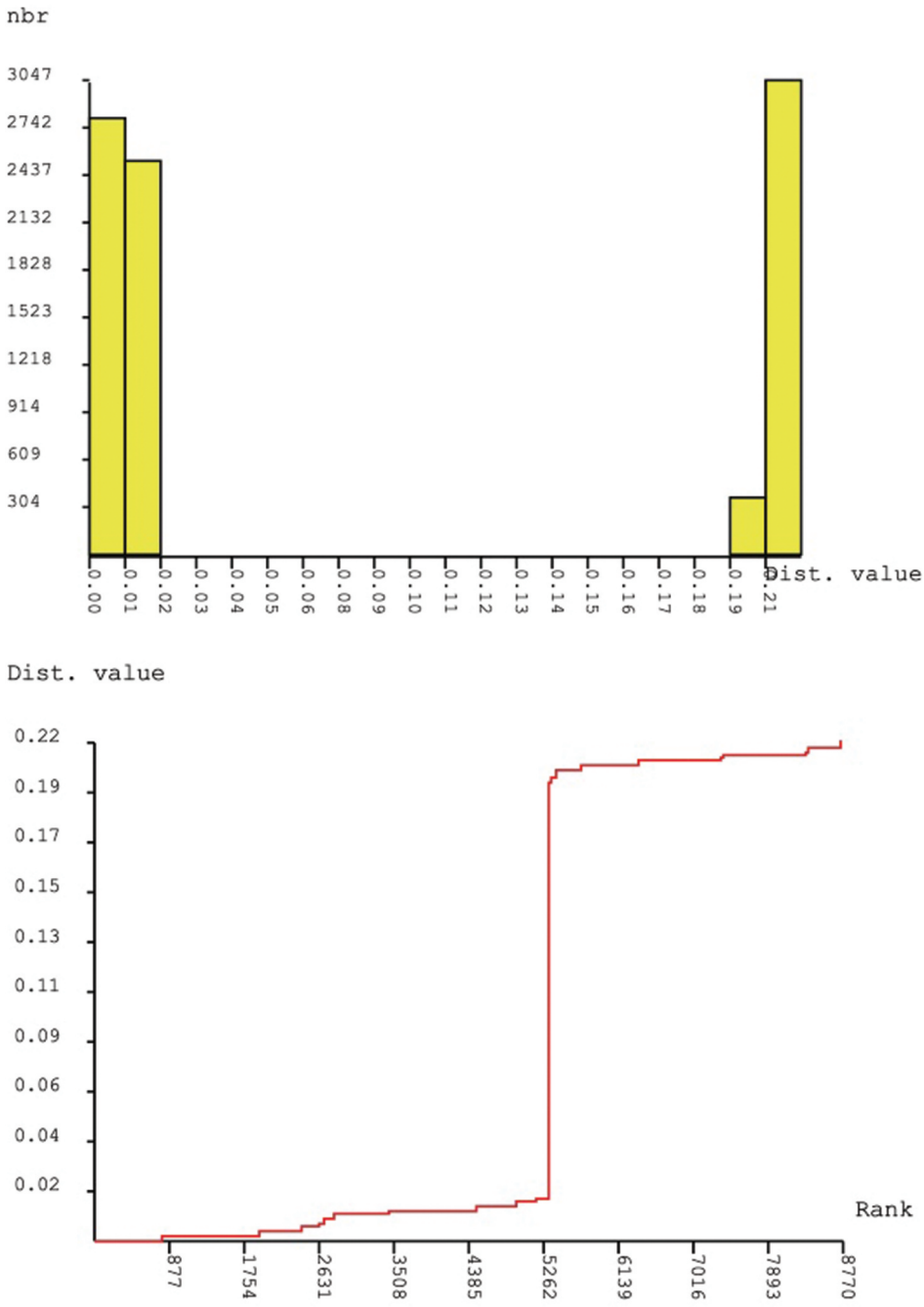

Figure 6. Results of the automatic barcoding gap discovery (ABGD) analysis using the K80 distance measure for cox1: (a) distribution of pairwise K80 distances among all samples shows two modes (low and high distance values) related to intraspecific and interspecific distances, respectively; (b) the same values plotted in ranked order show a steep slope at the barcoding gap value.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary material

Supplemental data for this article can be accessed here.

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