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Molecular Phylogenetics and Evolution 26 (2003) 348–353

MOLECULAR PHYLOGENETICS AND EVOLUTION

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Taxonomy of Iberian *Hoplia* (Col., Scarabaeoidea, Hopliinae) based on mtDNA analysis

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Received 24 July 2001; revised 10 December 2001

Abstract

The morphology of some *Hoplia* species (Scarabaeoidea: Hopliinae) is so variable that parapatric populations have often been considered different species or subspecies. In this study we analyze the nucleotide sequences of a fragment of mitochondrial gene cytochrome *c* oxidase subunit I (COI) of six species and two subspecies of Palaearctic *Hoplia* to reexamine the species limits. Based on the analysis of sequences from COI and morphological and ecological observations, we consider *Hoplia freyi* Baraud to be a junior synonym of *Hoplia chlorophana* Erichson and *H. philanthus ramburi* Heyden to be a junior synonym of *H. philanthus philanthus* (Fuessly). However, complete resolution of relationships among *H. philanthus* subspecies requires the addition of sequences from genes evolving faster than COI. Phylogenetic relationships among the species studied are discussed.

1. Introduction

Hoplia Illiger is a widespread genus comprising more than 250 species from the Holarctic, Ethiopian, and Neotropical regions (Hardy, 1977). However, of the 40 species inhabiting Europe, only 7 species and two subspecies are known to occur in the Iberian Peninsula. Morphological studies of *Hoplia* species show the existence of few diagnostic characters that separate species. For these reasons, several species and subspecies have been recognized based on highly variable morphological characters (Hardy, 1977).

Hoplia (*Hoplia*) *freyi* Baraud, 1967 was described based on five specimens collected in southeastern Spain. However, we found a great morphological variation, with overlapping of diagnostic characters between the Iberian endemic *Hoplia* (*H.*) *chlorophana* Erichson, 1848 and *H.* (*H.*) *freyi*.

Another example of controversy was the taxonomic status of *Hoplia* (*Decamera*) *philanthus* subspecies. To date, three subspecies have been described: *H.* (*D.*) *philanthus philanthus* Fuessly, 1775; *H.* (*D.*) *philanthus ramburi* Heyden, 1870; and *H.* (*D.*) *philanthus gagates* Bedel, 1911. *H.* (*D.*) *philanthus philanthus* is widely spread throughout western Europe and this subspecies is sympatric in the Iberian Peninsula with *H.* (*D.*) *philanthus ramburi* (Baraud, 1992; Tomás-Biosca and Galante, 1978). Moreover, *H.* (*D.*) *philanthus ramburi* is sympatric in the north of Africa with *H.* (*D.*) *philanthus gagates* which was described as the "black form" inhabiting the north of Africa (Baraud, 1985). We found a well-established population of *H.* (*D.*) *philanthus gagates* in the southeastern Iberian Peninsula. Morphological studies provided little character support for cited species and subspecies. To resolve problems, we applied molecular analysis to reexamine the species limits. Mitochondrial DNA (mtDNA) is well suited to identifying morphologically similar species because the mutation rate is high enough to provide numerous sequence differences between closely related species (Avise, 1994). The mitochondrial gene cytochrome *c* oxidase subunit I (COI) has been applied to phylogenetic problems in a wide range of hierarchical levels in insects (Brower, 1996; Frati et al., 1997; Funk, 1999; Howland and Hewitt, 1995), including closely related species (Beckenbach et al., 1993; Sper-

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doi:10.1016/S1055-7903(02)00302-0

ling and Hickey, 1994). The purpose of this study was to compare sequences of a fragment of the mtDNA COI to (1) assess phylogenetic relationships of some Iberian taxa of the subfamily Hopliinae, (2) demostrate that *H.* (*H.*) *freyi* and *H.* (*H.*) *chlorophana* are conspecific, and (3) clarify the taxonomic status of *H.* (*D.*) *philanthus* subspecies.

2. Materials and methods

2.1. Specimens

We examined the type material of *H. freyi*, *H. philanthus gagates*, and *H. philanthus ramburi* deposited in the National Natural History Museum (Paris, France). Material analyzed by molecular techniques is listed in Table 1. Additional Scarabaeoidea species that were sequenced as outgroups were *Anisoplia remota* Reitter, 1889 and *Anisoplia depressa* Erichson, 1847 (Rutelidae), *Melolontha melolontha* (L., 1758) (Melolonthidae), and *Aphodius immaturus* (Mulsant, 1842) (Aphodiidae). Voucher specimens were deposited in the entomological collection of Alicante University (Spain) (CEUA) and in the Paul Valéry University (Montpellier, France).

2.2. Molecular techniques

Total genomic DNA was extracted with the standard phenol–chloroform method. A double-stranded COI template suitable for sequencing was generated using the polymerase chain reaction (PCR) with universally conserved mtDNA COI primers C1-J-1718, C1-J-1859, C1-J-2183, TL2-N-3014 (Simon et al., 1994), and C1-N-2507 (Howland and Hewitt, 1995). PCR-amplified products were cleaned using Quiagen's PCR Purification Kit. The PCR product was cycle-sequenced with PE Biosystems Big Dye Terminator Cycle Sequencing Kit using a ABI 310 sequencer. All sequences were aligned by eye.

2.3. Data analysis

A heuristic parsimony analysis was performed with PAUP* 4. 0 (Swofford, 1998) under the Fitch criterion (equal weights; Fitch, 1971). Characters were successsively weighted (successive approximation weighting (SW); Farris, 1969) based on the rescaled consistency index, a base weight of 1000, and their maximum value if more than one tree was found. Afterward, a heuristic search was performed with TBR. Successive rounds of weighting/searching were performed until the same tree length was obtained in two successive rounds. We also inferred the phylogenetic relationships among all species using maximum-likelihood (ML) (Strimmer and von Haeseler, 1996).

3. Results and discussion

3.1. Analysis of molecular data

The final aligned sequences yielded 375 characters (deposited in GenBank; accession numbers are listed in Table 1).

Table 1
Species and specimens examined

Species	Collection locality	Collector	GenBank Accession No.
H. (*H.*) *chlorophana* 1	Luciana (Ciudad Real, Spain)	E. Micó and E. Galante	AY090510
H. (*H.*) *chlorophana* 2	Camping de Riopar (Albacete, Spain)	E. Micó and J.R. Verdú	AY090511
H. (*H.*) *chlorophana* 3	Campillo de Llerena (Badajoz, Spain)	E. Micó and E. Galante	AY090512
H. (*H.*) *chlorophana* 4	Frias de Albarracín (Teruel, Spain)	E. Micó and E. Galante	AY090508
H. (*H.*) *freyi*	Marjal de Pego (Alicante, Spain)	E. Micó and J.R. Verdú	AY090509
H. (*H.*) *bilineata*	Riopar (Albacete, Spain)	E. Micó and J.R. Verdú	AY090514
H. (*H.*) *africana*	Zaafrane (Tunis)	J.P. Lumaret	AY090513
H. (*H.*) *argentea*	Saint Guilhem le Désert (France)	O. Mountreuil	AY090515
H. (*D.*) *p. philanthus* 1	Javalambre (Teruel, Spain)	E. Micó and J.R. Verdú	AY090520
H. (*D.*) *p. philanthus* 2	Montes Aquilianos (León, Spain)	E. Micó and J.R. Verdú	AY090521
H. (*D.*) *p. philanthus* 3	Baldayo (La Coruña, Spain)	E. Micó and J.R. Verdú	AY090518
H. (*D.*) *p. ramburi* 1	Peña de Francia (Salamanca, Spain)	E. Micó and J.R. Verdú	AY090519
H. (*D.*) *p. ramburi* 2	Prioro (León, Spain)	E. Micó and J.R. Verdú	AY090522
H. (*D.*) *p. gagates* 1	Marjal de Pego (Alicante, Spain)	E. Micó	AY090516
H. (*D.*) *p. gagates* 2	Marjal de Pego (Alicante, Spain)	E. Micó	AY090517
Outgroups			
A. remota	Frias de Albarracín (Teruel, Spain)	E. Micó and E. Galante	AY090507
A. depressa	Prioro (León, Spain)	E. Micó and J.R. Verdú	AY090506
M. melolontha	Viol le fort (Montpellier, France)	O. Mountreuil	AY090505
Aph. inmaturus	Mont Ventoux (France)	O. Piau	AY090504

All codon positions have highly A + T-rich composition, as is typical of animal mtDNA molecular evolution (Brown, 1985). In the studied sequences, the third codon A + T bias is more than 84%. The proportion of informative sites is most important when all sequences are considered (26–28% without or with outgroup), but if we consider only the *Hoplia* sequences, this proportion reaches only 17% (with mostly two variants). Uncorrected pairwise distances within the species varied from 5.6% (for closely related species) to 20.3%. Intraspecific variation was not equal for the different species. Variation ranged from 2.1 to 4.8% in *H.* (*H.*) *chlorophana*, while for *H.* (*D.*) *philanthus* values were always below 1.6%. *Hoplia* species are not good flyers, which favors isolation among populations. However, the intraspecific rates obtained for *H. philanthus* were surprisingly low when geographically distant populations were considered (Fig. 1).

3.2. Phylogenetic analysis

Using the heuristic search, the three most parsimonious trees obtained had 288 steps, consistency index (CI) and retention index (RI) being equal to 0.67 and 0.75, respectively. After SW, one of these trees (Fig. 1 was retained (see Section 2) (weighted length = 141.88, CI = 0.84, RI = 0.88).

Based on the species sequences studied, *Hoplia* genus had 63 parsimony-informative sites (16.9%). Monophyly was not rejected and was supported by 99% bootstrap

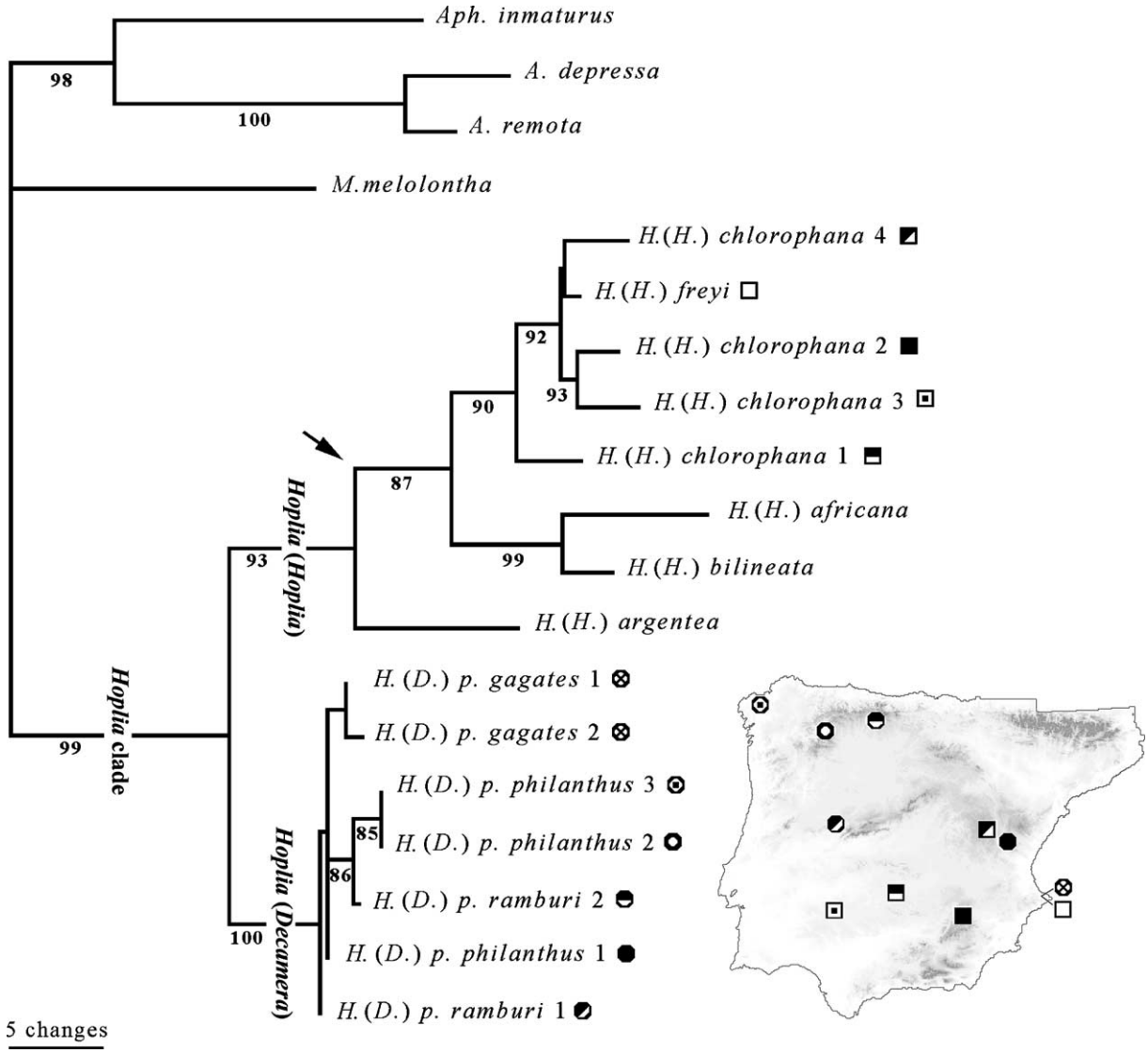

Fig. 1. Most parsimonious SW tree. Bootstrap values are indicated on the tree (1000 replications). Branches not found in the Fitch strict consensus are marked with an arrow. This tree is one of the three trees found with Fitch (unweighted) parsimony. On the right, map of collection sites of *H.* (*H.*) *clorophana*, *H.* (*H.*) *freyi*, and *H.* (*D.*) *philanthus* specimens.

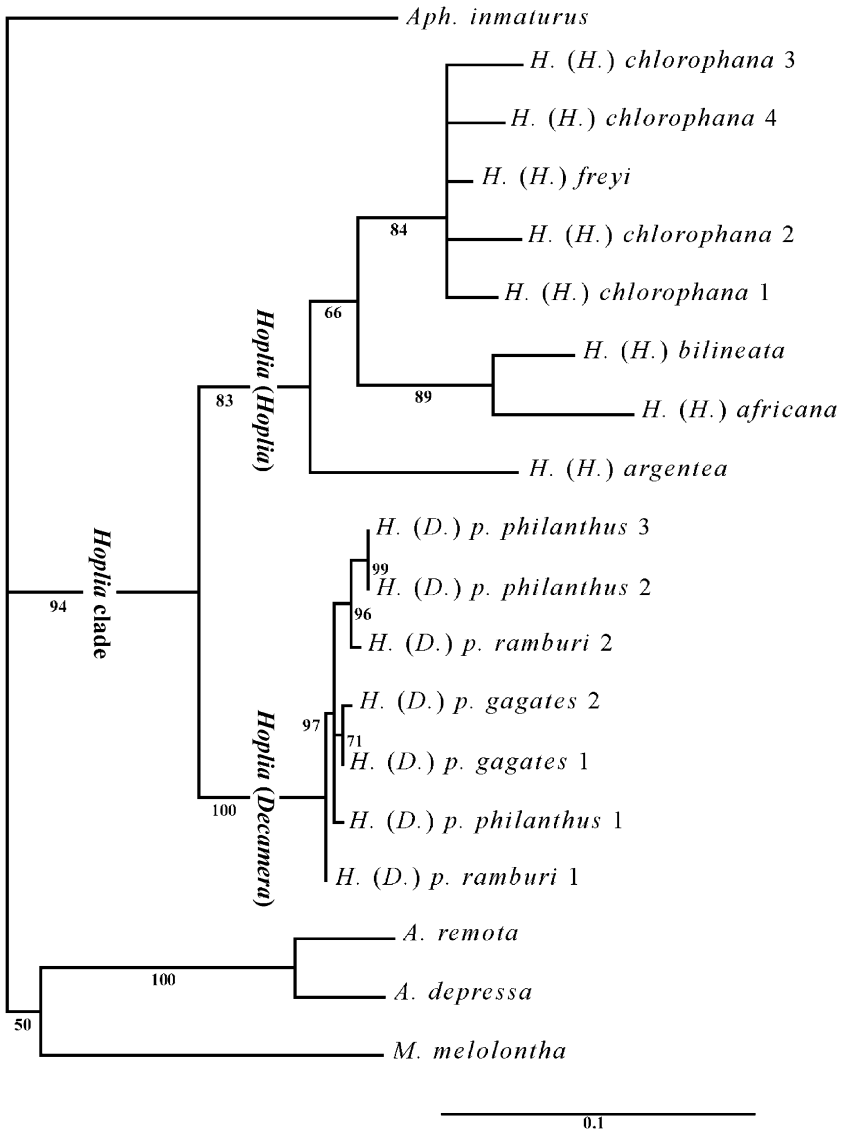

Fig. 2. Phylogenetic relationships of the studied species using the maximum-likelihood algorithm; tree constructed using a neighbor-joining method. The bootstrap values are shown above the branches (100 replications).

replicates and maximum-likelihood value (94%) (Figs. 1 and 2).

Parsimony and maximum-likelihood trees showed two main clades in the *Hoplia* species examined: (i) one clade constituted by the species of the subgenus *Hoplia* Illiger was strongly supported by the 93 and 83% bootstrap replicates and (ii) the other clade comprising *Decamera* Mulsant subgenus was supported by 100% replicates (Figs. 1 and 2).

The Hoplia (Hoplia) clade. This clade includes five species belonging to the subgenus *Hoplia*. The basal species was *H. argentea*, a species present in Europe except for the Iberian Peninsula and Britain (Baraud, 1992). This branch has been marked with an arrow in the SW tree to enhance the fact that this node appears unresolved in the Fitch strict consensus (Fig. 1). In the maximum-likelihood tree, it was supported by a low bootstrap value (66%) (Fig. 2).

The clade including *H. africana* and *H. bilineata* was clearly identified (Figs. 1 and 2). *H. africana* is present only in the north of Africa (Morocco, Algeria, Tunisia) while *H. bilineata* is distributed from the north of Africa to the southern Iberian Peninsula. The highest intraspecific distance within *H. chlorophana* was recorded from *H. chlorophana* 1. This distance was close to the limit of the range observed between closely related, morphologically distinct species (e.g., *H. bilineata*). Within the *H. chlorophana* clade, *H. freyi* showed low distances from other *H. chlorophana* specimens. This range of distances observed in the *H. chlorophana* group was equal to the intraspecific divergence shown in other Coleoptera (Funk, 1999). This result was congruent with

our morphological observations. The Iberian endemic *H. chlorophana* had a wide range throughout the Iberian Peninsula, while *H. freyi* was known only from two localities of southeastern Spain (Baraud, 1967). In the diagnosis of *H. freyi*, Baraud (1967) used morphological characters to differentiate both species, such as the overlapping degree of elytral scales and location of the basal teeth on the anterior tibiae. However, after studying 829 specimens of *H. chlorophana* which belong to 120 distinct populations, we recorded high variability for these characters. Another diagnostic character was the distal portion of the parameres, which were "symmetrical in *H. freyi* and slightly asymmetrical in *H. chlorophana*." However, after studying 17 specimens of *H. freyi* from 3 different populations, we consider that this character is easily masked by manipulation due to the light sclerotization of the apical ventral area of the parameres.

The Hoplia (Decamera) clade. The subgenus *Decamera* is separated from members of the subgenus *Hoplia* due to the male antennae bearing 10 segments instead of 9 segments. Intrataxon distances in the *Hoplia* (*Decamera*) clade were clearly lower than those in *H. chlorophana*. The position of the subspecies is not resolved within the *H. philanthus* clade. In fact, *H. philanthus ramburi* appeared intermixed with the nominative subspecies (Figs. 1 and 2). Molecular analyses were in agreement with morphological observations showing that the three different recognized morphotypes for *H. philanthus* belong to the same species. No molecular evidence supports the existence of the three subspecies, and only *H. philanthus gagates* could be maintained as a unit (Figs. 1 and 2).

Two morphological autapomorphies used to differentiate the *H. philanthus* subspecies were exclusively based on the color and shape of elytral scales and on the background color on the dorsum. The known distribution of *H. philanthus ramburi* is restricted to isolated populations in the north of Africa, in the southern Iberian Peninsula and in the middle and the north of Spain. This subspecies has the same ecological niche as that of *H. philanthus philanthus*. On the contrary, the autapomorphies distinguishing *H. philanthus gagates* show less variation than those of *H. philanthus ramburi*. *H. philanthus gagates* is totally black on the dorsum instead of dark brown; the scales are bluish, scarce, and piliform instead of oval. Body shape, especially the pronotum, appears more spherical. The morphology of 90 specimens from three different populations from the north of Africa and the eastern Iberian Peninsula did not show any variation in these characters. The habitat occupied by *H. philanthus gagates* is wetland. However, more data are needed to assess whether the ecological isolation is favoring the fixation of gene lineages of *H. philanthus gagates*.

3.3. Taxonomic implications

Results from morphological and molecular analyses allow the following conclusions. (i) *H.* (*H.*) *freyi* Baraud n. syn. is a junior synonym of *H.* (*H.*) *chlorophana* Erichson. This is highly supported (92% bootstrap support in SW tree; 84% bootstrap in ML tree) by molecular analyses and by morphological observations. Both parsimony and maximum-likelihood analyses showed a well-defined clade comprised of *H. chlorophana* and *H. freyi*. (ii) Based on our molecular analyses we hypothesize that the subspecies of *H. philanthus* are conspecific. Geographical, ecological, and morphological data allow us to assert that *H. philanthus ramburi* Heyden n. syn. is a junior synonym of *H. philanthus philanthus* Fuessly. We consider *H. philanthus gagates* Bedel a valid subspecies until more geographical, ecological, and molecular data are provided. Due to the surprisingly low divergence obtained in this group we believe that it is necessary to conduct a larger survey which would include sampling many populations and different genes to reconstruct the phylogeny of *H. philanthus* and its subspecies in the Iberian Peninsula.

Acknowledgments

We thank G. Sthalls for technical and logistical help and for discussion and comments on an earlier version of the manuscript. We thank O. Montreuil for the specimen of *Melonlontha melolontha*. We are indebted to the anonymous reviewers whose comments greatly improved the manuscript. K. Burke helped with an early English version. The research was supported in part by the Spanish Ministry of Science and Technology (BOS2000-0148) and by the French Entomological Society Germaine Cousin for O. Piau.

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