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DNA barcoding a highly diverse group of parasitoid wasps (Braconidae: Doryctinae) from a Mexican nature reserve

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Abstract

Background and aims. The preliminary results of a DNA barcoding study of the doryctine fauna of parasitoid wasps from the Chamela–Cuixmala Biosphere Reserve in Mexico, a region dominated by tropical dry forest, are presented. So far, three field trips have been carried out to the reserve and 468 specimens have been collected, of which 407 *cox1* sequences were obtained.

Materials and methods. The general mixed Yule-coalescent model was applied to a phylogram to investigate the number of evolutionary units that can be detected from the DNA sequence data examined.

Results. A total of 185 barcoding species assigned to 20 identified doryctine genera were discriminated using the above model, 115 of which belong to the speciose genus *Heterospilus*, pointing out the extraordinary species richness of this subfamily of insects in a Mexican tropical dry forest.

Conclusion. On the basis of the DNA barcodes generated, *Ptesimogastroides* Braet & van Achterberg is proposed to be a junior synonym of *Ptesimogaster* Marsh syn. nov. *Neoheterospilus* was also found deeply nested within a large *Heterospilus* clade, suggesting the paraphyly of the latter genus.

Keywords: *DNA barcoding, Doryctinae, Hymenoptera, Braconidae,* cox1, *tropical dry forest*

Introduction

DNA barcoding (Hebert et al. 2003) has proved to be a valuable tool for the rapid identification of species, especially in the case of megadiverse, poorly known fauna. In particular, this molecular technique has shown to be useful in many cases in assessing the actual diversity of small, morphologically conserved invertebrate taxa (e.g. Smith et al. 2008, 2009; Monaghan et al. 2009). In addition to considerably accelerating the process of species delimitation in those groups, DNA barcoding analyses also inherently help to solve some of their taxonomic problems. For instance, DNA barcoding studies (in animals, usually a fragment of the *cox1* mitochondrial [mt] DNA gene) may reveal conspecificity among specimens of distinct sexes that were previously considered to be allospecific (e.g. Sheffield et al. 2009), allow association of different semaphoronts of a given species (e.g. Gossner and Hausmann 2009), detect cryptic species (e.g. Desneux et al. 2009; Li et al. 2010), or can be used to select candidate taxa for phylogenetic analyses with a vast taxon sampling (Hajibabaei et al. 2007). Moreover, although regarded with caution, DNA barcoding could also help to give insights into the taxonomic placement of problematic taxa based on the tree-building methods that are performed.

With over 1000 described species and more than 180 recognized genera, the braconid wasp subfamily

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Doryctinae represents a prime example of an understudied highly diverse group of insects. Doryctine wasps are known to attack mainly xylophagous or bark-boring coleopteran larvae; however, other host groups (e.g. species of Lepidoptera and Diptera) and biologies (e.g. gall association, termitophyly) have also been recorded for members of this subfamily (Belokobylskij 1992; Wharton and Hanson 2005). Higher level classification within the Doryctinae has been the subject of extensive confusion due to its extensive morphological homoplasy, and the erection of genera based only on the combination of characters or trends, which probably has arisen in a number of non-monophyletic supraspecific taxa (Belokobylskij et al. 2004; Zaldívar-Riverón et al. 2008).

Here, we show the preliminary results of an ongoing DNA barcoding study of the doryctine species richness from the Chamela–Cuixmala biosphere reserve, a region dominated by tropical dry forest that is located near the Pacific coast of Jalisco, Mexico. We employ the general mixed Yule-coalescent (GMYC) model to estimate species boundaries using the standard animal DNA barcoding marker (~650 bp of the *cox1* mtDNA gene). Our results obtained after only three collecting trips carried out so far have not only unveiled an extraordinary species richness for the Doryctinae but also allowed for some taxonomic inferences within the group.

Materials and methods

Study site and specimen sampling

Specimens belonging to the braconid wasp subfamily Doryctinae were mostly collected in the Chamela biological station of the Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). This station is located within the Chamela–Cuixmala biosphere reserve, near the Pacific coast in the state of Jalisco, Mexico. Insects were collected in two different vegetation types, tropical dry and tropical subdeciduous forests, the first being the predominant type of vegetation in the region. Three field trips of 6–8 days duration each were carried out during June, September, and November 2009. In all the trips, specimens were collected with two malaise traps, two to four light traps, 100–300 yellow pan traps, and a minimum of four sweep nets for at least 4 h per day.

A 615–658 bp fragment corresponding to the standard animal DNA barcoding marker (cytochrome *c* oxidase subunit I mtDNA gene) was amplified for the collected samples using both LepF1/LepRI (Hebert et al. 2004) and LCO1490/HCO2198 (Folmer et al. 1994) primers. All specimens were preserved in absolute ethanol and kept at −20°C until they were processed. For most of the specimens, a single leg was removed, placed in a 96-well lysis plate, and posted to the University of Guelph for DNA extraction, amplification, and sequencing. DNA extractions and amplifications were carried out at the Instituto de Biología, UNAM, for about one-quarter of the specimens. PCR amplification products of these samples were placed in 96-well lysis plates containing 6.25 μl of 10% trehalose each well and also posted to the University of Guelph for DNA sequencing (see laboratory protocols in Smith et al. 2009). Sequences were edited with Sequencher 4.0.5 (Gene Codes Corp.) and aligned manually based on their translated amino acids.

All of the *cox1* sequences generated are deposited in GenBank (accession numbers GU715182-288, HM420734-5, HM434309-544, HM882254, HQ200960-201008, 201239-54). These sequences and all the specimen information are available in the project file "Parasitoid Wasps (Braconidae: Doryctinae) of Chamela–Cuixmala Biosphere Reserve" (ASDOR project) in the projects section of the Barcode of Life Data Systems (www.barcodinglife.org).

Species boundaries analyses

All the collected specimens were identified to genus level using the relevant literature (Marsh 1997, 2002; Braet et al. 2003; Belokobylskij 2006; Gomes and Penteado-Dias 2007) and subsequently mounted after generating their DNA sequences. Species boundaries were assessed using the GMYC approach (Pons et al. 2006; Fontaneto et al. 2007), which requires a fully resolved topology with branch length estimates. A Bayesian analysis using BEAST 1.5.3 (Drummond and Rambaut 2007) was therefore carried out in order to reconstruct a fully resolved topology with branch length estimates, removing first all the duplicate haplotypes with Collapse 1.2 (Posada 2004). The analysis performed used a relaxed lognormal clock, and branch lengths were estimated using a coalescent prior and a GTR + I + Γ model of evolution (Lanave et al. 1984; Yang 1994) with unlinked codon positions. The latter model was selected using the program jModelTest (Posada 2008) and the Akaike Information Criterion (Akaike 1974). The coalescent prior gives more conservative results, as it is more likely to ignore a coalescent—speciation transition since the GMYC employs a coalescent as the null model to explain branching patterns (Pons et al. 2006; Monaghan et al. 2009). Moreover, the latter prior has been found to perform better for estimating branch lengths than the Yule prior for relaxed lognormal clock estimates, therefore, giving more accurate species boundaries' estimates (Monaghan et al. 2009).

The Bayesian analysis was run for 20 million generations, sampling trees every 1000 generations. Stationarity was detected to occur after 700,000 generations using Tracer 1.4 (Rambaut and Drummond 2007), although we followed a conservative

approach and deleted the trees sampled from the first 10 million generations. The remaining sampled trees were used to build a maximum clade credibility tree with TreeAnnotator v.1.5.3 (Rambaut and Drummond 2008).

The GMYC approach for species delimitation was carried out using the SPLITS package for the R statistical environment (from http://r-forge.r-project.org/projects/splits), running both the single and the multiple threshold optimizations (Monaghan et al. 2009) in order to compare their performance with the data set examined.

Results and discussion

Species diversity

A total of 468 specimens were collected during the three field trips performed, of which we were able to generate barcoding sequences (between 483 and 658 bp in length) for 407 of them. The number of haplotypes used for the analyses was 275. Application of the single and the multiple threshold GMYC models using the phylogram reconstructed with BEAST with a lognormal coalescent approach yielded 186 and 178 putative species ("barcoding" species), (CI = 182–190, 174–183; -lnL of null model = 23929.2, 3929.2; -lnL of GMYC model = 23995.7, 4016.7, respectively). Of these species, 54 and 79 were recovered as separate clusters (i.e. clades with two or more haplotypes) and 132 and 99 as singletons (i.e., represented by a single haplotype), respectively. Inspection of the results obtained from both analyses revealed that, even when it resulted in a lower number of barcode species, the multiple-threshold GMYC model frequently considered the existence of two or more species within various clusters of sequences that evidently belonged to a single lineage. We, therefore, only included the results derived from the single-threshold GMYC model.

The 185 barcode species delimited with the single-threshold GMYC model were assigned to 20 identified doryctine genera (Table I), of which 115 species belonged to *Heterospilus* Haliday. Specimens belonging to two additional genera, *Notiospathius* Matthews and Marsh and cf. *Barbalhoa* Marsh, were also collected, although failure to amplify the barcoding region for their assigned specimens prevented their inclusion in the analyses. The barcoding species accumulation curve including the samples from the three collecting trips that were carried out clearly reveals that the number of evolutionary units discriminated in the site of study has not reached an asymptote (Figure 1); and therefore, a considerable number of species are still expected to be discovered during subsequent trips. This species diversity, however, still needs to be confirmed using other sources of evidence, such as using an additional nuclear marker and/or confirming it with morphological examination of the barcoding species involved (Monaghan et al. 2009).

Our preliminary results support previous DNA barcoding studies in other geographic regions that

Table I. Number of specimens and barcode species recovered for each identified genus.

Genera	Number of specimens	Number of haplotypes	Number of barcoding species
Allorhogas Gahan	34	20	16
cf. *Barbalhoa* Marsh*	3	–	–
Callihormius Ashmead	14	13	12
Coiba Marsh	4	4	2
Ecphylus Foerster	26	22	14
Glyptocolastes Ashmead	3	2	2
Hansonorum Marsh	10	5	2
Heterospilus Haliday	307	171	115
Iare Barbalho & Penteado-Dias	17	15	6
Janzenia Marsh	2	1	1
Masonius Marsh	2	2	1
Neoheterospilus Belokobylskij	10	2	1
Notiospathius Matthews & Marsh*	4	–	–
Odontobracon Cameron	2	2	2
cf. *Ondigus* Braet & van Achterberg	2	1	1
Panama Marsh	3	2	1
Platydoryctes Barbalho & Penteado-Dias	2	2	2
Psenobolus Reinhard	1	1	1
Ptesimogaster Marsh	2	1	1
Rhaconotus Ruthe	5	4	3
Spathius Nees	14	4	1
Whitfieldiellus Marsh	1	1	1
Total	468	275	185

* Genera whose *cox1* sequences could not be recovered.

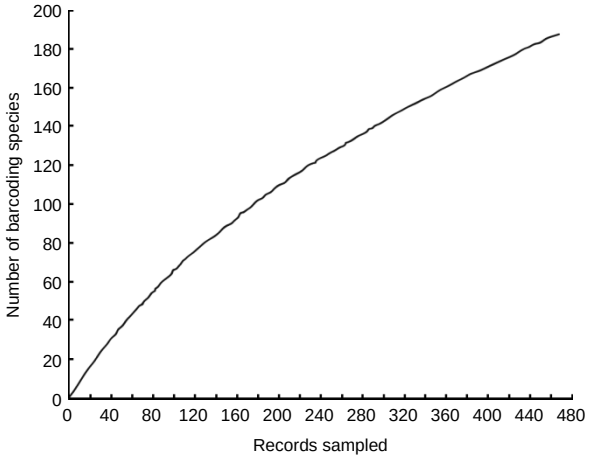

Figure 1. DNA barcode accumulation curve (obtained from the Barcode of Life Data Systems; www.barcodinglife.org) for the doryctine wasp species collected during three field trips carried out in June, September, and November 2009.

have revealed the existence of an extraordinary but highly overlooked species diversity of small invertebrates not only in the tropics (e.g. Smith et al. 2008; Monaghan et al. 2009) but also in temperate zones (Sheffield et al. 2009; Smith et al. 2009). Regarding our group of study, the parasitoid wasp subfamily Doryctinae is known to be particularly diverse in the tropics (Belokobylskij 1992; Marsh 2002), although it has been virtually neglected in the Mexican territory since only 33 described species have been reported to occur in this country, five of which belong to *Heterospilus* (Yu et al. 2005). Our ongoing study will give insight into the actual species richness of this speciose group of insects in a Mexican tropical dry forest, which has traditionally been considered to have a lower biodiversity than other tropical ecosystems.

Taxonomic inferences

Our DNA barcoding sequences detected that the specimens of two of the genera that were identified, *Ptesimogaster* Marsh and *Ptesimogastroides* Braet and van Achterberg (Figure 2a,b), share a same haplotype and therefore they might be conspecific, pending additional molecular evidence. In any case, this finding reveals that, whether these two taxa are conspecific or not, they actually are very closely related, and we therefore propose *Ptesimogastroides* to be a junior synonym of *Ptesimogaster* syn. nov. (type species: *P. parkeri* Marsh 1965). The described species of *Ptesimogastroides* are only distinguished from the species of *Ptesimogaster* by the absence of a distinctive antero-ventral basal tubercle on the hind coxa (Braet et al. 2003) (Figure 2c,d). This result supports other studies (Zaldívar-Riverón et al. 2007, 2008) that have shown that the latter morphological feature, traditionally considered a major character to separate groups of genera within the Doryctinae, is considerably variable and thus cannot be employed alone to separate genera nor to propose new genus group names in the subfamily. Interestingly, the specimens collected from the latter two genera are a male and a female, so further

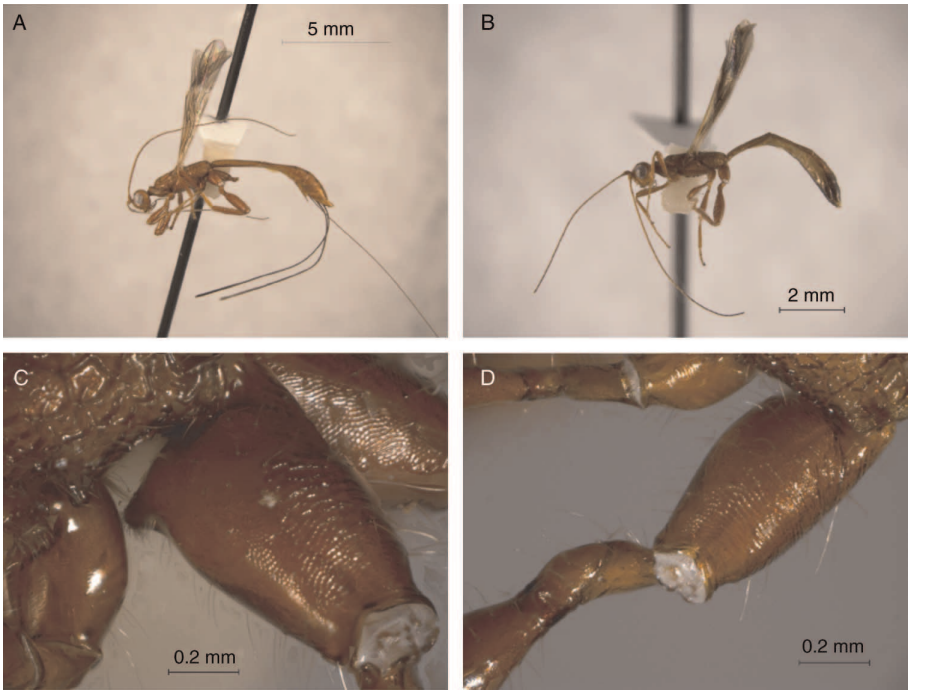

Figure 2. Lateral view of the body of (a) *Ptesimogaster* sp. and (b) *Ptesimogastroides* sp. Hind coxa of (c) *Ptesimogaster* sp. and (d) *Ptesimogastroides* sp.

collections will help to determine whether the presence or absence of a tubercle in the hind coxa actually represents sexual dimorphism or individual variation within a species.

The phylogenetic analysis performed with the *cox1* sequences recovered a large *Heterospilus* clade, but with the specimens assigned to *Neoheterospilus* Belokobylskij deeply nested within it. Despite being regarded with caution when inferring evolutionary relationships when it is analyzed separately due to its high substitution rate, it has been observed that the *cox1* gene possesses reliable phylogenetic signal at different taxonomic levels; therefore, it is not rare that barcoding studies may accurately recover clusters of genera, tribes, and even families (e.g. Sheffield et al. 2009). The results obtained in this work point out the paraphyletic condition of the speciose genus *Heterospilus* at least with respect to *Neoheterospilus*, which is congruent with the morphological features displayed by the two genera involved. The species of *Neoheterospilus* are only distinguished from the ones of *Heterospilus* by having a highly modified, unusually shaped ovipositor (Belokobylskij 2006; Martínez and Zaldívar-Riverón, in press). This conclusion, however, needs to be confirmed with additional molecular markers.

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