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EXPLORING DNA BARCODES OF NEOTROPICAL AND AFROTROPICAL SPECIES OF *ECCOPSIS* ZELLER (LEPIDOPTERA: TORTRICIDAE)

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ABSTRACT. Originally described from Africa, the genus *Eccopsis* Zeller (Lepidoptera: Tortricidae) currently includes 25 Afrotropical and five Neotropical species. Adult morphological characters suggest that the Afrotropical and Neotropical species might not be congeneric. Here we present the first DNA sequences for Neotropical *Eccopsis* and use these data in a maximum likelihood (ML) analysis to evaluate the monophyly of the genus, and to examine the utility of DNA barcodes in separating the South American *E. galapagana* Razowski & Landry, 2008 and *E. razowskii* Vargas, 2011. Intraspecific and interspecific pairwise distances (K2P) were 0–0.5% and 4.9–5.2%, respectively, and each species was recovered as a distinct, well supported group of sequences (i.e., species) in the ML analysis. An analysis including barcodes of Afrotropical *Eccopsis* (four species), Afrotropical *Paraeccopsis* (one species), and Neotropical *Eccopsis* (two species) failed to recover *Eccopsis* as monophyletic. Consistent with previous suggestions based on adult morphology, this study highlights the necessity to reassess the congeneric status of Afrotropical and Neotropical species of *Eccopsis*.

Additional key words: *Acacia macracantha*, *Eccopsis galapagana*, *Eccopsis razowskii*, Fabaceae, *Prosopis alba*

As currently defined, the genus *Eccopsis* Zeller, 1852 (Lepidoptera: Tortricidae: Olethreutinae: Olethreutini) is represented in both the Afrotropical and Neotropical regions (Brown 2014, Gilligan et al. 2014a). The Afrotropical fauna includes 25 described species, including the type species, *Eccopsis* wahlbergiana Zeller, 1852, from South Africa (Gilligan et al. 2014a). The genus was first reported from the Neotropics by Razowski and Wojtusiak (2008), who described a species of *Eccopsis* from the mountains of Ecuador, which subsequently was transferred to *Megalota* Diakonoff, 1966 by Brown (2014). Later, three additional Neotropical species were described in *Eccopsis*, two from the Galapagos (Razowski et al. 2008) and one from Chile (Vargas 2011). Brown (2014) transferred two other species to the genus, resulting in a total of five described *Eccopsis* in the Neotropics. All five appear to be closely related to each other and very similar to Afrotropical *Eccopsis*. However, the generic assignment of the Neotropical species recently was questioned based on adult morphological characters (Brown 2014).

Two species of *Eccopsis* are known from the Atacama Desert of northern Chile: *E. galapagana* Razowski & Landry, 2008 and *E. razowskii* Vargas, 2011. *Eccopsis galapagana* was described from the Galapagos Islands, Ecuador, and it was subsequently discovered in Chile (Vargas 2011) and Colombia (Gallego et al. 2012). It is apparently widespread in western South America where its larvae feed on Fabaceae (Vargas 2011, Gallego et al. 2012). In Colombia, *E. galapagana* can be a serious pest in silvopastoral systems of *Prosopis juliflora* under outbreak conditions (Gallego et al. 2012). In contrast, *E. razowskii* appears to have a more restricted geographic range; it is known only from the coastal valleys of northern Chile where its larvae feed on *Acacia macracantha* Willd. (Vargas 2011).

DNA barcodes are useful for exploring biodiversity and taxonomy, especially in concert with other character sources (Brown et al. 2014a, Gilligan et al. 2016, Escobar-Suárez et al. 2017, Razowski et al. 2017). Barcodes also can be used to identify immature stages of insects, including Lepidoptera, providing knowledge of

their trophic interactions when rearing larvae to obtain the adults is difficult or impossible (Gossner & Hausmann 2009, Hausmann & Parra 2009, Frye & Robbins 2015). This application is important in studying trophic ecology (Hrcek et al. 2011) or species of economic concern (Shashank et al. 2015), such as *E. galapagana* (Gallego et al. 2012).

The objectives of this study are to provide the first DNA barcode sequences of *Eccopsis* for the Neotropical Region and to provide a preliminary study of the relationships between Afrotropical and Neotropical *Eccopsis*. We also assess the usefulness of DNA barcodes in separating and identifying *E. galapagana* and *E. razowskii*.

MATERIALS AND METHODS

Specimens

Larvae of *E. galapagana* and *E. razowskii* were collected on *Prosopis alba* in the city of Arica, and on *Acacia macracantha* in the Azapa and Chaca valleys, in the Atacama Desert of northern Chile, from March 2014 to January 2015 (Table 1). The larvae were brought into the laboratory in plastics vials, where they were kept at room temperature. New leaves were added periodically until larvae were ready to pupate. Some pupae were preserved in ethanol 95% at -20°C for DNA extraction; others were kept in plastic vials to obtain adults, which were deposited as vouchers in the Colección Entomológica de la Universidad de Tarapacá (IDEA), Arica, Chile. In addition, two specimens of *E. galapagana* reared from *Prosopis pallida* from the Tumbes Region in northwestern Peru were included (vouchers in the T. M. Gilligan collection, Colorado, USA).

DNA extraction and sequencing

For Chilean specimens, genomic DNA was extracted from pupae following procedures described in Huanca-Mamani et al. (2015). PCR amplification and sequencing of the DNA barcode fragment of the COI gene were performed by a commercial facility (Macrogen, South Korea) using the primers LEP-F1 (5′-ATTCAACCAATCATAAAGATAT-3′) and LEP-R1 (5′-TAAACTTCTGGATGTCCAAAAA-3′) developed by Hebert et al. (2004). PCR conditions were those described in Vargas et al. (2014). For Peruvian specimens, genomic DNA was extracted from legs, amplified using the Hebert et al. (2004) primers, and sequenced according to procedures described in Gilligan et al. (2014b).

Data analysis

The software MEGA6 (Tamura et al. 2013) was used to align the sequences with MUSCLE (Edgar 2004), to calculate the mean nucleotide composition, and to calculate pairwise distances among sequences according the Kimura 2-parameter (K2P) model (Kimura 1980). The number of haplotypes, the number of variable sites, and the number of parsimony informative sites were determined using the software DnaSP (Librado & Rozas 2009).

A maximum likelihood (ML) analysis (Felsenstein 1981) was performed to assess the evolutionary relationships of the two Neotropical *Eccopsis* with four Afrotropical representatives of the genus. Additional DNA barcode sequences (658 bp) were downloaded from GenBank (Benson et al. 2013) and BOLD (Ratnasingham & Hebert 2007), including those of congeneric species and some of species belonging to the closely related genera *Cosmorrhyncha* Meyrick, 1913, and *Paraeccopsis* Aarvik, 2004; also, one sequence of a representative Tortricinae, *Eugnosta percnoptila* (Meyrick, 1933), was used to root the tree (Regier et al. 2012). The ML analysis and the selection of the best model to describe the substitution pattern were performed in MEGA6 following the procedures described by Hall (2013). The General Time Reversible model with invariable sites (GTR + I) was selected previous to ML analysis according the Bayesian Information Criterion (BIC). The bootstrap method (Felsenstein 1985) was used with 1,000 replicates to assess the statistical support of the clades.

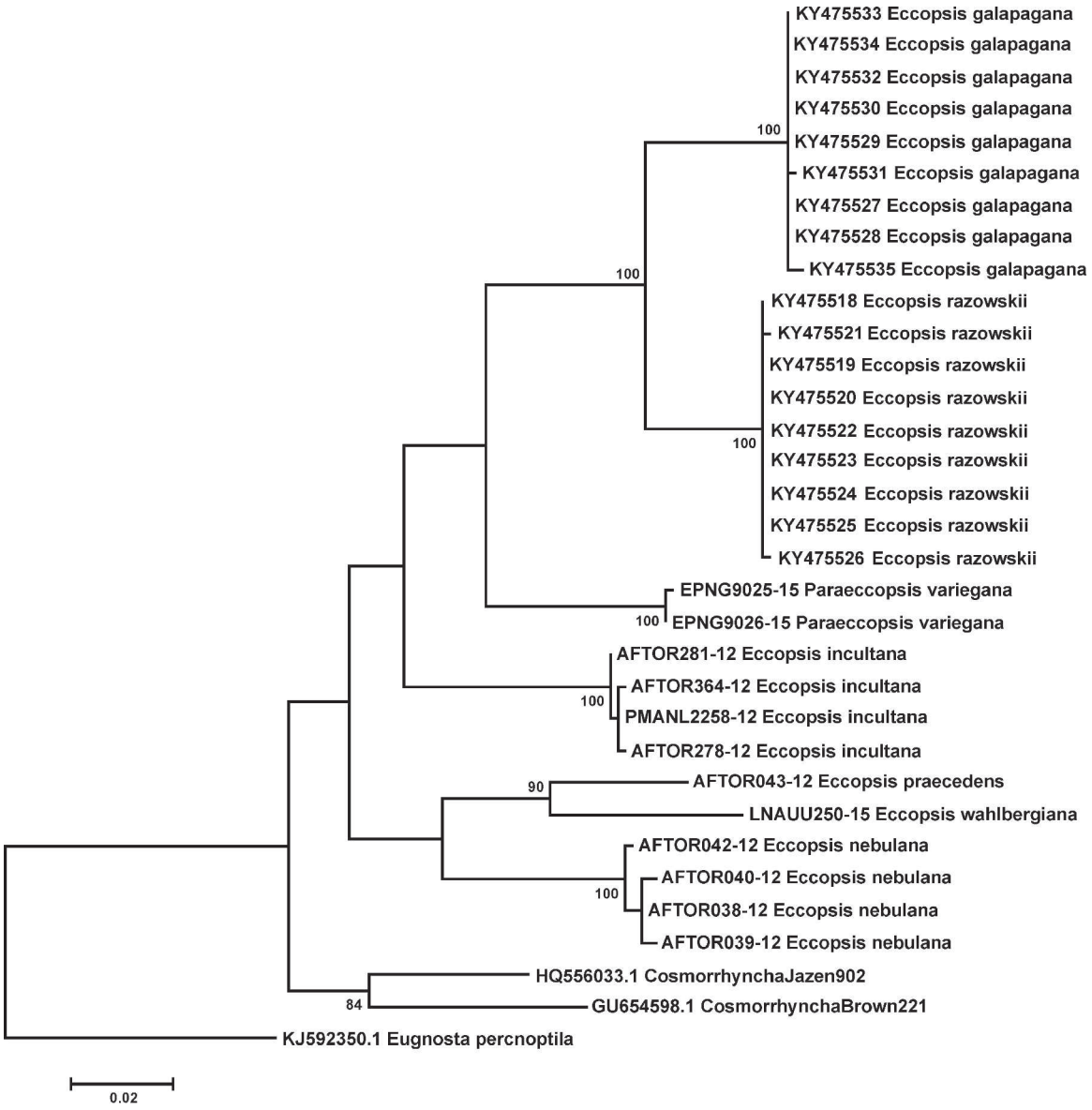
RESULTS

DNA barcodes

Eighteen sequences of DNA barcodes (658 bp) were obtained for *E. galapagana* (n = 7 from Chile; n = 2 from Peru) and *E. razowskii* (n = 9), the mean nucleotide composition of which was 39.14% (T), 15.73% (C), 30.54% (A) and 14.59% (G). Pairwise distances (K2P) among the 18 sequences of the two South American *Eccopsis* were between 0.0–0.5% at the intraspecific level, and 4.9–5.2% at the interspecific level. Three haplotypes were detected in the *E. galapagana* sample, which were differentiated by three mutations: two transitions (T-C, site 238; and G-A, site 493) and one transversion (G-C, site 620). Three haplotypes were found in the *E. razowskii* sample, which were defined by two mutations: one transversion (A-T, site 281) and one transition (A-G, site 424) (Table 2).

ML analysis

The alignment for the ML analysis was composed of 33 DNA barcode sequences (658 bp), including the 18 newly reported here and 15 downloaded from GenBank and BOLD (Table 1). The mean nucleotide composition of all these sequences was 38.9% (T), 15.5% (C), 30.9%



This does have enough resolution

FIG. 1. Maximum likelihood tree of *Eccopsis* and *Paraeccopsis* species based on sequences of the DNA barcode fragment (658 bp) of the cytochrome c oxidase subunit I (COI) gene. Bootstrap values >70 are shown at nodes.

(A) and 14.7% (G). Overall, the alignment had 186 variable sites, 148 of which were parsimony informative; no insertions or deletions were found. The lowest pairwise distances (K2P) among the Neotropical *Eccopsis* and *Paraeccopsis* were 9.3% with *E. galapagana* and 8.5% with *E. razowskii*.

The nodes representing each of the two Neotropical species of *Eccopsis* were strongly supported, and the cluster formed by these two species was also strongly supported (Fig. 1). However, an *Eccopsis* group including both Afrotropical and Neotropical representatives was not supported; instead, the genus was found to be polyphyletic. Although single gene trees are no substitute for rigorous phylogenetic analyses, the results do provide limited insight into relationships, or at least similarity, among the species. The type species of *Eccopsis* (*E. wahlbergiana*) clustered with two other Afrotropical representatives: *E. praecedens* Walsingham, 1897 and *E. nebulana* Walsingham, 1891. Although *E. wahlbergiana* clustered with *E. praecedens* with high support, the association of this clade with *E. nebulana* was only weakly supported (67% bootstrap). *E. incultana*

TABLE 1. Voucher data of the DNA barcode sequences (658 bp) used in the analyses of this study.

Species	Country	Locality	Voucher	Accession	Reference
Eccopsis razowskii	Chile	Azapa	IDEA003-02-01	KY475518	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-24	KY475519	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-25	KY475520	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-26	KY475521	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-27	KY475522	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-28	KY475523	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-29	KY475524	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-30	KY475525	This study
Eccopsis razowskii	Chile	Chaca	IDEA003-02-31	KY475526	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-01	KY475527	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-02	KY475528	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-03	KY475529	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-04	KY475530	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-05	KY475531	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-06	KY475532	This study
Eccopsis galapagana	Chile	Arica	IDEA003-03-07	KY475533	This study
Eccopsis galapagana	Peru	Tumbes Region	TMG-802	KY475534	This study
Eccopsis galapagana	Peru	Tumbes Region	TMG-803	KY475535	This study
Paraeccopsis variegana	Kenya	Mpala Ranch		EPNG9025-15	BOLD
Paraeccopsis variegana	Kenya	Mpala Ranch		EPNG9026-15	BOLD
Eccopsis incultana	Nigeria	Int. Inst. Trop. Ag.		AFTOR278-12	BOLD
Eccopsis incultana	Nigeria	Int. Inst. Trop. Ag.		AFTOR281-12	BOLD
Eccopsis incultana	Kenya	Mpala Res. Centre		AFTOR364-12	BOLD
Eccopsis incultana	Nigeria	Int. Inst. Trop. Ag.		PMANL2258-12	BOLD
Eccopsis nebulana	Kenya	Ololua Forest		AFTOR038-12	BOLD
Eccopsis nebulana	Kenya	Muhaka Forest		AFTOR039-12	BOLD
Eccopsis nebulana	Kenya	Arabuko-Sokoke Forest		AFTOR040-12	BOLD
Eccopsis nebulana	Kenya	Muhaka Forest		AFTOR042-12	BOLD
Eccopsis praecedens	Kenya	Kamwana Forest		AFTOR043-12	BOLD
Eccopsis wahlbergiana	Somalia	Baidoa		LNAUU250-15	BOLD
Cosmorrhyncha sp. (Brown221)	Costa Rica	Area de Conservacion Guanacaste		GU654598.1	GenBank
Cosmorrhyncha sp. (Janzen902)	Costa Rica	Area de Conservacion Guanacaste		HQ556033.1	GenBank
Eugnosta percnoptila	Kenya	Kereta Forest		KJ592350.1	GenBank

(Walker, 1863) was not clustered with any other Afrotropical *Eccopsis*. Although *P. variegana* Agassiz & Aarvik, 2014 was placed as a sister group of the Neotropical group of *Eccopsis*, the statistical support of this relationship was low.

DISCUSSION

These are the first sequence data for Neotropical *Eccopsis* (i.e., *E. galapagana* and *E. razowskii*) and the first study to examine relationships between the Neotropical and Afrotropical members of the genus using DNA sequences. Although a number of barcodes of Afrotropical *Eccopsis* are available on BOLD and in GenBank, the only previously published DNA barcodes for *Eccopsis* are two Afrotropical species (Brown et al. 2014b). The sequences of *E. razowskii* are particularly useful because eight of the specimens used for DNA extraction were collected at the type locality (Chaca Valley). The barcode data for *E. galapagana* may have slightly less fidelity to that species because it was described from the Galapagos Islands.

The high genetic divergence at the interspecific level compared with the low divergence at the intraspecific level suggests that DNA barcodes can be successfully used to separate *E. galapagana* and *E. razowskii*. Furthermore, the sequences of each of the two species were recovered as groups reciprocally monophyletic with high statistical support in the ML analysis (Fig. 1). As already reported for other study systems involving immature stages of Lepidoptera (Gossner & Hausmann 2009, Vargas et al. 2014, Rivera-Cabello et al. 2015, Frye & Robbins 2015, Shashank et al. 2015), DNA barcodes could be useful in studies focused on the identification of immature stages of the Chilean species of *Eccopsis*, such as surveys of larvae on additional Fabaceae to determine host breadth.

The high statistical support found in the ML analysis for the clade formed by *E. galapagana* and *E. razowskii* is an interesting result in favor of the probable monophyly of the Neotropical species currently included in *Eccopsis* (Brown 2014). However, additional Neotropical representatives should be included in future analyses. Furthermore, it is probable that a number of Neotropical species are waiting to be discovered and studied; thus, additional work on the alpha taxonomy of the South American fauna and additional taxon sampling are required to reach more meaningful phylogenetic conclusions.

Although sequences of only six species of *Eccopsis* were included in the ML analysis, the preliminary results argue against the monophyly of the genus as currently defined, and this corroborates the conclusions of Brown (2014) based on morphology. There is little doubt that Afrotropical and New World members of *Eccopsis* are closely related. However, New World *Eccopsis* appears to be the sister-group to the African *Paraeccopsis* based on forewing shape and maculation (i.e., the forewing is longer and narrower in both genera than in Afrotropical *Eccopsis*, with a somewhat *Lobesia*-like forewing pattern). Also, polymorphism or extreme individual variation in forewing markings is the norm in New World *Eccopsis* and *Paraeccopsis*, whereas it is absent in Afrotropical *Eccopsis*. New World *Eccopsis* and *Paraeccopsis* also share the absence of the hindwing anal roll characteristic of many Olethreutini including *Eccopsis*. In the male genitalia of *Eccopsis*, *Cosmorryncha*, and other Neopotamiae group genera (sensu Aarvik 2004), the apex of the uncus is usually conspicuously bilobed with each lobe bearing a patch of ventrally-projecting setae, whereas it is blunt, rounded, or only slightly emarginate apically in New World *Eccopsis* and *Paraeccopsis*, often with a "crown" of setae rather than two patches. And whereas species of many genera of the Neopotamiae group have a digitate process from the costa of the valva (e.g., *Eccopsis*, *Cosmorryncha*, *Megalota*, *Metendothenia*), this process is entirely absent in New World *Eccopsis* and *Paraeccopsis*.

Despite the low statistical support (52%), the African *P. variegana* was recovered as the nearest species to the

TABLE 2. Nucleotide variation among haplotypes of the DNA barcode fragment (658 bp) of the cytochrome c oxidase subunit I (COI) gene of *Eccopsis galapagana* (n = 9) and *E. razowskii* (n = 9) from Peru and Chile. ([a]) "-" indicates nucleotide identity to the first haplotype of the respective species. ([b]) Ts: transition, Tv: transversion.

Haplotype	Variable sites([a,b])			n
	Eccopsis galapagana			
	238	493	620	
	Ts	Ts	Tv	
H1EG	T	G	G	7
H2EG	-	-	C	1
H3EG	C	A	-	1
	Eccopsis razowskii			
	281	424		
	Tv	Tv		
H1ER	A	A	7	
H2ER	-	G	1	
H3ER	T	-	1	

two Neotropical *Eccopsis* based on the lowest K2P divergence. Although our results did not capture the monophyly of Afrotropical *Eccopsis*, because *E. incultana* did not clustered with any "congeneric" species, Afrotropical *Eccopsis* appear to be monophyletic based on morphology. The lack of support along the "backbone" of the phylogenetic tree is not unusual given the relatively short length of COI and its relatively rapid rate of evolution (McDonagh et al. 2016). Other data also support the conclusion that *Eccopsis*, as currently defined, is not monophyletic. Gallego et al. (2012) indicated a biological difference, as the Neotropical species appear to be restricted to Fabaceae, whereas many African representatives are known to be polyphagous (Diakonoff 1977, Aarvik 2004, Gallego et al. 2012, Brown et al. 2014b). Recently, Vargas-Ortiz et al. (2017) highlighted the potential usefulness of the external morphology of the immature stages to help solve taxonomic problems in *Eccopsis*.

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