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PHYLOGENETIC RELATIONSHIPS WITHIN THE GENUS *EUPHERUSA* INFERRED FROM MTDNA SEQUENCES

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Resumen. El objetivo de este trabajo fue evaluar la monofilia del género Eupherusa y esclarecer las relaciones entre las cuatro especies que lo conforman. Para analizar la monofilia de *Eupherusa* se utilizaron ocho especies de otros géneros de colibríes y se reconstruyó su filogenia usando secuencias del gen mitocondrial ND2 (1041 pb) con diferentes métodos, incluyendo Máxima Parsimonia, Inferencia Bayesiana y Máxima Verosimilitud. Encontramos un total de 13 diferentes haplotipos y los diferentes análisis confirman la monofilia de *Eupherusa* y apoyan que el clado de *Elvira* y *Microchera* representa su grupo hermano. Las relaciones dentro de *Eupherusa* revelan que las dos especies endémicas de México, y que se distribuyen en la ladera oeste del país (E. poliocerca y *E. cyanophrys*) están más relacionadas entre sí, aunque mantienen independencia filogenética entre ellas, mientras que las dos especies restantes, una con una distribución muy amplia del lado oriente de México hasta Centroamérica (*E. eximia*) y la otra con una distribución restringida a Centroamérica (*E. nigriventris*), están más relacionadas entre ellas, aunque también respetando su independencia como linajes evolutivos independientes.

Abstract. We evaluated the monophyly of the hummingbird genus *Eupherusa* and reviewed the phylogenetic relationships of its four species. To assess the monophyly of the genus we used eight species of hummingbirds from genera other than *Eupherusa*, in maximum parsimony, maximum likelihood and Bayesian analyses. We sequenced ND2 (1041 bp mtDNA), and identified 13 different haplotypes. The different analyses confirmed the monophyly of *Eupherusa* and suggested that the clade formed by *Elvira* and *Microchera* is its sister group. Within *Eupherusa*, our analyses revealed two sister clades reflecting species geography: *E. poliocerca* and *E. cyanophrys* from western Mexico and *E. nigriventris* and *E eximia* from eastern Mexico.

Key words: Molecular systematics, Phylogeny, Trochilidae, *Eupherusa*, Mexico, Central America, Hummingbird.

INTRODUCTION

The species number and composition of the hummingbird genus *Eupherusa* has changed over time. Initially, Gould (1857) included only *E. eximia* (originally named *Ornismya eximia* DeLatree 1843) and *E. poliocerca*. Later, Ridgway (1911) considered that *Eupherusa* included three species: *E. poliocerca* and divided *E. eximia* in two species (*E. eximia* and *E. egregia*). In 1945, Peters transferred *Callipharus nigriventris* to *Eupherusa* and rejected that *E. egregia* was a different species from *E. eximia*. Afterwards, Rowley & Orr (1964) discovered *E. cyanophrys*. *Eupherusa* includes small hummingbirds with the bill about as long as the head, maxillary

tomium serrated terminally, nasal operculum mostly unfeathered, tail more than half as long as wing, rounded, with broad rectrices, rufous secondaries, and lateral rectrices mostly white (at least the inner web), the adult males show bright metallic green below with white under tail-coverts, and the females show pale gray or grayish white below (Ridgway 1911). According to the American Ornithologists' Union (1998), *Eupherusa* includes four species (Fig. 1). *E. cyanophrys* is distributed in the mountains of southern Oaxaca. *E. poliocerca* is distributed along the Pacific slope of Guerrero (Sierra Madre del Sur) and western Oaxaca, and southern Mexico. *E. eximia* is found on the eastern slope of Mexico, with three described subspecies: *E. e. nelsoni* is distributed in eastern and southeastern Mexico, *E. e. eximia*, distributed in extreme eastern Mexico south through the highlands to central Nicaragua, and *E. e. egregia*, from the highlands of Costa Rica and western Panama. Finally, *E. nigriventris* is found in the Highlands of Caribbean slope, from central Costa Rica to western Panama.

The objective of this paper is to evaluate the monophyly of *Eupherusa* and to provide a phylogenetic hypothesis for its four described species, based on comparisons of 1041 base pairs of the mitochondrial gene ND2.

MATERIAL AND METHODS

We obtained tissue samples from four species of *Eupherusa* genus (*E. eximia*, *E. poliocerca*, *E. nigriven-*

FIG. 1. Geographic distribution of *Eupherusa* genus. *E. poliocerca* (1), *E. cyanophrys* (2), *E. eximia* (3) and *E. nigriventris* (4).

tris and *E. cyanophrys*), as well as selected species of the Emerald group that could be part of one natural clade (McGuire *et al.* 2007, Table 1); we used *Amalizia tzacatl* as outgroup. We also included sequences from the Genbank for *E. nigriventris*, *Microchera abocoronata*, *Thalurania furcata*, *T. colombica*, *Chalybura buffonii* and *C. urochrysia* (Table 1).

We extracted DNA from tissue samples using the *Qiagen DNeasy* extraction kit, following the manufacturer's protocols. We amplified and sequenced the full length of NADH dehydrogenase subunit 2 (ND2, 1041 bp). Amplification of ND2 was conducted using the primers L5219 and H6313 (Sorenson *et al.* 1999); amplification products were cleaned with Exo-Sap. Sequencing reactions used dye-labeled terminators (BigDye chemistry, *Applied Biosystems*); the products of sequencing reactions were cleaned with 70% alcohol and diluted with water. All new sequences have been deposited in Genbank under Accesion numbers XXXXXXX

Sequences were aligned and proofread using Sequencher 4.8 (Gene Codes Corporation 2007) and ClustalX (Higgins & Sharp 1988). We corroborated the mitochondrial origin of our sequences

TABLE 1. List of the species, GenBank accession numbers and vouchers/sample IDs.

Taxon	GenBank (accession no.)	Collection No.
Thalurania colombica, T. furcata, *Chalybura buffonii, C. urochrysia,* *Microchera albocoronata,* *Elvira chionura, E. cupreiceps,* *Amazilia tzacatl* *Eupherusa eximia*	AY830524, AY830525, EU042537, EU042538, EU042571, EU042548, AY830478, EU983391 EU042552	
Eupherusa nigriventris		B16051 B16055
Eupherusa eximia		CHIMA161 CHIMA181 CHIMA318 CHIMA378 OMVP558 OMVP505
Eupherusa cyanophrys		CONACYT04_202 CONACYT04_231 CONACYT04_240 PLU030
Eupherusa poliocerca		ATO031 ATO034 ATO043 BEHB200 BEHB215 OMVP655 OMVP657 SIT043 SIT049

by combining at least two of the following methods: amplifying overlapping gene segments, amplifying or sequencing one region with different primer sets, sequencing both DNA strands or using multiple individuals of a single species.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP 4.0b (Swofford 2002) unless otherwise stated. MP analyses used a heuristic search using a TBR branch-swapping option and with all positions equally weighted; support for each node was obtained by 1000 bootstrap replicates (Felsenstein 1985). ML was conducted in Garli (Zwickl 2006), using a heuristic search and nodal support was estimated via 100 bootstrap replicates. We used jModeltest (Posada 2003) to evaluate the model parameters for the ML searches. The model of molecular evolution that best fitted our sequences was GTR+I+G (Lset base: freqA=0.2097, freqC=0.0771, freqG=0.3738, freqT=0.3394; nst = 6; rates = gamma; shape = 0.9010; ncat = 4; pinvar = 0.4000).

We also constructed a phylogenetic tree using Bayesian Inference (BI) with the same model of evolution that best explained our data obtained with jModelTest (see above). BI analyses were conducted using MrBayes 2.0 (Huelsenbeck & Ronquist 2001). We ran two independent analyses. Each analysis consisted of four chains, random starting trees, and uniform prior distribution of parameters. The chains were run for ten million generations, sampling trees every 250 generations. The asymptote was determined visually, 1,000,000 number of burn-in trees discarded, and the remaining trees were used to estimate Bayesian posterior probabilities. We considered clades strongly supported if they were present in ~95% of the sample trees (Huelsenbeck & Ronquist 2001, Wilcox *et al.* 2002).

RESULTS

Of the 1041 bp of the ND2 sequenced, 732 (70.3%) were invariant and 309 (29.7%) were variable; 183 (17.6%) variable sites were potentially parsimony informative. The nucleotide composition was: C = 10%, A = 24%, T = 31%, and G = 34%. The model of molecular evolution that best fitted our data was GTR+I+G.

The MP analysis with 1000 bootstrap replicates found 17 most parsimonious trees, from which we constructed a strict consensus tree (L = 1986, CI = 0.3666, HI = 0.6334). The 17 most parsimonious trees were variants of the same theme and exhibited the same major groupings. The consensus tree had the same topology as the one obtained in ML and BI analyses (Fig. 2). In this topology, *Eupherusa* is monophyletic (MP: 100; ML: 96; BI: 1.00 pp) and, in agreement with McGuire *et al.* (2007), its sister clade is the group comprising *Elvira* and *Microchera*.

Within *Eupherusa* we found four clades corresponding to the currently recognized species: 1) *E. poliocerca* (MP: 100; ML: 100; BI: 1.00 pp), 2) *E. cyanophrys* (MP: 100; ML: 100; BI: 0.99 pp), 3) *E. nigriventris* (MP: 100; ML: 98; BI: 1.00 pp), and 4) *E. eximia* (MP: 100; ML: 100; BI: 0.98 pp). In all three analyses (MP, ML and BI) *E. poliocerca* was the sister taxon of *E. cyanophrys* (MP: 100; ML: 92, BI: 1.00 pp), whereas *E. nigriventris* was the sister taxon of *E. eximia* (MP: 100; ML: 100; BI: 0.98 pp), with strong support for these clades.

DISCUSSION

Eupherusa traditionally has been considered monophyletic, mainly because the species share a cinnamon patch in the secondary wing feathers. Because of this trait, the genus has been relatively stable in terms of nomenclature for a long time, although the number of species increased (Gould 1857, Ridgway 1911, Peters 1945, Rowley & Orr 1964). Our ND2 data, with multiple individuals per species, supports the monophyly of the traditionally recognized *Eupherusa*.

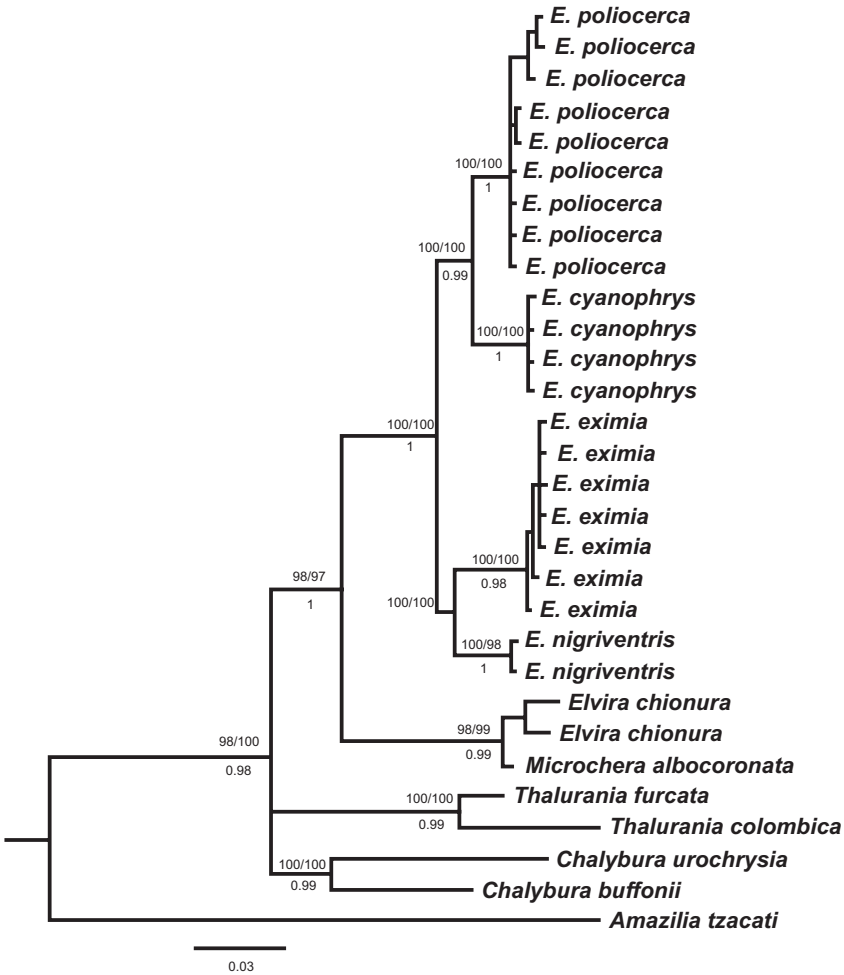

FIG. 2. Bayesian tree using full gene of mtDNA gene ND2, using the model described in the text. Branching patterns from Maximum Likelihood and Maximum Parsimony analyses agree with the tree depicted. Values above each node correspond to parsimony and maximum likelihood bootstraps, below the node correspond to posterior probabilities.

We discovered two sister clades, one including *E. poliocerca* and *E. cyanophrys* and the other including *E. eximia* and *E. nigriventris*. It is interesting to note that the two species endemic to western Mexico (*E. poliocerca* and *E. cyanophrys*) form a well supported clade. *E. poliocerca* has a disjunct distribution, with populations in the states of Guerrero and Oaxaca, but no genetic distinction could be detected among them, so we propose that they represent a single evolutionary lineage. *E. cyanophrys* has a very restricted distribution, being found only in the Sierra de Miahuatlán in the State of Oaxaca, in an area subject to habitat destruction. These factors led us to propose that *E. cyanophrys* is an endangered species.

E. eximia also displays a disjunct distribution and three subspecies have been proposed:

E. e. nelsoni, *E. e. eximia* and *E. e. egregia*. However, with our data we were not able to detect these three evolutionary clades, although they present clear morphological differences (the males of *E. e. eximia* in their ventral part are more green yellow and the black color at the end of the external rectrices is more diffuse compared with *E. e. egregia*, were the black tips are very clearly marked). If further studies support the existence of the three subspecies, it is possible that their genetic isolation is very recent.

Traditionally, the genera *Elvira* and *Chalybura* have been considered closely related to *Eupherusa* (Rowley & Orr 1964). However, our study supports the suggestion of McGuire *et al.* (2007) that the clade formed by *Elvira* and *Microchera* is the sister group of *Eupherusa*, whereas *Chalybura* is more distantly related (Fig. 1).

According to the topology of our phylogenetic tree (Fig. 2), the genus *Eupherusa* originated in Central America, and the invasions from Central America to Mexico, combined with changes in climate and ecological processes produced by large-scale tectonic event (Coates & Obando 1996), promoted further speciation (Bleiweiss 1998). Previous studies (e. g. *Lampornis*; García-Moreno *et al.* 2008) suggest that these factors were also relevant for the diversification of other hummingbird genera.

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