

12-1-2015

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### Recommended Citation

Eberhard, J., Iñigo-Elias, E., Enkerlin-Hoeflich, E., & Cun, E. (2015). Phylogeography of the military macaw (*Ara militaris*) and the Great Green Macaw (*A. Ambiguus*) based on MTDNA sequence data. *Wilson Journal of Ornithology*, 127 (4), 661-669. <https://doi.org/10.1676/14-185.1>

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## PHYLOGEOGRAPHY OF THE MILITARY MACAW (*ARA MILITARIS*) AND THE GREAT GREEN MACAW (*A. AMBIGUUS*) BASED ON MTDNA SEQUENCE DATA

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**ABSTRACT.**—The Military Macaw (*Ara militaris*) and the Great Green Macaw (*A. ambiguus*) are species whose close relationship is reflected in their morphological similarity as well as their geographic ranges. Military Macaws have a disjunct distribution, found in Mexico as well as several areas in South America, while Great Green Macaws have two or more disjunct populations from Honduras to eastern Ecuador. We used mitochondrial sequence data to examine the phylogenetic relationships between these two species, and also among representative samples across their ranges. Our data clearly support recognition of the two species as being distinct evolutionary lineages, and while we found significant phylogeographic structure within *A. militaris* (between samples collected in eastern and western Mexico), we did not find any evidence of lineage divergence between *A. ambiguus* from Costa Rica and Ecuador. Received 12 December 2014. Accepted 30 May 2015.

**Key words:** disjunct distribution, Great Green Macaw, Military Macaw, phylogeny, phylogeography.

The Military Macaw (*Ara militaris*) and the Great Green Macaw (*A. ambiguus*), sometimes named Buffon's Macaw, are both large macaws that are closely related and possibly conspecific (Fjeldså et al. 1987, Collar et al. 1994), but typically classified as separate species (Forshaw 1989; AOU 1998; Juniper and Parr 1998; Collar et al. 2014a, b). Both species are mostly green with bright red forehead patches. *A. militaris* is distinguished from *A. ambiguus* by its slightly smaller size, a smaller and completely black bill, overall darker plumage, dull red (rather than orange) at the base of the central rectrices, and differences in their vocalizations (Ridgway 1916, Forshaw 1989, Collar et al. 2014a). The Military Macaw has an extremely broad but fragmented distribution (Fig. 1), being found on the Pacific slope of Mexico from Sonora to Chiapas, with isolated populations scattered through the lowlands of the Balsas depression within the states of Mexico and Morelos, and on the Atlantic slope from Nuevo León to San Luís Potosí and Querétaro; it is absent from Central America, and patchily distributed in

South America, primarily east of the Andes from northwestern Colombia and northwestern Venezuela to north-western Argentina (Ridgway 1916; Chapman 1917; Alvarez del Toro 1980; Ridgely 1981; Hilty and Brown 1986; Forshaw 1989; Juniper and Parr 1998; Ñigo-Eliás 1999, 2000; CONANP 2005; Urbina-Torres et al. 2009, 2012; Marín-Togo et al. 2012; Rivera-Ortíz et al. 2013; BirdLife International 2014). The Great Green Macaw is found from eastern Honduras and Nicaragua through Costa Rica and Panama to northwestern Colombia (*A. a. ambiguus*), and also in western Ecuador (*A. a. guayaquilensis*; Forshaw 1989; Juniper and Parr 1998; Collar et al. 2014a, b). The two species are generally allopatric, but their ranges overlap slightly in northwestern Colombia, with sympatry documented in Orihueca, Cerro Quimarí, and Cerro Murrucucú (Ridgely 1981, Hilty and Brown 1986, Fjeldså et al. 1987, Rodríguez-Mahecha and Hernández-Camacho 2002).

The Great Green Macaw is primarily found in lowland humid forest, but sometimes in more deciduous forest, and often forages in relatively open, partially cleared areas (Fjeldså et al. 1987, Forshaw 1989, Juniper and Parr 1998). The Military Macaw tends to be found in tropical dry and semi-deciduous forests, often in foothill regions, but may range into the lowlands and pine forests (Ridgely 1981; Forshaw 1989; Juniper and Parr 1998; Ñigo-Eliás 1999, 2000; Marín-Togo et al. 2012; Rivera-Ortíz et al. 2013). Although the Great Green Macaw is often considered a humid forest species, and the Military Macaw a deciduous forest

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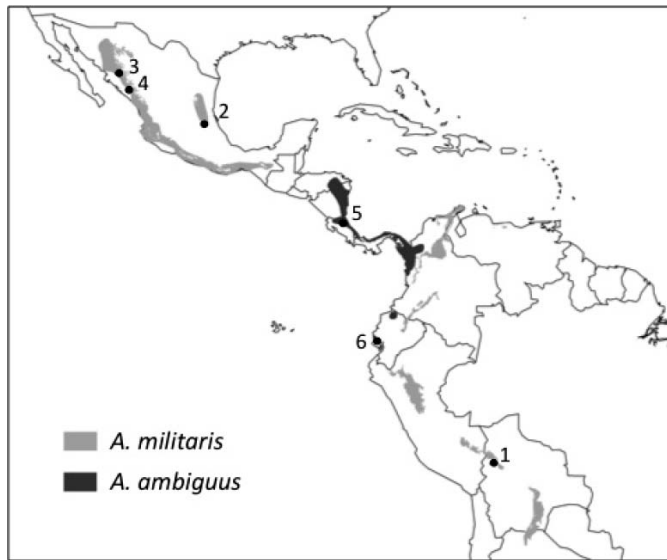


FIG. 1. Distribution of *Ara militaris* and *Ara ambiguus*, redrawn after Ridgely et al. 2011. Numbered points indicate the collection localities of samples included in this study, and correspond to those listed in Table 1.

species, there is substantial overlap in the types of habitat and altitudinal gradients that they prefer (Ridgway 1916, Chapman 1917, Ridgely 1981, Hilty and Brown 1986, Fjeldså et al. 1987, Forshaw 1989).

Both species have been subdivided into a number of subspecies based primarily on plumage characters and distribution. Three subspecies are generally recognized for *A. militaris* (*A. m. militaris*, *A. m. mexicanus*, and *A. m. bolivianus*; Forshaw 1989, Clements et al. 2014, Collar et al. 2014b), but the subspecies distinctions are not well-supported (Juniper and Parr 1998, Collar et al. 2014b). *Ara ambiguus* has been divided into two subspecies (*A. a. ambiguus* and *A. a. guayaquilensis*; Forshaw 1989, Juniper and Parr 1998, Collar et al. 2014a), but there is evidence that *guayaquilensis* may not be valid (Fjeldså et al. 1987). As described, *A. a. guayaquilensis* is confined to western Ecuador, but the large amount of morphological variation in specimens examined by Fjeldså et al. (1987) led to the suggestion that birds described as *guayaquilensis* might be intermediates between *A. ambiguus* and *A. militaris*. Fjeldså et al. (1987) suggested three possibilities regarding the relationship of *guayaquilensis* to the nominate *A. ambiguus* and *A. militaris*: *guayaquilensis* is derived from *ambiguus* and converged towards *militaris*; *guayaquilensis* is a relict of a cline that

formerly connected *A. ambiguus* and *A. militaris*; or *guayaquilensis* is a hybrid population.

Here we present molecular genetic data bearing on these taxonomic and biogeographic uncertainties, which have been difficult to resolve using morphological data. In addition to the goal of resolving these questions, this study was motivated by the need for information on evolutionary relationships that can be used to guide conservation efforts. Both the Military and Great Green macaws are threatened species, listed on CITES Appendix I (Snyder et al. 2000). The Great Green Macaw is classified as “endangered,” and the Military Macaw is listed as “vulnerable,” by the IUCN (International Union for the Conservation of Nature) according to criteria outlined by Collar et al. (1994). Both species’ populations are decreasing, primarily due to habitat loss and capture for the pet trade (BirdLife International 2014).

## METHODS

The samples used in this study include feathers collected from wild birds in the field as well as museum material (see Table 1). As noted in the table, source material included frozen tissue (muscle) and feathers (both emergent and full-grown). It is important to acknowledge that ideally, a study like this one would have museum skins as vouchers for all samples. Because of the limited representation

TABLE 1. List of samples sequenced in this study. Sample names correspond to those shown on the phylogenetic tree. Museum abbreviations are as follows: LSU (Louisiana State University Museum of Natural Sciences Collection of Genetic Resources) and STRI (Smithsonian Tropical Research Institute Molecular Labs). Tissue ID numbers listed are the accession numbers assigned to each sample or specimen by the corresponding museum or collection. Sample types are either tissue (frozen muscle or liver) or feather (both full-grown and pin-feathers were used). Locality numbers refer to sampling localities mapped in Figure 1.

Species	Museum/ Collection	Sample name	Tissue ID number	Collector	Locality map no.	Locality	Sample type
<i>Ara militaris</i>							
<i>A. m. militaris</i>	LSU	Ami22667	B-22667	M. R. Blair	1	Bolivia: La Paz dept.; Prov. B. Saavedra, 73 km by road E Charazani, near Rio Comata	tissue
<i>A. m. mexicanus</i>	STRI	Ami3-5	stri-x-144	E. Ñiño-Eliás	2	Mexico: Querétaro; Sierra Gorda	feather
<i>A. m. mexicanus</i>	STRI	Ami4-5	stri-x-221	E. Ñiño-Eliás	2	Mexico: Querétaro; Sierra Gorda	feather
<i>A. m. mexicanus</i>	STRI	Ami-11	stri-x-170	A.B. Mancera & A. Miller	3	Mexico: Chihuahua; Guerachi, R. Sinturosa, SW of Guachochi	feather
<i>A. m. mexicanus</i>	STRI	Ami-15	stri-x-169	E. Ñiño-Eliás	4	Mexico: Sinaloa; Mineral de Nuestra Señora de la Candelaria, Cosalá	feather
<i>Ara ambiguus</i>							
<i>A. a. ambiguus</i>	STRI	AamP99	stri-x-164	Proy. Lapa Verde	5	Costa Rica: 10° N, 84° W	feather
<i>A. a. ambiguus</i>	STRI	Aam2.8	stri-x-165	Proy. Lapa Verde	5	Costa Rica: 10° 42.33 N, 84° 6.579 W	feather
<i>A. a. guayaquilensis</i>	–	AamPB3	stri-x-172	B. López & P. Cun	6	Ecuador: Guayas; Bosque Protector Cerro Blanco (2° 07' 30" S, 80° 04' 40" W)	feather
<i>A. a. guayaquilensis</i>	–	AamPB4	stri-x-166	P. Cun	6	Ecuador: Guayas; exact locality unknown (Fund. ProBosque)	feather
<i>A. a. guayaquilensis</i>	–	AamPB8	stri-x-167	P. Cun	6	Ecuador: Guayas; exact locality unknown (Fund. ProBosque)	feather

of these macaws in museum tissue collections, and the difficulty of obtaining collecting and import/export permits for these taxa (even for feather samples), we had to be opportunistic in our sample collection, in many cases taking advantage of ongoing field work to secure samples for DNA extraction.

Total cellular DNA extractions for some of the samples were done by incubating the samples overnight in CTAB buffer (Murray and Thompson 1980) and Proteinase K, followed by a standard phenol-chloroform extraction and dialysis. For other samples, a commercial DNA extraction kit was used (DNEasy, QIAGEN Inc., Valencia, CA, USA). Three mitochondrial DNA (mtDNA)

fragments were amplified and sequenced for this study: a 550 bp portion of the COI gene and the complete ND2 gene (1,041 bp) were sequenced for all of the samples, for a total of 1,591 bp of mitochondrial sequence per sample. For the COI gene we used primers COIa and COIf (Palumbi 1996); for the ND2 gene we used primers L5216 and H6313 (Sorenson et al. 1999), and H5581 (JRE and T. F. Wright, unpubl. data). An additional 924 bp of sequence data from the complete ATPase6 and ATPase8 genes were obtained for a subset of the samples (see Table 2), to attempt to improve the resolution of relationships within the *militaris* and *ambiguus* clades. The ATPase6 and ATPase8 genes were amplified using primers

TABLE 2. Accession numbers of sequences generated during this study, as well as additional GenBank sequences included in the phylogenetic analyses.

Species	Sample name	Gene region	GenBank accession no.	Reference
<i>Ara militaris</i>				
<i>A. m. militaris</i>	Ami22667	ATP	KP411029	Present study
		COI	KP411038	Present study
		ND2	KP411048	Present study
<i>A. m. mexicanus</i>	Ami3-5	ATP	KP411027	Present study
		COI	KP411036	Present study
		ND2	KP411046	Present study
<i>A. m. mexicanus</i>	Ami4-5	ATP	KP411028	Present study
		COI	KP411037	Present study
		ND2	KP411047	Present study
<i>A. m. mexicanus</i>	Ami-11	ATP	KP411026	Present study
		COI	KP411035	Present study
		ND2	KP411044	Present study
<i>A. m. mexicanus</i>	Ami-15	ND2	KP411045	Present study
		COI	KR677388	Present study
<i>Ara ambiguus</i>				
<i>A. a. ambiguus</i>	AamP99	ATP	KP411022	Present study
		COI	KP411031	Present study
		ND2	KP411040	Present study
<i>A. a. ambiguus</i>	Aam2.8	ATP	KP411021	Present study
		COI	KP411030	Present study
		ND2	KP411039	Present study
<i>A. a. guayaquilensis</i>	AamPB3	ATP	KP411023	Present study
		CO	KP411032	Present study
		ND2	KP411041	Present study
<i>A. a. guayaquilensis</i>	AamPB4	ATP	KP411024	Present study
		COI	KP411033	Present study
		ND2	KP411042	Present study
<i>A. a. guayaquilensis</i>	AamPB8	ATP	KP411025	Present study
		COI	KP411034	Present study
		ND2	KP411042	Present study
<i>Ara ararauna</i>	Arara1	ND2	HQ629720	Schirtzinger et al. 2012
<i>Ara ararauna</i>	Arara2	ND2	KF017463	Urantowka et al 2014
<i>Ara glaucogularis</i>	Arglau1	ND2	HQ270481	Kirchman et al. 2012
<i>Ara glaucogularis</i>	Arglau2	ND2	KF017464	Urantowka et al. 2014
<i>Ara macao</i>	Armac	ND2	EU327601	Wright et al. 2008
<i>Cyanopsitta spixii</i>	Cyspix	ATP	DQ143259	Tavares et al. 2006
		COI	EU621610	Wright et al. 2008
		ND2	EU327614	Wright et al. 2008

CO2GQL, A8PWL and CO3HMH (Eberhard and Bermingham 2004). In all cases, the fragment was first amplified via the polymerase chain reaction (PCR) in 25 µl reactions using AmpliTaq or AmpliTaq Gold (Applied Biosystems, Grand Island, NY, USA). In most cases, the PCR was run for 30 cycles at an annealing temperature of 56 °C; some samples were amplified using five cycles at an annealing temperature of 50 °C followed by 30 cycles at 56 °C, and in a few cases, PCR products were re-amplified with 30 cycles at either 50 °C or 56 °C. Amplification products were cleaned and purified using either GELase

(Epicentre Technologies, Madison, WI, USA) or a polyethylene glycol and ethanol precipitation. PCR fragments were then sequenced using dRhodamine (Applied Biosystems/Perkin-Elmer, Grand Island, NY, and Waltham, MA, USA) cycle sequencing reactions and an ABI 377 automated sequencer, or BigDye chemistry (v3.1 Applied Biosystems) and an ABI 3130 automated sequencer. The amplification primers were used for sequencing both the heavy and light strands of the PCR fragments. Sequences generated by the automated sequencer were aligned and proofread using Sequencher (v.3.1.1, GeneCodes Corp.,

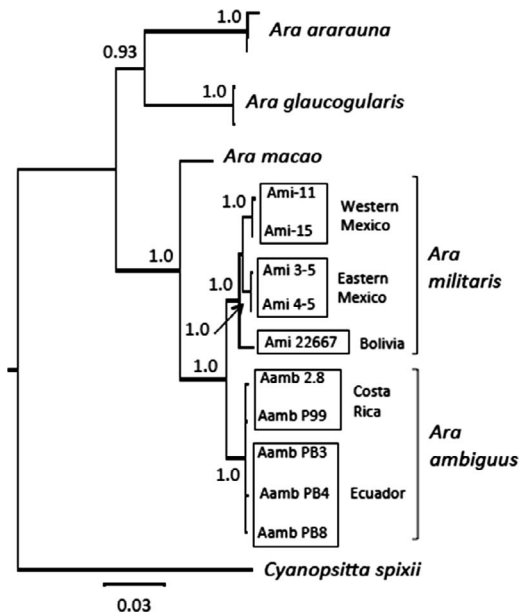


FIG. 2. Phylogenetic hypothesis obtained in a Bayesian analysis using 1,041 bp of sequence data from the mitochondrial ND2 gene. Clade support values  $>0.90$  are shown. Sample names for the *Ara militaris* and *A. ambiguus* samples correspond to those listed in Table 1.

Ann Arbor, MI, USA). Sequences for ATPase6 and ATPase8, COI and ND2 were available in GenBank for several additional members of the genus *Ara*, and were included in the phylogenetic analyses (see Table 2). Genbank sequences for *Cyanopsitta spixii* were used as an outgroup.

Phylogenies were reconstructed using a Bayesian maximum-likelihood (MB) approach using the Markov chain Monte Carlo method implemented in MrBayes (version 3.2.2; Ronquist and Huelsenbeck 2003, Ronquist et al. 2012), as well as parsimony (MP) and maximum likelihood (ML) as implemented in PAUP\* version 4.0b10 (Swofford 2003). Phylogenetic analyses of the ND2-only dataset included the largest number of taxa, and the COI+ND2 and the ATPase+COI+ND2 datasets included sequentially smaller sets of taxa. Analysis of the ND2-only dataset allowed us to include as many members of the genus *Ara* as possible, to provide some information regarding relative genetic distances among lineages of *Ara* macaws as a context for the genetic distances observed among members of the *Ara militaris/ambiguus* complex. On the other hand, the COI+ND2 and the ATPase+COI+ND2 datasets included more sequence data per taxon, and were analyzed in an effort to obtain better

resolution of relationships among closely related lineages.

An appropriate evolutionary model for the MB and ML analyses was selected for each dataset using jModelTest (version 2.1.6; Guindon and Gascuel 2003, Durriba et al. 2012) under the Akaike Information Criterion. For the COI+ND2 dataset, the HKY+I model was selected, while a GTR+I model was selected for the ND2 and ATPase+COI+ND2 datasets. In order to explore whether data partitioning might improve the phylogenetic analysis, PartitionFinder v1.1.1 (Lanfear et al. 2012) was used to choose a partitioning scheme (partitioned by gene and codon position) and model of sequence evolution for each of the datasets, and the suggested partitioning scheme was implemented in additional tree searches using MrBayes. Each of the unpartitioned MB analyses consisted of two parallel runs, each with one cold chain and three heated chains, 100,000,000 generations sampled every 1,000 generations, and a burn-in of 0.25; the partitioned tree searches were run for 10,000,000 generations. For each MB tree search, we verified that the standard deviation of split frequencies was  $<0.01$ , and we used Tracer v1.6 (Rambaut and Drummond 2013) to generate trace plots in order to verify that the run

had reached stationarity and that ESS (effective sample size) estimates were  $>200$  for all of the parameters. A majority-rule consensus of the post-stationarity trees was generated to provide a phylogenetic hypothesis, with associated posterior probability values indicating the support for internal branches. Bootstrap analysis for ML (100 replicates) and MP (1,000 replicates) trees were performed to assess nodal support. PAUP\* was also used to compute pairwise distances (uncorrected  $p$ -distances) among taxa. *Cyanopsitta spixii*, which is closely related to the genus *Ara* (Wright et al. 2008), was used as the outgroup for all analyses.

## RESULTS

The phylogeny obtained in the Bayesian analysis of the unpartitioned ND2 dataset, which included the largest number of terminal taxa, is shown in Figure 2. Topology of the cladograms obtained in MB analyses of the COI+ND2 and the ATPase+COI+ND2 datasets were consistent with the ND2 gene tree (see Fig. S1 and Fig. S2 in Supplemental Materials), and the resolution of relationships within the *militaris/ambiguus* clade was not improved by inclusion of the additional gene sequences. The implementation of data partitioning schemes did not result in trees with topologies that differed from those of trees obtained without data partitioning, and levels of branch support were nearly identical to those found using the unpartitioned datasets. For each dataset, identical cladograms were also obtained in the ML and MP analyses, and bootstrap values were comparable to the Bayesian support values at most nodes. In all cases, support was strong (1.0 or 100%) for the monophyly of the *Ara militaris/ambiguus* complex.

In all phylogenetic analyses, the *Ara militaris* samples (one *A. m. militaris* from Bolivia and, depending on the analysis, either three or four *A. m. mexicanus* from Mexico) form a well-supported clade (MB support values of 1.0, 0.99, and 0.97 for the ND2, COI+ND2 and ATPase+COI+ND2 datasets respectively). Within-clade structure reflects differentiation of the South American *militaris* from those of Mexico, as well as differentiation between Mexican samples representing western Mexico (Ami-11 from Chihuahua and Ami-15 from Sinaloa) and eastern Mexico (Ami3-5 and Ami4-5, both from Querétaro). The *Ara ambiguus* samples (two from Costa Rica and three from

Ecuador) also form a well-supported clade (MB support values of 1.0, 1.0, and 0.93 for the ND2, COI+ND2 and ATPase+COI+ND2 datasets respectively), but within this clade, the Costa Rican samples (*A. a. ambiguus*) do not cluster apart from the Ecuadorean ones (*A. a. guayaquilensis*), and the genetic distances between them are tiny.

A close relationship between the *militaris* and *ambiguus* clades is reflected by the relatively short genetic distance that separates them as well as a strong support value at the node that connects them. The sequence divergence (ND2  $p$ -distances) among *militaris* samples ranged from 0.0% to 1.4% and among *ambiguus* samples the range was much smaller (0–0.2%). The distance between the Bolivian *militaris* sample and the Mexican ones (mean: 1.3%) was nearly twice as great as the mean divergence between eastern and western Mexican *militaris* (mean: 0.07%). The range in genetic distances between *militaris* and *ambiguus* samples was 1.6–2.2%. These distances are much smaller than the divergences (range: 4.6–4.9%) between *militaris/ambiguus* samples and *Ara macao*, their nearest relative among the taxa included in the analyses.

## DISCUSSION

Our phylogenetic analysis of mitochondrial sequence data shows varying amounts of differentiation among the previously described taxa within the *Ara militaris/ambiguus* complex. Analyses using all three of our datasets show that the *militaris* and *ambiguus* clades are reciprocally monophyletic, indicating that they are evolutionarily independent lineages. Taken together with the clear plumage differences between macaws belonging to these clades, as well as their allopatry, it is reasonable to consider the two as separate species according to a phylogenetic species concept (Cracraft 1983, McKittrick and Zink 1988). Low nodal support at the base of the *militaris* clade supports the consideration of Mexican and South American *militaris* as conspecific, even though the mean genetic (ND2) distance between the Mexican and Bolivian samples (1.3%) was nearly as large as the some of the distances between *ambiguus* and *militaris* (range: 1.6–2.2%).

Our data do not indicate any significant phylogenetic structure within the *ambiguus* clade, with no differentiation between the samples representing populations in Costa Rica and Ecuador, which

have been described as different subspecies. On the other hand, differentiation within the *militaris* clade is evident, with modest genetic distance separating the Bolivian sample from the Mexican ones. Macaws from the two sampled regions in Mexico, west of the Sierras Madres (Sinaloa and Chihuahua) and east of the Sierras Madres (Querétaro) are clearly differentiated, being reciprocally monophyletic and separated by genetic distances that are approximately half as large as the distances between Mexican and Bolivian samples. Our data support the recognition of two Mexican clades as evolutionarily significant units (ESUs; Moritz 1994), but given the limited morphological variation within *militaris* and our limited sampling, we refrain from making suggestions regarding subspecies nomenclature.

Due to the lack of voucher specimens for our *ambiguus* samples, we are unable to evaluate the status of *A. a. guayaquilensis*, however the overall lack of phylogenetic structure and negligible genetic differentiation within the *ambiguus* clade—even between the geographically distant populations from Costa Rica and Ecuador—suggest that *guayaquilensis* is unlikely to be phylogenetically distinct. Our data indicate that of the three hypotheses proposed by Fjeldså et al. (1987) regarding the relationship of *guayaquilensis* to the nominate *A. ambiguus* and *A. militaris*, it is most likely that *guayaquilensis* is derived from *ambiguus* and converged towards *militaris*. The hypothesis that *guayaquilensis* is a relict of a cline that formerly connected *A. ambiguus* and *A. militaris* is not supported by our analyses, and our mitochondrial data do not allow us to evaluate the third hypothesis, that *guayaquilensis* is a hybrid population.

On the other hand, the phylogenetic structure found within the *militaris* clade indicates that more complete geographic sampling, in order to include representatives from across that species' broad and fragmented range, would likely reveal additional phylogenetic structure. This is important from a conservation standpoint, since evolutionarily independent lineages should be managed as evolutionarily significant units (Ryder 1986, Moritz 1994). Given the differentiation that we observed within Mexico, when wild-caught *Ara militaris* are confiscated by government authorities, their geographic provenance should be ascertained before the animals are bred in captivity or reintroduced to the wild. Further phylogeographic studies that expand the limited geographic sampling in our study, as well as genetic screening of

confiscated individuals, should be conducted in order to facilitate the preservation of evolutionarily distinct lineages within *Ara militaris*.

The contrast between the within-Mexico divergences in *militaris* and the lack of structure within *ambiguus* is interesting, and suggests that mountain barriers have been important causes of lineage divergence (e.g., between *militaris* from eastern and western Mexico that are separated by the Sierras Madres, and between the cis- and trans-Andean lineages), and that there has been fairly recent gene flow across the range of *ambiguus*. The highly fragmented ranges of these species may be a relatively recent phenomenon, such that genetic divergence is primarily observed between populations separated by major mountain ranges.

#### ACKNOWLEDGMENTS

We thank the following persons and institutions for contributing samples that were included in this analysis: A. Miller and A. B. Mancera; P. Wright and G. Powell (Proyecto Lapa Verde, Costa Rica); K. Berg, E. Horstman and B. López (Fundación Pro-Bosque, Ecuador); and R. Brumfield and D. Dittmann (Louisiana State Museum of Natural History Collection of Genetic Resources). M. González assisted with sequencing of samples. JRE thanks F. Sheldon and R. Brumfield for access to laboratory facilities, and Andrés Cuervo for assistance with the distribution map. The manuscript benefitted from the comments and suggestions of two anonymous reviewers. Feather samples of *Ara militaris* from Mexico where obtained in the 1990s with permits from the Dirección General de Vida Silvestre (SEMARNAP) to EEIE and EEH through research projects with graduate students. Support for the *Ara militaris* work in Mexico was provided by the Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO), the Fondo Mexicano para la Conservación de la Naturaleza (FMCN) and Fundación Ara. JRE received support from the LSU Department of Biological Sciences.

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