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"REEVALUACION DEL ESTATUS TAXONÓMICO DEL COMPLEJO *Amazilia viridifrons* (AVES: TROCHILIDAE) UTILIZANDO HERRAMIENTAS MOLECULARES"

T E S I S

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RESUMEN

La taxonomía del colibrí frente-verde *Amazilia viridifrons* ha sido ambigua e inestable. Algunas veces se le ha considerado conespecífica con *Amazilia violiceps*, algunas veces como especies separadas y más recientemente se ha sugerido que *Amazilia viridifrons sensu* AOU 1998 está conformada por varios taxones independientes. Proponemos una hipótesis sobre los límites de la especie del colibrí frente-verde *Amazilia viridifrons* utilizando secuencias de los genes mitocondriales ND2 (primer fragmento, 571 pb) y ND3 (352 pb). Nuestros resultados sugieren que *Amazilia viridifrons* y *A. violiceps* son especies separadas pero hermanas. Análisis previos morfológicos y nuestros resultados con caracteres moleculares sugieren que *A. wagneri* puede constituir una especie separada. La propuesta de que *A. v. villadai* (Oaxaca) es una unidad diagnosticable independiente no se apoyó, incluso comparte haplotipos con *A. viridifrons* (Guerrero) lo que sugiere que ambas poblaciones pertenecen a la misma especie. Las relaciones de este complejo con otros miembros del género *Amazilia* sugieren también que se necesita de un análisis más completo de la filogenia del género.

Abstract

The taxonomy of the Green-fronted hummingbird *Amazilia viridifrons* has been ambiguous and changing. Sometimes it has been considered conespecific with the Violet-crowned hummingbird (*A. violiceps*), sometimes as a separate species, and more recently it has been suggested that *A. viridifrons sensu* the American Ornithologists' Union (AOU 1998) is composed of several independent taxa. Here, we provide a hypothesis regarding the species limits and phylogeny of the Green-fronted hummingbird complex using mtDNA genes [partial ND2 (571 bp) and ND3 (352 bp)] sequences. Our results suggest that *A. viridifrons* and *A. violiceps* represent separate, sister species. Previous morphological analyses and our molecular data also show that the Cinnamon-sided hummingbird *A. wagneri* may constitute a separate species, though further analyses are needed to confirm this. *A. v. villadai* (Oaxaca) was not supported as a different taxon; instead, it shared haplotypes with the samples of *A. viridifrons* (Guerrero) suggesting that they belong to the same species. In addition, unsuspected relationships of this complex with other *Amazilia* species suggest that a thorough analysis of the phylogeny of the species assigned to this genus is still needed.

INTRODUCCIÓN GENERAL

Los colibríes pertenecen a la familia *Trochilidae* y junto con los vencejos (subfamilias *Apodidae* y *Hemiprocnidae*) conforman el orden Apodiformes (Sibley y Monroe 1990; Howell y Webb 1995; Johnsgard 1997; AOU 1998; Schuchmann 1999; Johansson et al. 2001; Dickinson 2003). Su distribución es muy amplia, ya que se les localiza desde el nivel del mar hasta los 4000 m.s.n.m. pero se restringen al Continente Americano (Johnsgard 1997; Bleiweiss et al. 1997; Schuchmann 1999). Los colibríes son uno de los grupos de aves más complejos e interesantes, ya que presentan una gran adaptabilidad al medio que los rodea, así como convergencia y una marcada selección sexual en algunos caracteres que ha obstaculizado su adecuada identificación y clasificación (Ornelas 1996; Gerwin y Zink 1998; Schuchmann 1999). Por esta razón, los trabajos con caracteres morfológicos (e.g. Zusi y Bentz 1982) no han podido resolver muchas dudas y se ha recurrido a herramientas moleculares que poco a poco han ido resolviendo diversas preguntas (e.g. Gerwin y Zink 1989; Gill y Gerwin 1989; Sibley y Monroe 1990; Bleiweiss et al. 1994a y b, 1997, 1998a,b, c, y d; Gerwin y Zink 1998; Hernández-Baños 1998; García-Moreno 1999; Wiens y Penkrot 2002; Cortés-Rodríguez 2003, García-Deras 2003; Altshuler et al. 2004, García-Moreno et al. 2004; García-Moreno et al. 2006).

La familia *Trochilidae* está dividida en dos subfamilias: Phaetornithinae y Trochilinae (Bleiweiss 1994b, 1998d; Schuchmann 1999). La primera está conformada por 34 especies pertenecientes a seis géneros mientras que la segunda lo está por 294 especies y 102 géneros. Los miembros de la subfamilia Trochilinae difieren de los Phaethornithinae en varias características morfológicas como en su típico tendón humeral, en que muestran una pigmentación predominantemente café, grisácea y rojiza, la variedad en la forma de sus picos va desde los rectos hasta los ligeramente curvos (los Phaethornithinae los presentan marcadamente curvos) y la mayoría de las especies tienen un dimorfismo sexual marcado siendo las hembras las de apariencia opaca y grisácea y a menudo carecen de colores iridiscentes (Schuchmann 1999).

En los machos, los colores iridiscentes están limitados básicamente a la parte superior de su cuerpo como la garganta y crestas y en ocasiones pueden presentar plumas largas modificadas de la cola de colores vistosos e iridiscentes. Son típicamente territorialistas y desafortunadamente, aún se conoce muy poco acerca de su comportamiento reproductivo. Los límites a nivel de género así como las relaciones entre las especies de esta subfamilia aún están poco estudiadas y entendidas (Schuchmann 1999).

El género *Amazilia,* perteneciente a esta subfamilia, es uno de los grupos que presenta mayor número de especies (32 especies y 81 subespecies, según Dickinson 2003). A través del tiempo, su taxonomía ha sido ambigua y cambiante (Ridgway 1911; Peters 1945; AOU 1998) y la monofilia entre los grupos no es clara, por lo que se ha propuesto que varias especies de este género pueden pertenecer a otros géneros (e. g. Schuchmann 1999). Ornelas et al. (inédito) propusieron una filogenia del género *Amazilia* utilizando caracteres moleculares, como los genes mitocondriales 12s, ND5 y ND2, además de usar nuevos caracteres como las vocalizaciones y la iridiscencia, sin embargo, sus resultados aún no han sido publicados.

En el caso del colibrí frente verde, *Amazilia viridifrons* (Elliot 1871), la taxonomía ha sido inestable y sus relaciones filogenéticas son aún inciertas (e.g. Phillips 1964; Howell 1993; AOU 1998; Peterson y Navarro 2000, Dickinson 2003). Se ha sugerido que *Amazilia viridifrons wagneri* puede ser reconocida como una especie separada (Phillips 1964, Howell 1993, Peterson y Navarro 2000, Dickinson 2003), debido a que presenta diferencias morfológicas y se ha propuesto una nueva subespecie *A. v. villadai* (Peterson y Navarro 2000) que también pudiera ser una unidad diagnostica independiente.

El objetivo de este trabajo es reevaluar el estatus taxonómico del complejo *Amazilia viridifrons* y proponer una hipótesis sobre su filogenia utilizando secuencias de los genes mitocondriales ND2 (primera mitad, 571 p.b.) y ND3 (352 p.b.). Los resultados de este trabajo se presentan a continuación en la forma del artículo de publicación.

A review of the Green-fronted hummingbird *Amazilia viridifrons* (Aves: Trochilidae) using mitochondrial genes.

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The Green-fronted Hummingbird (*Amazilia viridifrons sensu* AOU 1998) is a Mexican endemic species that inhabits southern Mexico in arid to semiarid scrub, thorn forest and semiopen areas with scattered trees in Guerrero, Oaxaca and Chiapas (Fig. 1). It shows a disjunct distribution (900-1400m) from central Guerrero to central Oaxaca and Chiapas (60-1300m). This species does not have long migrations but may present altitudinal movements (Howell and Webb 1995; Schuchmann 1999, Dickinson 2003).

The taxonomic history of A. *viridifrons* has been complex and changing. It has been linked to the taxonomic history of the Violet-crowned hummingbird *A. violiceps* as some authors placed them together as the same species (e.g. Peters 1945). After the original description of *A. viridifrons* by Elliot in 1871, several subspecies have been described, as well as some synonyms have been proposed (Phillips 1964; Peterson and Navarro 2000). Authors as Ridgway (1911) and Cory (1918) recognized *A. violiceps* and *A. viridifrons* as separate species. Later, Griscom (1934) synonimized five forms to *A. violiceps*: *A. viridifrons, A. guerrerensis, A. verticalis* and *A. violiceps,* arguing that they only differ due to the sex and age. Friedmann et al. (1950) again recognized *A. violiceps* and *A. viridifrons* as different species, being the latter a monotypic species. Phillips (1964) considered *A. viridifrons* as a subspecies of *A. violiceps* and described a new subspecies: *A. violiceps wagneri*. Howell (1993) suggested to elevate to species Phillips' subspecies *A. wagneri* and described a new subspecies of *A. viridifrons*: *rowleyi* considering coloration characters, but ignoring size and shape. This made him no to notice the strong differentiation of the Chiapas population (Fig. 1) (Peterson and Navarro 2000). These previous authors created artificial taxa of *A. viridifrons* and *A. violiceps* based on small and incomplete series of specimens and in a very inclusive version of the biological species concept (e.g. Mayr 1942, 1969).

The AOU (1998) recognizes *A. violiceps* and *A. viridifrons* as species. For the latter, AOU (1998) recognizes two groups: *viridifrons* and *wagneri* and comments that *A. wagneri* may constitute another separate species. Schuchmann (1999) based on morphological characters but not on a cladistic analysis, divided the genus *Amazilia* into four genuses: *Amazilia, Agyrtia, Polyerata* and *Saucerottia*. In this division, Schuchmann transferred *A. viridifrons* and *A. violiceps* along with *A. cyanocephala* and *A. candida* to the genus *Agyrtia*. Recently, Peterson and Navarro (2000) developed a phylogenetic analysis of the *A. viridifrons* complex using standard phenotypic characters such as bill length, wing chord and tail length as well as plumage coloration and found that *A. violiceps* (Guerrero and Oaxaca) and *A. viridifrons* (Guerrero, Oaxaca and Chiapas) are different species. These authors did not recognize Howell's subspecies *rowleyi* because the plumages of the specimens reviewed were worn and the colors faded so they synonimized *rowleyi* with *wagneri*. Also, Peterson and Navarro (2000) recognized *A. wagneri* (south of Oaxaca) as a valid species and described a new subspecies: *A. v. villadai* (eastern Oaxaca to western Chiapas, possibly into the west of Guatemala) which can be a clearly diagnosable, monophyletic unit, probably a phylogenetic species.

The goals of this project are to review the taxonomic status of this complex, test if *A. wagneri* and *A. v. villadai* could be consider as a valid species and to test the monophyly of the complex using mitochondrial DNA (mtDNA) sequences.

MATERIALS AND METHODS

*Sampling and outgroups. - S*amples of *A. viridifrons*, *A.wagneri* and *A. v.villadai* were collected in specific localities (Fig. 1). The rest of samples were collected along their distribution range or were taken from the tissue collection of the Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autónoma de México (MZFC). All collected samples and voucher specimens were deposited in the MZFC. Monophyly of *A. viridifrons* was tested including samples of several other species of *Amazilia (*Peters 1945 and AOU 1998). *Amazilia cyanocephala* was used as the outgroup (following AOU 1998) to root all the trees obtained. A list of the specimens sampled, their localities, voucher numbers, and GenBank accession numbers is given in Table 1.

PCR and sequencing.- DNA extractions were made using the kit DNeasy (Qiagen) following the manufacturer's protocols. We amplified and sequenced the first fragment of the NADH dehydrogenase subunit 2 (ND2, 571 bp) and the complete NADH dehydrogenase subunit 3 (ND3, 352 bp) for a total of 923 bp. Primers L5215 (TATCGGGCCCATACCCCGAAAAT) (Hackett, 1996) and H5788 (ATT CANCCYAGGTGGGAGATGG) (García-Moreno, pers. comm.) were used to amplify ND2. Primers L10647 (TTYGAAGCMGCMTGATACTG) (Mindell et al. 1998) and H11151 (Chesser 1999) were used to amplify ND3 (position numbers are given in relation to the chicken mitochondrial genome; Desjardin and Morais 1990). PCR reaction volume of 50 μl were used, including 0.5 ml of taq polymerase, 5 μl of 10mM solution of each primer, 4 μl of 25 mM $MgCl_2$ solution, 5 μl of 20x reaction buffer, 5 μl of mM dNTP mixture and 3 μl DNA template. The PCR protocol for ND2 was as follows: 2 min at 94°C, followed by 10 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and 72°C for 30 s, this was followed by another 35 cycles of 94°C for 15 s, 50°C for 15 s and 72°C for 30 s, and a final extension step of 3 min at 72°C and 4°C ∞. The PCR protocol for ND3 was as follows: 35 cycles of denaturation at 92°C for 1 min, 50°C for 1 min and 72°C for 1 min. This was followed by a 7 min extension at 72°C and 4°C-∞. Products were purified using a Millipore PCR purification kit following the manufacturer's protocols. Standard, 20μl sequencing reactions were performed using

2μl of BigDye (ABI) and 20–40 ng of purified and concentrated PCR product. Products of these reactions were purified using Sephadex G50 columns (Sigma) and resolved on a Perkin-Elmer ABI 373 automated sequencher at the Instituto de Biología, UNAM. The sequences were visually aligned using Chromas 1.45 (32-bit) (http://brcweb.bio.cornell.edu) and they were corrected with Clustal X 1.81 (Thompson et al. 1999).

Phylogenetic analyses.- We performed combined phylogenetic analyses of mtDNA sequences (both ND2 and ND3) using the maximum parsimony (MP), maximum likelihood (ML), and Bayesian MCMC (BI) methods. The MP analyses were conducted using PAUP 4.0b10 (Swofford 2003) with a heuristic search using TBR branch-swapping option, all positions equally weighted and unordered. Support for each node was obtained by 100 bootstrap replicates (Felsenstein 1985). For ML and BI analyses we used Modeltest v. 3.06 (Posada and Crandall 1998) to determine the model of evolution that best explained our data. ML was also conducted in PAUP 4.0b (Swofford 2003) using a heuristic search and nodal support was estimated with 100 bootstrap replicates.

Bayesian analyses (BI) were conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2002). Two character partitions were defined, corresponding to ND2 and ND3, allowing for different evolutionary rates in each partition. We made two independent searches, running four Markov chains for 5 $x10^6$ generations and sampling every 250 generations; the chains reached the stationary phase quickly, and we discarded conservatively the first 400 trees as burn-in and computed a consensus tree and posterior probabilities for each node based on the remaining 20000 trees (Huelsenbeck et al. 2002). We considered that clades were strongly supported if they were present in ≥95% of the sampled trees (Huelsenbeck and Ronquist 2001; Wilcox et al. 2002).

Because the Bayesian method infer sets of trees proportional to their posterior probability rather than a single estimate of phylogeny, statistical methods for testing phylogenetic hypotheses are not practical (Brandley et al. 2005). We therefore employed a Bayesian approach to hypothesis testing and built 95% credible sets of unique trees. We did this to determine whether alternative phylogenetic hypotheses not supported with high posterior probabilities (Pp<0.95) could be rejected by the data. If a phylogenetic hypothesis of interest was absent in all of the trees of the 95% credible set, it could be rejected statistically (see Buckley et al. 2002; Reeder 2003; Brandley et al. 2005).

We analyzed general genetic structure of populations via analysis of genetic variance (AMOVA). In particular, we calculated Φ_{st} values to detect possible geographic genetic structure. Populations were not characterized as subgroups a priori (Sgariglia and Burns, 2003); rather, this step was achieved using ARLEQUIN ver. 2.000 (Schneider et al., 2000). We constructed an unrooted minimum-spanning haplotype network using Network 4.1.1.2 (Bandelt et al. 1999; www.fluxus-engineering.com, MJ algorithm).

RESULTS

Ingroup sequence variation.- We obtained 20 sequences that represent specimens assigned to *A. viridifrons, A. violiceps* and outgroups, with length of 923 base pairs. Of these bases, 718 were invariant, 205 were variable at some point in the data set, and 135 were phylogenetically informative. The mean nucleotide composition was T= 24.8%, C= 33.8%, A= 29.3%, and G= 12%. Overall, we saw only 2 pairs of identical haplotypes; as such, eight haplotypes were observed among *A. viridifrons* populations, four of *A. violiceps* and six more representing outgroup taxa. Transitions dominated the observed changes (37), as opposed to transversions (11) and nucleotide diversity was 0.07990.

Significant geographic structure was indicated by the AMOVA (Table 3), suggesting that geographic substructuring among *viridifrons-violiceps* populations is statistically

significant and greater than that expected at random. Indeed, >90% of the total variation in the overall ingroup dataset, and a significant Φ_{st} = 0.90150, is assorted among, rather than within, populations, indicating substantial geographic genetic subdivision of populations.

We estimated model parameters for likelihood searches using Modeltest (Posada and Crandall 1999), which compares goodness of fit of models using the likelihood ratio test statistic (Huelsenbeck and Rannala 1997). The best-fitting model for our dataset using AIC was HKY+I+G, with a ts/tv ratio = 4.2814, with invariants = 0.4447 and a gamma distribution α shape parameter =0.5930. For our analyses we used the base frequency values estimated by the model (pA = 0.3005, pC = 0.3345, pG = 0.1175, pT = 0.2474).

Haplotype diversity.— The haplotype network structure (Figure 5) coincided closely with all trees (MP, BI and ML) showing *A. viridifrons* well differentiated from *A. violiceps*. *Amazilia wagneri* is located closer to the latter. *A. rutila* and *A. yucatenensis* are closely related to *A. viridifrons* as *A. cyanocephala* and *A. beryllina* are to *A. violiceps*. Dots represent missing or extant haplotypes between the groups (Bandelt et al. 1999).

The distance matrix (Table 2) show little variation due to the fact that they are sister taxa.

Phylogenetic analyses.— ML, BI, and MP analyses, including all individuals and characters, converged on the same basic topology (Figures 2-4), differing only in small rearrangements of individuals within major clades. The MP tree (Figure 2) showed *A. beryllina* as basal to all *Amazilia* group. Two major clades are evident: 1) a group with *A. candida, A. rutila*, *A. tzacatl* and *A. yucatanensis* (bootstrap = 74) and 2) a monophyletic clade with *A. viridifrons* and *A*. *violiceps* (bootstrap= 100). This second clade has two subclades; in one we can see *A. viridifrons* samples mixed with *A. v. villadai* with no geographic structrure (from Guerrero and Oaxaca near the Isthmus, bootstrap = 59). *A. wagneri* (central Oaxaca) appears as its sister species (bootstrap= 57). The other subclade grouped all the samples of *A. violiceps* (bootstrap = 96).

The ML tree (Figure 3) has a very similar topology and bootstrap values to the MP tree, the same two major clades are consistent in both trees. The subclade of *A. viridifrons* shows geographic structure as the samples from Oaxaca (*A. v. villadai*) are grouped together as well as the samples from Guerrero (*A. viridifrons*). Again, *A. wagneri* appears as its sister species (bootstrap= 73) and *A. violiceps* is located in the other subclade (bootstrap= 71) where the samples of Zacatecas are grouped showing also a geographic structure.

The Bayesian tree (Figure 4) is also very similar in topology to the MP/ML trees. We can see the same two major clades and almost the same arrangement in the *viridifrons* clade: the samples from Oaxaca (*A. v. villadai*) are grouped together while the samples from Guerrero are grouped together with the exception of one sample, CON0404 that appears as sister to this subclade and then *A. wagneri* as the sister of this group (Pp = 0.95). *A. violiceps* is again grouped in a subclade (Pp = 1.00) where a geographic structure is showed.

These results together corroborate the idea that *A. viridifrons* and *A. violiceps* are separate but sister species. *A. viridifrons, A. violiceps* and *A. wagneri* indeed form a monophyletic group (bootstrap = 100/91, Pp=1.00). The majority of the nodes received high posterior probabilities (≥ 95%), and bootstrap values >70%. The geographic structure recovered in the ML and BI trees coincides with the haplotype network (Figure 3) and suggest that there exists geographic differentiation between *A. viridifrons* (including *villadai*), *A. violiceps* and *A. wagneri*.

DISCUSSION

Two major goals of systematics are delimiting species and reconstructing their phylogenetic relationships. Traditionally, morphological data have been used to delimit species but many recent studies have used DNA sequences, particularly, mtDNA to test

traditional morphology-based taxonomies (Wiens and Serviedo 2000; Wiens and Penkrot 2002; Edwards et al. 2005). Species are fundamental units in studies of systematics, biodiversity and ecology but their delimitation has been controversial (Wiens and Servedio 2000; Zink 2006), different methods have been applied and different characters have been studied (Wiens and Servedio 2000; Wiens and Penkrot 2002)

In avian systematics, species-level taxonomy was largely formed at the beginning of the 20th century based on incomplete series of specimens and prequantitative methods (Peterson and Navarro 2006); more recently, there are studies that use mtDNA sequence data in species delimitation (e.g. Fry and Zink 1998; Zink and Blackwell-Rago 2000; Zink et al. 2001; Liebers and Helbig 2002; García-Deras 2003; Sgariglia and Burns 2003; Zink et al. 2005; Navarro et al. in prep.) where more specimens and taxa are included as well as further analyses of population genetics (e.g. Fst, mismatch distribution, Nm) and nested clade analyses (see Templeton 1998).

Numerous hummingbird genera are based in bill and plumage characters that are also used to distinguish one hummingbird species from other; this may not reflect levels of genetic distances or phylogenetic relationships (Bleiweiss et al. 1997) giving as a result an artificial classification as is the case of genus *Amazilia*.

The genus *Amazilia* belongs to a clade named Emeralds along with tiny bee-like forms from genera *Chlorostilbon, Thalurania* and *Orthorhynchus*. The Emeralds are widely distributed across high and low elevations in North and South America and the Caribbean and they would qualify as a major radiation within hummingbirds simply by inclusion of the genus *Amazilia* which present an extraordinary variety of forms (Bleiweiss et al. 1997). According to the results of Bleiweiss et al. (1997) the clade where are placed the Emeralds (*Chlorostilbon* and *Amazilia*) are not monophyletic but this question will be answer when every species of both and more genera are included.

The monophyly of the clade *A. viridifrons* (including *villadai*) + *A. wagneri* + *A. violiceps* was clearly supported by our results (MP bootstrap = 100, ML bootstrap = 91, Pp=1.00) and the AMOVA also supported the distinctiveness of *A. viridifrons* from *A. violiceps* as Ridgway (1911) and Peterson and Navarro (2000) had proposed. Schuchmann's proposal (1999) about changing *A. viridifrons (sensu lato*) and *A. violiceps* to genus *Agyrtia* was not supported by our results as it was not our goal, but we agree with the proposal of the AOU South American Classification Committee of not moving them until there is enough evidence to change some species of this genus (Remsen et al. in prep).

For *A. viridifrons*, little gene variation is seen in the genetic distances matrix (Table 2) and despite the fact of the low number of samples, the haplotype diversity was high, which might suggests that every population is different from the other and that they do not share haplotypes. There were only two haplotypes shared between *A. viridifrons* and *villadai* (CONACYT1289 and CONACYT986) because they belong to the same taxon, the rest are unique haplotypes. Our results show that samples from Guerrero (*A. v. viridifrons*) and Oaxaca (*A. v. villadai*, close to the Tehuantepec Isthmus) belong to the same taxon, *A. viridifrons*. This suggests that *A. viridifrons* has a disjunct distribution in Guerrero and Oaxaca and that it is distinguishable from *A. wagneri* from the Sierra de Miahuatlán in Oaxaca.

Taxonomic consistency and accurate delimitation of species affect conservation priorities being critical to bird conservation (Peterson and Navarro 2006). The debate about species concept turns important because in some cases it depends on whether concept is used to recognize species that conservation priorities will focused in (Peterson and Navarro 1999; Peterson and Navarro 2006; Zink 2006). Traditionally, the biological species concept (BSC) has been applied and bird conservancy adopted the taxonomy proposed by followers of this concept and led them to focus attention on species' distributions without concern for geographic variation or species limits (Peterson and Navarro 1999); when thorough studies are made applying alternative species concept (e.g. phylogenetic or evolutionary species concept) strategies and

management plans can be implemented to protect these taxa (Peterson and Navarro 1999).

Amazilia viridifrons including *villadai* and *wagneri* is considered a Mexican endemic and threatened species (NOM-059-ECOL-2001). Though recognizing it as a separate species can help its conservation because recently, *A. wagneri* has only been recorded in a specific locality near the Isthmus of Tehuantepec (Cerro Piedra Larga). This site has been proposed as an Important Areas for Bird Conservation (AICAS in Spanish) due to the numerous endemism present in the area (Arizmendi and Márquez-Valdelamar 2000).

Cerro Piedra Larga, Oaxaca is a mass of Mesozoic origin located west of the Isthmus of Tehuantepec; it presents patches of cloud forest in high-elevation canyons (Peterson et al. 2004) and tropical decidous forest in lower parts (García- Deras et al. pers. obs.). It represents the last island of humid montane forest before the barrier of the Isthmus of Tehuantepec and lies isolated between two mountain ranges (Peterson et al. 2004) being a place for *in situ* speciation.

CONCLUSIONS

The genus *Amazilia* overall (*sensu* AOU 1998) is probably polyphyletic but at least the taxa included in this study formed a monophyletic clade. With further data we would be able to determine which clades belong to *Amazilia* and which ones deserve a change in their taxonomy.

Delimitation of species using mtDNA sequence data has important advantages over other type of characters such as morphological. Some methods as the proposed by Wiens and Penkrot (2002) and used here can be used to delimit species even when some of the included species are represented by only one or two individuals.

The current data, even with the limitation imposed by incomplete taxon sampling, clearly support the distinctiveness of *A. viridifrons* from *A. violiceps* as Ridgway (1911), AOU (1998) and Peterson and Navarro (2000) proposed. It is possible to see both species in central Guerrero. Their proposal that *A. v. villadai* may constitute another species is not supported by our data as this taxon shares haplotypes with *A. v. viridifrons* suggesting that they belong to the same species.

Although we only obtained only one sample of *A. wagneri* it is clear that it is both morphologically and molecularly distinct from the other *A. viridifrons* as seen in the haplotype network (Figure 5) and may constitute another species. Another important criterion that may guarantee its status as a separate species is its restricted distribution (Fig. 1) to the coast of Oaxaca where the habitat is currently disturbed. *A. wagneri* is a threatened species and very difficult to see and to collect in the field. It seems that the disturbance of its habitat has diminished its population as we had seen in our field work.

Recognizing *A. wagneri* as a separate species as our results suggest can be of critical importance to its conservation, a major step was accomplished when Cerro Piedra Larga had been proposed as a protected area. Recently, a fully review of the check-lists of protected areas was made by expert ornithologists; conservation priorities and

strategies for management for every protected area should be the new goal to be accomplished.

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FIGURE LEGENDS

Figure 1. General distribution of Green-fronted hummingbird (*Amazilia viridifrons*) and Violet-crowned hummingbird (*A. violiceps*). White triangles refer to localities of collecting *A. viridifrons* and the white circle to the site of *A. wagneri.*

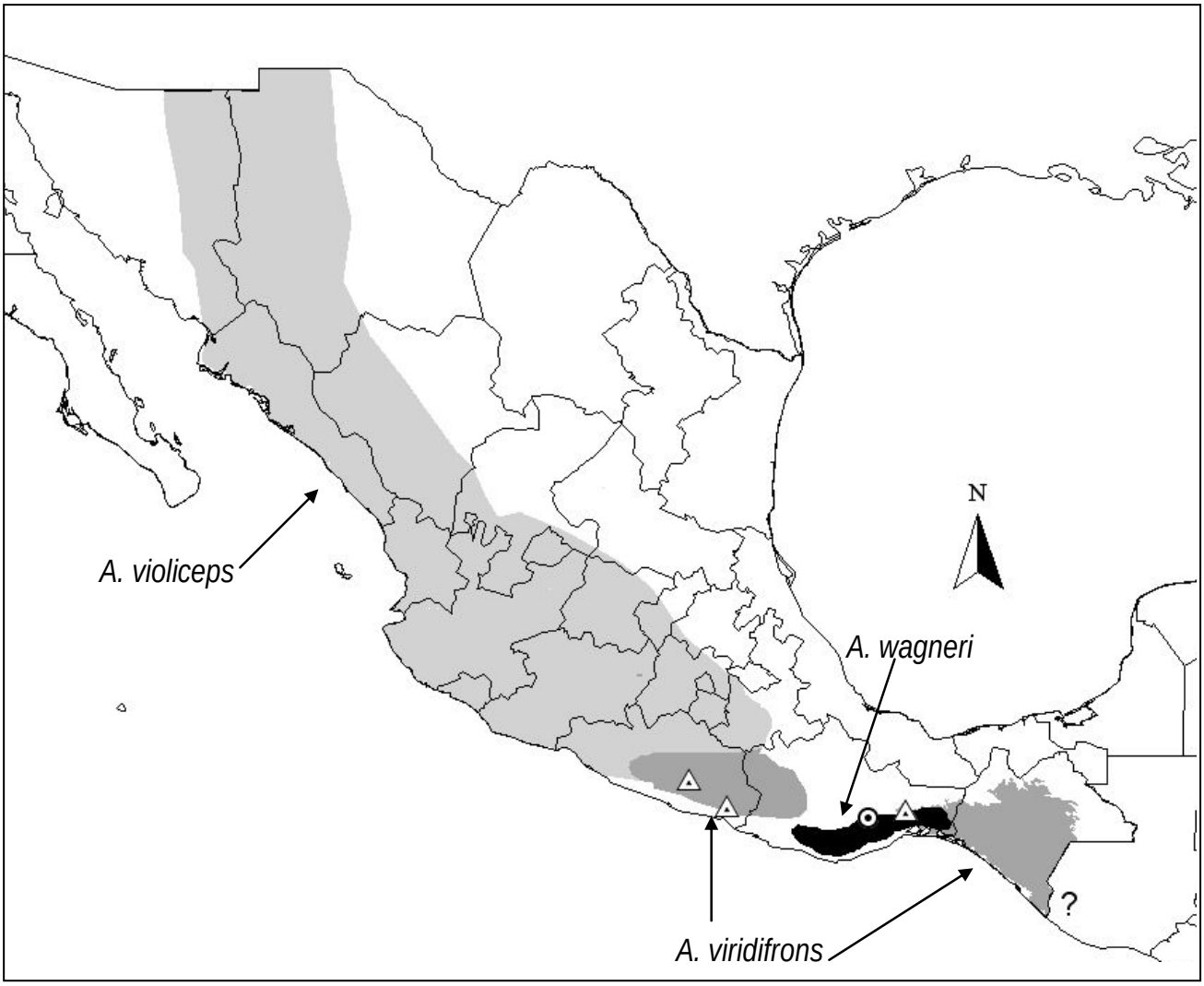

Figure 2. MP bootstrap tree. L = 329 CI =0.6991 HI = 0.3009 RC = 0.4991. Values above the node correspond to bootstrap. Abbreviations refer to Mexican states: GRO = Guerrero, OAX = Oaxaca, ZAC= Zacatecas, GUA= Guanajuato.

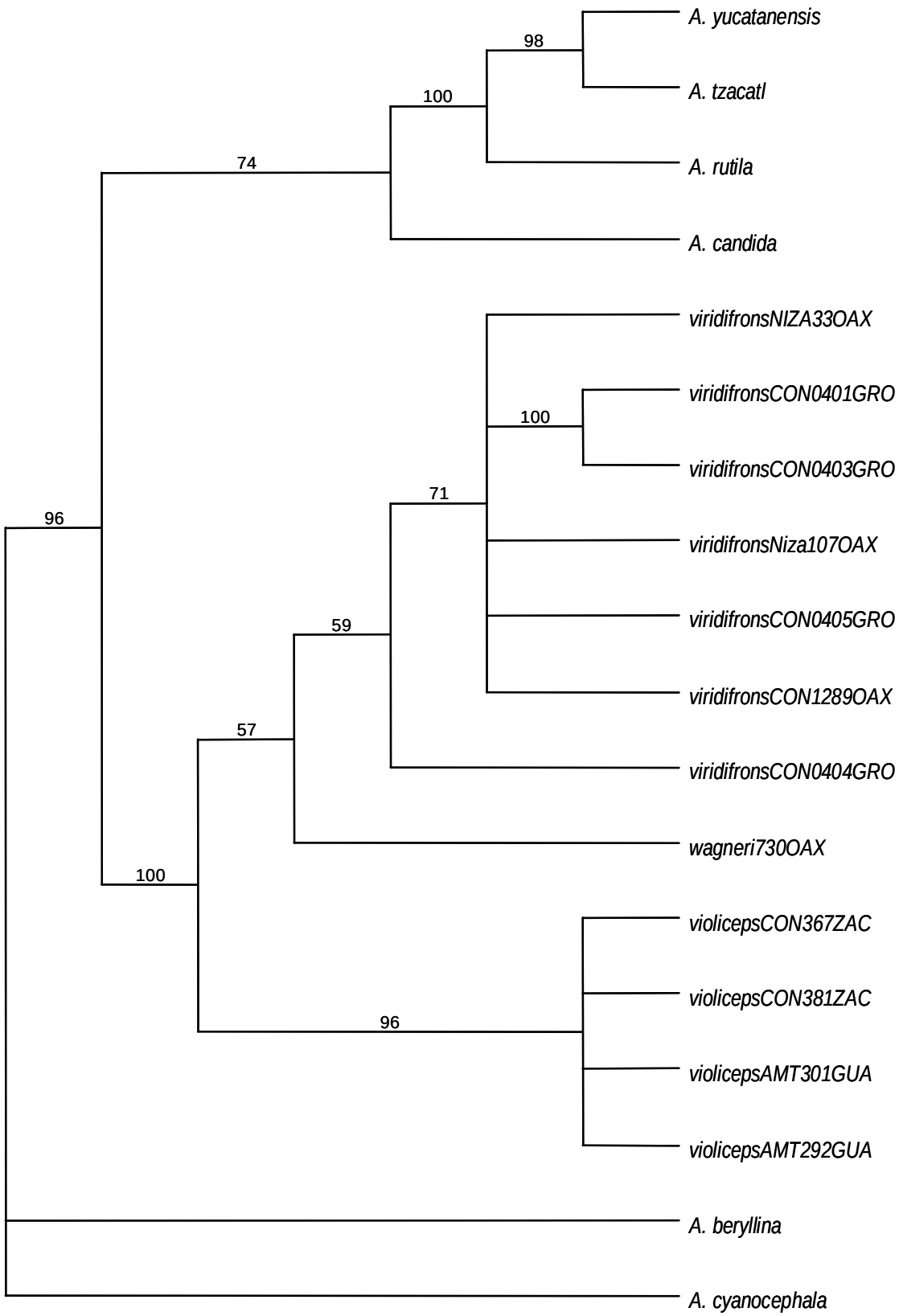

Figure 3. ML bootstrap tree. Values above the node correspond to bootstrap. Abbreviations refer to Mexican states: GRO = Guerrero, OAX = Oaxaca, ZAC= Zacatecas, GUA= Guanajuato.

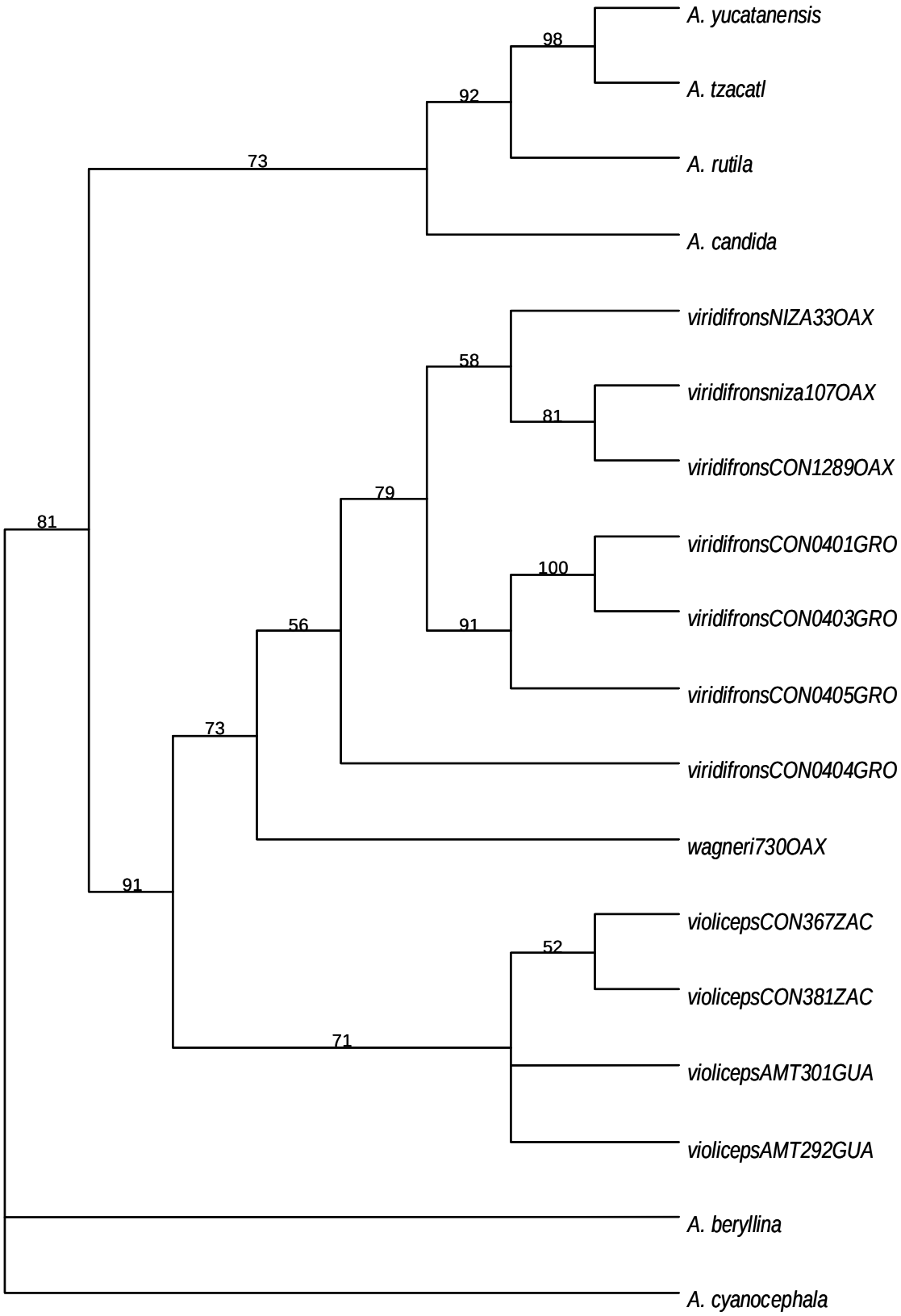

Figure 4. Bayesian consensus tree. Values above the node correspond to posterior probabilities. Abbreviations refer to Mexican states: GRO = Guerrero, OAX = Oaxaca, ZAC= Zacatecas, GUA= Guanajuato.

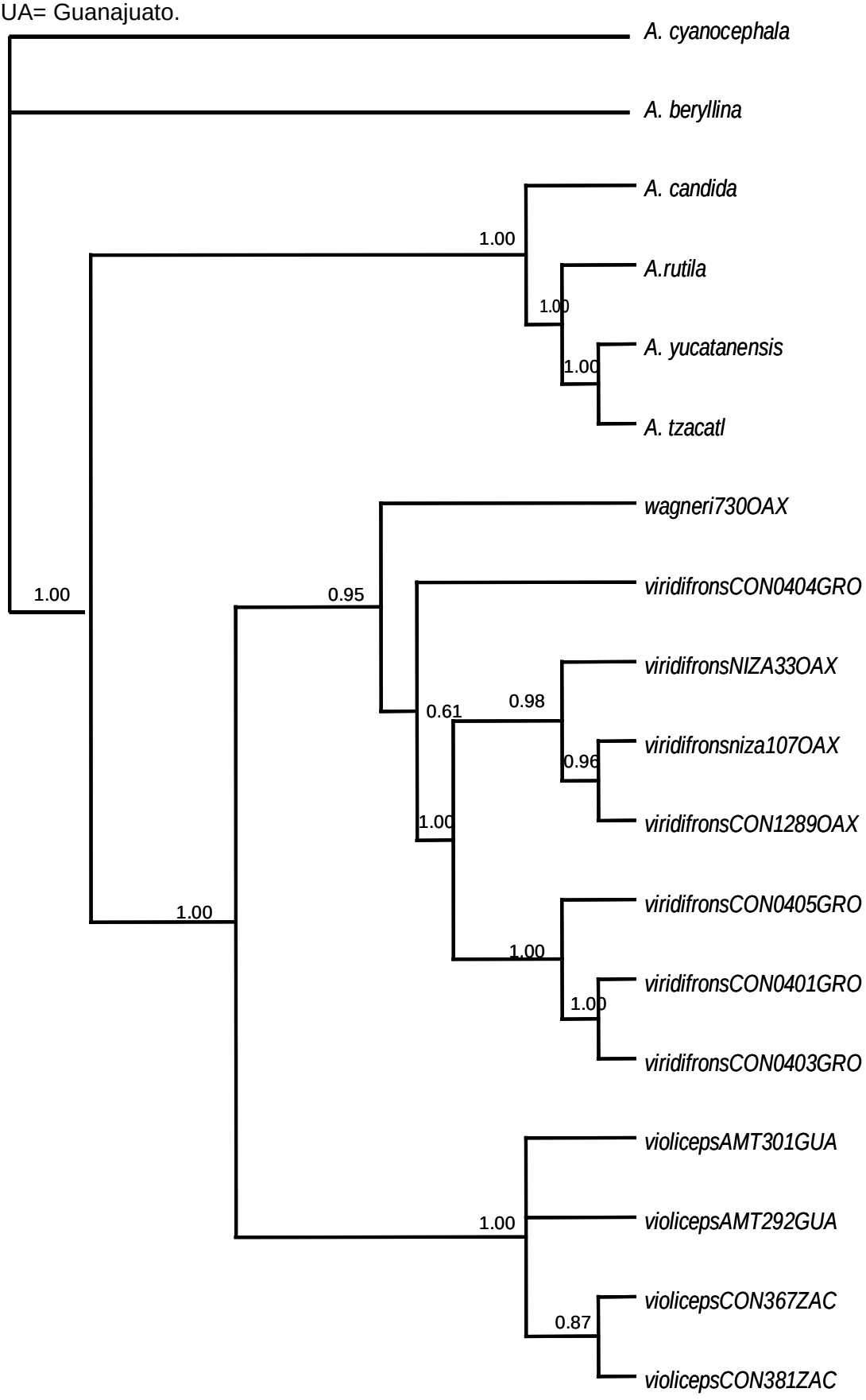

Figure 5. Haplotype network of the *Amazilia* samples in the present study. Black dots refer to either extant or missing haplotypes. Abbreviations refer to Mexican states: GRO = Guerrero, OAX = Oaxaca, ZAC= Zacatecas, GUA= Guanajuato.

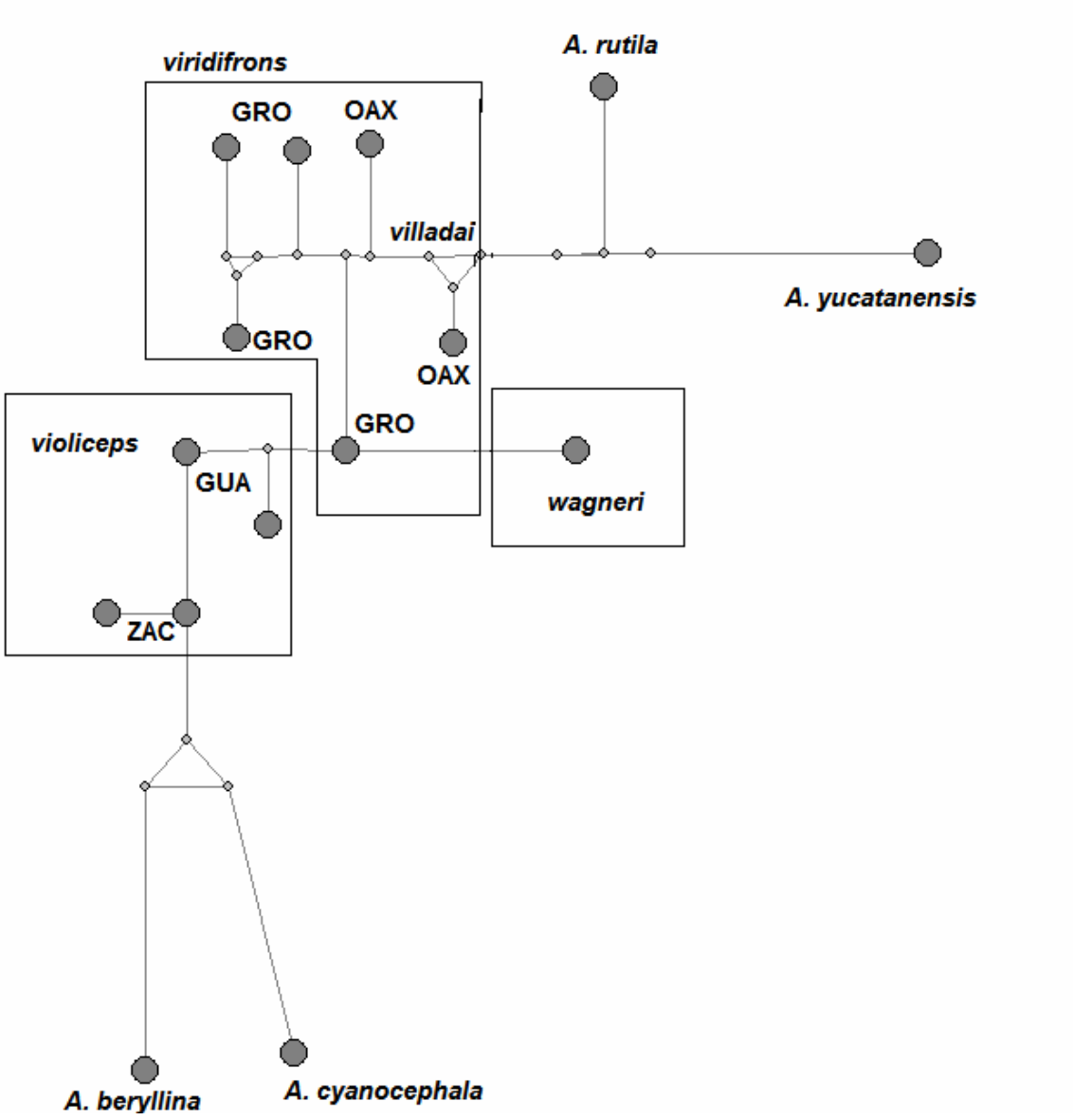

Table 1. List of samples used in this study, localities, vouchers/sample IDs, GenBank Accession numbers.

Species	Catalogue No.	Locality	GeneBank Accession Numbers
Amazilia beryllina	OMVP 1012	Oaxaca, Sierra de Miahuatlán, Distrito Juquila, 4 km E Peñas Negras	XXXXXX
Amazilia cyanocephala	PUE 095	Puebla, Cuitchat, 8 km NE Cuetzalan	XXXXXX
Amazilia candida	CHIMA 314	Oaxaca, Chalchijapa, a 20 km NE del pueblo	XXXXXX
Amazilia rutila	B 552	Quintanta Roo, Puerto Morales	XXXXXX
Amazilia tzacatl	B 1943	Yucatán, Dzilán de Bravo	XXXXXX
Amazilia yucatanensis	HGOSLP 132	Campeche, Tachich a 15 Km W	XXXXXX
Amazilia violiceps	CONACYT 367	Zacatecas, Rancho Chalchisco, 10 Km W de Jalpa	XXXXXX
Amazilia violiceps	CONACYT 381	Zacatecas, Rancho Chalchisco, 6 Km SO de Jalpa	XXXXXX
Amazilia violiceps	AMT 292	Guanajuato, Tinaja de Pastores, Yuriria	XXXXXX
Amazilia violiceps	AMT 301	Guanajuato, Tinaja de Pastores, Yuriria	XXXXXX
Amazilia viridifrons villadai	CONACYT 1289	Oaxaca, Nizanda, El Naranjo	XXXXXX
Amazilia viridifrons villadai	NIZA 33	Oaxaca, Nizanda, El Naranjo	XXXXXX
Amazilia viridifrons villadai	NIZA 107	Oaxaca, Nizanda, El Naranjo	XXXXXX
Amazilia v. viridifrons	CONACYT 986	Guerrero, El Carmen 2km NE San Luis Acatlán	XXXXXX
Amazilia v. viridifrons	CONACYT 04-01	Guerrero, Acahuizotla	XXXXXX
Amazilia v. viridifrons	CONACYT 04-02	Guerrero, Acahuizotla	XXXXXX
Amazilia v. viridifrons	CONACYT 04-03	Guerrerp, Acahuizotla	XXXXXX
Amazilia v. viridifrons	CONACYT 04-04	Guerrero, Acahuizotla	XXXXXX
Amazilia v. viridifrons	CONACYT 04-05	Guerrero, Acahuizotla	XXXXXX
Amazilia wagneri	OMVP 0730	Oaxaca, Cerro Piedra Larga	XXXXXX

Table 2 Matrix of genetic distances

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.A.yucatanensis																			
2.A. tzacatl	0.0352																		
3.A. rutila	0.0678	0.0534																	
4. A.viridifrons0402	0.1225	0.1019	0.0951																
5. A. wagneri	0.1153	0.1160	0.1156	0.0145															
6.A.viridifronsN33	0.1255	0.1117	0.0981	0.0086	0.0233														
7.A. viridifrons1289	0.1011	0.1113	0.1013	0.0202	0.0328	0.0115													
8.A. viridifrons986	0.1215	0.1113	0.0977	0.0202	0.0352	0.0115	0.000												
9.A. viridifrons0401	0.1160	0.1658	0.1149	0.1410	0.0796	0.1375	0.0505	0.1372											
10.A. viridifrons0403	0.1269	0.1813	0.1242	0.1173	0.0673	0.1139	0.0459	0.1204	0.0254										
11.A. viridifrons107	0.0935	0.0848	0.0925	0.0441	0.0458	0.0351	0.0176	0.0350	0.0470	0.0447									
12.A. viridifrons0405	0.1066	0.1254	0.1066	0.0231	0.0340	0.0202	0.0131	0.0320	0.0448	0.0368	0.0243								
13.A. viridifrons0404	0.1028	0.1019	0.1017	0.0000	0.0248	0.0086	0.0087	0.0202	0.0529	0.0436	0.0198	0.0087							
14.A. violiceps367	0.1066	0.1051	0.1042	0.0086	0.0352	0.0115	0.0187	0.0231	0.0660	0.0565	0.0277	0.0209	0.0120						
15.A. violiceps381	0.1080	0.1051	0.1029	0.0086	0.0364	0.0115	0.0198	0.0231	0.0672	0.0577	0.0289	0.0221	0.0132	0.0011					
16.A. violiceps301	0.1054	0.1019	0.1030	0.0057	0.0341	0.0144	0.0198	0.0260	0.0648	0.0553	0.0289	0.0198	0.0110	0.0011	0.0022				
17.A. violiceps292	0.1054	0.0985	0.1004	0.0028	0.0341	0.0115	0.0198	0.0231	0.0648	0.0553	0.0289	0.0198	0.0110	0.0033	0.0022	0.0022			
18.A. beryllina	0.1038	0.1225	0.1053	0.0727	0.0918	0.0698	0.0829	0.0826	0.1137	0.1154	0.0905	0.0827	0.0804	0.0779	0.0792	0.0791	0.0816		
19.A. cyanocephala	0.1002	0.1195	0.1004	0.0792	0.0945	0.0888	0.0916	0.0917	0.1205	0.1182	0.1008	0.0942	0.0881	0.0893	0.0906	0.0881	0.0907	0.0368	
20.A.candida	0.1399	0.1329	0.1152	0.1442	0.1558	0.1547	0.1577	0.1577	0.1973	0.2053	0.1503	0.1579	0.1442	0.1402	0.1402	0.1369	0.1405	0.1402	0.1222

Table 3. Summary of results of AMOVA, showing the distribution of genetic variation among groups, populations, and individuals of *Amazilia viridifrons* and *A. violiceps*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	2	59.189	9.24786	90.15
Within populations	8	8.083	1.01042	9.85
Total	10	67.273	10.25827	

CONCLUSIONES GENERALES

A pesar del conocimiento que se tiene sobre el grupo de los colibríes aún sigue faltando información sobre su historia natural, su taxonomía y evolución que nos pueda ayudar a entender mejor su historia evolutiva. Con el descubrimiento de fósiles de aves parecidas a un colibrí como el de Mayr (2004) será posible ajustar la calibración de relojes moleculares que nos permitan estimar el tiempo de divergencia de los *Trochilidae*.

La delimitación de especies usando DNA mitocondrial es una herramienta importante en los estudios sistemáticos así como lo es la adecuada aplicación de conceptos de especie ya que las especies son las unidades básicas de estudio de la Sistemática, Biodiversidad y Conservación.

En medida que se tengan más taxones y ejemplares disponibles para estudios moleculares se podrá conjuntar con estudios previos como el de Zusi y Benz (1982) y con otros caracteres (ej. cantos, coloración, etc.) para delimitar de manera más robusta las especies y así proponer una filogenia del grupo de los colibríes y se podrá además determinar su historia evolutiva, al menos México.

Determinar con tanta información como sea posible el estatus taxonómico de las especies es importante, no solo para conocer su historia evolutiva y por ende su clasificación, sino también porque son individuos de una especie lo que vemos en el campo y lo que está sujeto a amenazas y solo con una documentada revisión podremos protegerlas de la extinción al implementar planes de manejo en las áreas de distribución de las especies.

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