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Molecular systematics of the Amazonian endemic genus *Hylexetastes* (Aves: Dendrocolaptidae): taxonomic and conservation implications

ROXIRIS A. AZUAJE-RODRÍGUEZ,[1] JASON D. WECKSTEIN,[2] JANICE H. DISPOTO,[2] SWATI PATEL,[3] JOSEPH A. CACIOPPO,[4] JOHN M. BATES,[5] SOFIA MARQUES SILVA[1,6] & ALEXANDRE ALEIXO[1,7]*

[1]*Programa de Pós-Graduação em Zoologia, Museu Paraense Emílio Goeldi/Federal University of Pará, Belém, Brazil*
[2]*Department of Ornithology, Academy of Natural Sciences of Drexel University and Department of Biodiversity, Earth, and Environmental Sciences, Drexel University, Philadelphia, PA, USA*
[3]*Department of Mathematics, Tulane University, New Orleans, LA, USA*
[4]*Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana-Champaign, IL, USA*
[5]*Life Sciences Section, Integrative Research Center, The Field Museum, Chicago, IL, USA*
[6]*Research Center in Biodiversity and Genetic Resources/InBIO, University of Porto, Porto, Portugal*
[7]*Department of Zoology, Museu Paraense Emílio Goeldi, Belém, Brazil*

Hylexetastes woodcreepers are endemic to the *terra firme* forests of the Amazon basin. Currently, most taxonomic sources recognize two species of *Hylexetastes* (*H. perrotii* and *H. stresemanni*), each divided into three subspecies. Some authors maintain that the *H. perrotii* subspecies should be elevated to full species status. In particular, *Hylexetastes perrotii brigidai* is endemic to the eastern Amazon, the second Amazonian area of endemism (Xingu) most affected by deforestation and habitat degradation. Consequently, the taxonomic status of *H. p. brigidai* is of particular concern for conservation. Thus far, only morphological characters have been evaluated for the taxonomic delimitation of species and subspecies of *Hylexetastes*. We present a molecular phylogenetic analysis of all subspecies to help delimit *Hylexetastes* interspecific limits. Fragments of two mitochondrial (*Cytb* and ND2) and three nuclear genes (FGB5, G3PDH and MUSK) from 57 *Hylexetastes* specimens were sequenced. An ecological niche model was estimated to describe more accurately the potential distributions of taxa and to evaluate their vulnerability to ongoing deforestation. Phylogenetic analyses support the paraphyly of the polytypic *H. perrotii* as currently delimited and the elevation of *Hylexetastes perrotii uniformis* to full species rank, as well as the presence of three evolutionary significant units (ESUs) within this newly delimited species, including one grouping all *H. p. brigidai* specimens. Alternatively, under lineage-based species concepts, our results support at least five evolutionary species in *Hylexetastes*: *H. stresemanni*, *H. undulatus*, *H. perrotii*, *H. uniformis* and *H. brigidai*. Each of these taxa and ESUs are distributed in different interfluvial areas of the Amazon basin, which have different degrees of disturbance. Because they occupy the most heavily impacted region among all *Hylexetastes* ESUs, regular assessments of the conservation statuses of *H. p. brigidai* and both *H. uniformis* ESUs are paramount.

Keywords: ecological niche models, evolutionary significant units, phylogeography, species delimitation, taxonomy.

*Corresponding author.
Email: aleixo@museu-goeldi.br
Twitter: @AlexandreAlei20

There remains a misconception that taxonomic studies may overly complicate species conservation relative to studies focused on monitoring endangered species in their habitats (Mace 2004,

Garnett & Christidis 2007, Aleixo 2009a,b). However, taxonomic studies can help delimit divergent evolutionary entities and enable us to recognize species that may be endangered, both of which are critical steps in facilitating the preservation of these lineages (Kahindo *et al.* 2007, Frankham *et al.* 2008). Furthermore, the establishment of priority areas for species conservation is highly sensitive to the taxonomic treatment used for species delimitation (Peterson & Navarro-Siguenza 1999). Therefore, conservation of species is inhibited when they are not correctly delimited within an evolutionary context (Mace 2004). Today, species delimitation can take into account a variety of information related to the taxa under study, such as evolutionary history, morphology, genetics, natural history and geographical distribution (e.g. De Queiroz 2007, Aleixo *et al.* 2013, Carstens *et al.* 2013, Portes *et al.* 2013, Engel *et al.* 2014, Halley *et al.* 2017).

In some Neotropical species, speciation is hypothesized to have resulted from range expansion followed by isolation, which often corresponds to specific geological barriers (Ribas *et al.* 2012, Smith *et al.* 2014, Fabre *et al.* 2017). In particular, changes in river formations are among the main sources of geographical isolation and drivers of diversification that affect vertebrate species in Amazonia (Ayres & Clutton-Brock 1992, Bates *et al.* 2004, Ribas *et al.* 2012, De Oliveira *et al.* 2016). These processes have generated a mosaic of distinct areas of endemism of different size and conservation status (Cracraft 1985, Bates & Demos 2001, Da Silva *et al.* 2005, Borges 2007) across the Amazon basin.

Genetic data have not only been important in the reconstruction of biogeographical and phylogenetic history but also are useful for detecting cryptic species, which may be prevalent among Amazonian taxa (Bickford *et al.* 2007, Batista *et al.* 2013, Rocha *et al.* 2015, Ferreira *et al.* 2017). Genetic information facilitates the identification of divergent lineages that may be considered full species, according to the general lineage species concept (De Queiroz 2005, Aleixo 2007, 2009a). Moreover, genetic diversity is necessary for species to evolve and adapt to environmental changes (Frankham *et al.* 2008) and, hence, is a major focus of conservation biology (Heywood & Watson 1995). Furthermore, the description of patterns of genetic diversity enables the identification of evolutionary significant units (ESUs), which are genetically differentiated meta-populations within what is currently considered a single species, and are also potentially in need of distinct conservation plans. A single species may have one or more ESUs (Ryder 1986, Moritz 1994, Hey *et al.* 2003, Frankham *et al.* 2008, Aleixo 2009a).

The recognition of threatened or endangered ESUs, subspecies or species needs to be complemented by distributional data to facilitate planning an efficient system of protected areas and generating criteria to prioritize conservation of these taxa (Ryder 1986, Moritz 1994). Ecological niche modelling estimates the geographical distribution of focal species based on occurrence and environmental data (Phillips *et al.* 2006, Excoffier & Lischer 2010), even among poorly studied species (Franklin 2010, Carstens *et al.* 2013). These models allow an evaluation of the distributional limits and degree of environmental differentiation of each taxon (Martinez-Gordillo *et al.* 2010, Tocchio *et al.* 2014, Ruedas *et al.* 2017) and add critical data to effective conservation planning.

The woodcreeper genus *Hylexetastes* (Dendrocolaptidae) is endemic to Amazonia with poorly documented interspecific limits and controversial taxonomy. *Hylexetastes* is a well-supported monophyletic genus (Derryberry *et al.* 2011), with two species currently recognized by many taxonomic sources (Marantz *et al.* 2003, Birdlife International 2017, Clements *et al.* 2017, Remsen *et al.* 2017): one distributed in western Amazonia (Bar-bellied Woodcreeper *Hylexetastes stresemanni*, with three subspecies described: *H. s. insignis*, *H. s. stresemanni* and *H. s. undulatus*) and another occurring in eastern Amazonia (Red-billed Woodcreeper *Hylexetastes perrotii*, with three subspecies described: *H. p. perrotii*, *H. p. uniformis* and *H. p. brigidai*). However, *H. p. uniformis* (Uniform Woodcreeper) and *H. p. brigidai* (Brigida's Woodcreeper) are regarded as full species by some authors, based on slight differences in the colours and patterns of the throat and belly plumage (Fig. 1; Da Silva *et al.* 1995, Piacentini *et al.* 2015, Gill & Donsker 2017).

Uniform and Brigida's woodcreepers are not considered globally threatened (BirdLife International 2017) but their conservation status is uncertain, given these species are uncommon across their ranges (Ridgely & Tudor 1994, Stotz *et al.* 1996). Moreover, Brigida's Woodcreeper is endemic to the Xingu area of endemism in

eastern Amazonia (Marantz *et al.* 2003, IUCN 2017), a region heavily affected by Amazonia's infamous arc of deforestation and by habitat degradation due to anthropogenic disturbance (Da Silva *et al.* 2005, Soares-Filho *et al.* 2005, Bird *et al.* 2011, Moura *et al.* 2013, Barlow *et al.* 2016, INPE 2017). Recognition of this taxon as an ESU or a full species would have important implications for its conservation: Brigida's Woodcreeper is listed as vulnerable in the Brazilian national checklist of threatened species (ICMBIO 2016).

To date, only morphological characters have been used to evaluate *Hylexetastes* interspecific limits (Cory & Hellmayr 1925, Zimmer 1934, Peters 1951, Da Silva *et al.* 1995, Piacentini *et al.* 2015, Marantz *et al.* 2003, Remsen *et al.* 2017). Thus, to independently assess the taxonomic uncertainties within *Hylexetastes*, we gathered genetic data to construct a population-level phylogenetic hypothesis for the genus. Using our phylogenetic hypothesis, we also estimated ecological niche-based models (ENMs) to distinguish the potential distribution of each identified taxon and evaluate the vulnerability of each species.

METHODS

Sampling

We sequenced DNA extracted from vouchered frozen or ethanol-fixed muscle tissues (n = 56) and an unvouchered blood sample (n = 1) from *Hylexetastes* specimens belonging to all described taxa in the genus (Marantz *et al.* 2003), distributed as follows: *H. s. undulatus* (n = 9); *H. s. stresemanni* (n = 1); *H. s. insignis* (n = 2); *Hylexetastes perrotii perrotii* (n = 15); *H. p. uniformis* (n = 24); and *H. p. brigidai* (n = 6). These samples and their respective voucher specimens had been deposited in several ornithological collections (Fig. 2a, Table S1). *Xiphocolaptes promeropirhynchus*, which is known to be the sister group to the genus *Hylexetastes* (Derryberry *et al.* 2011), was used as the outgroup.

DNA extraction, amplification and sequencing

Total DNA was extracted from tissue samples using the Genomic DNA Purification Kit (Wizard®; Promega, Madison, WI, USA) or

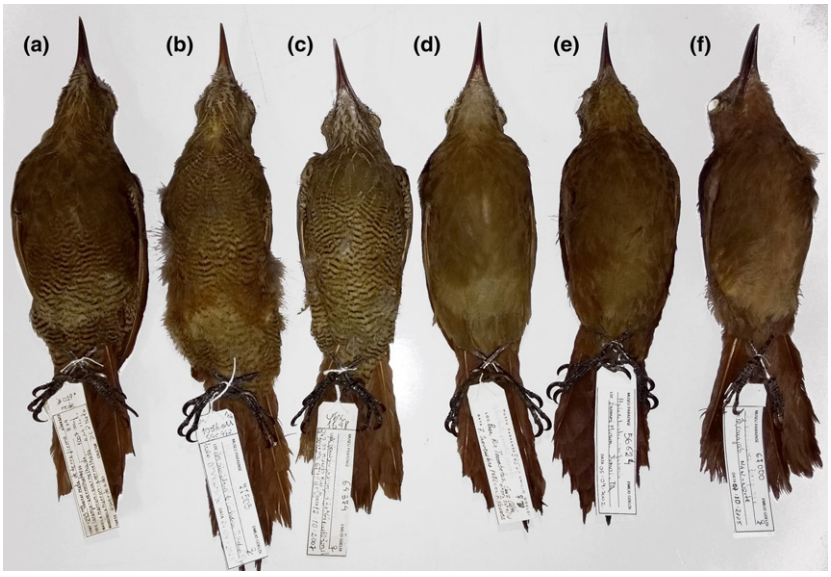


Figure 1. Ventral views of representative specimens illustrating plumage differences among *Hylexetastes* subspecies as recognized by current taxonomy (Marantz *et al.* 2003). From left to right: (a) *Hylexetastes stresemanni stresemanni* (MPEG 50548); (b) *Hylexetastes stresemanni insignis* (MPEG 71503); (c) *Hylexetastes stresemanni undulatus* (MPEG 64374); (d) *Hylexetastes perrotii perrotii* (MPEG 34540); (e) *Hylexetastes perrotii uniformis* (MPEG 56624); (f) *Hylexetastes perrotii brigidai* (MPEG 67000). Note differences in the coloration of the throat and belly among the subspecies.

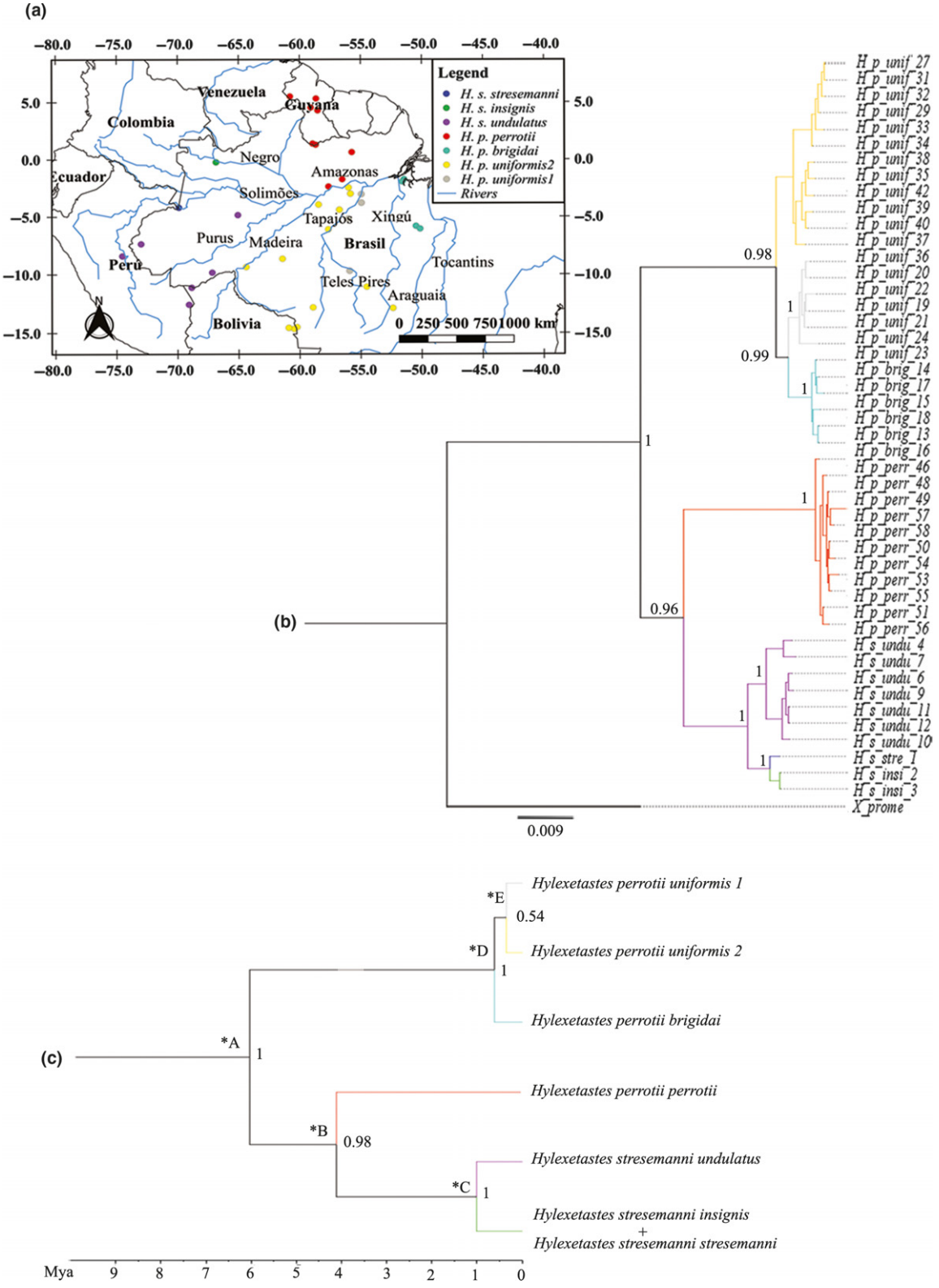
Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). Fragments of two mitochondrial DNA (mtDNA) genes (NADH dehydrogenase subunit 2 (ND2) and cytochrome b (*Cytb*)) and three nuclear genes (*β*-fibrinogen intron 5 (FGB5), glyceraldehyde-3-phospho-dehydrogenase intron 11 (G3PDH) and muscle-specific receptor tyrosine kinase intron 3 (MUSK)) were amplified by polymerase chain reaction (PCR; see Table S2 for primer and PCR conditions). Amplification products were visualized by electrophoresis on a 1% agarose gel containing 10 000× SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA) and purified using ExoSAP (Thermo Fisher Scientific, Waltham, MA, USA) or polyethylene glycol (PEG-8000; Hawkins *et al.* 1994). We conducted cycle-sequencing using the Big Dye Terminator v.3.01 or v.3.1 kit, following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). After an ethanol precipitation or PureSEQ™ (Aline Biosciences, Woburn, MA, USA) cleanup, purified sequencing reaction products were run on either an AB 3130 or AB 3730xl capillary sequencer. These procedures were performed in the Laboratory of Molecular Biology of the Museu Paraense Emílio Goeldi (MPEG), Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum, and the Laboratory of Molecular Systematics and Evolution at the Academy of Natural Sciences of Drexel University (where PCR-purification and sequencing was completed by Functional Biosciences, Madison, WI, USA).

Data processing and phylogenetic analyses

Nucleotide sequences obtained were manually edited using the software BioEdit (Hall 1999) or GENEIOUS (ver. 8.1.3 and 8.1.8, Biomatters Ltd) and aligned using the default settings of the CLUSTAL-W algorithm (Thompson *et al.* 1994) implemented in BioEdit. Heterozygous positions for the nuclear genes were coded according to the IUPAC code. MESQUITE v.3.0.4 was used to concatenate mtDNA and nuclear sequences. The codon reading frame was set to translate the mtDNA sequences to amino acid sequences to check the sequence alignment for inappropriate stop codons, an indication of errors (Maddison & Maddison 2017). PARTITION FINDER v.1.1.1 was used to determine the best evolutionary model for each gene, and best partitioning scheme using the Bayesian information criterion (BIC; Lanfear *et al.* 2012; Table S3). MrBayes v.3.2 was used to construct phylogenetic trees using the concatenated multilocus alignment under the criterion of Bayesian inference (Ronquist & Huelsenbeck 2003) and run for 5 million generations, using four heated chains, sampled every 500 generations. We discarded 10% of the sampled trees as burn-in.

To estimate a species tree and divergence times from the multilocus tree, we first assigned individual alleles to a 'species' using the well-supported and geographically structured mtDNA concatenated tree (see Results) and tested whether these lineages were evolutionarily independent using BPP v.3.2 (Yang 2015). A joint species delimitation and species tree analysis was conducted to test the delimitation of the clades recovered by the mtDNA concatenated tree (see below). We ran the reversible-jump Markov chain Monte Carlo (rjMCMC) analysis, with algorithm 0 and e = 2, for 500 000 generations (sampling interval of five) and a burn-in of 100 000. Priors for ancestral population sizes and divergence times may influence posterior probability distributions (Yang 2015), so we tested different combinations for these priors, considering relatively large and small ancestral population sizes: $\theta \sim G$ (1, 10) and $\theta \sim G$ (2, 2000),

Figure 2. Distribution of samples and resulting phylogenetic hypothesis obtained for the specimens of *Hylexetastes* sequenced in this study. (a) Map of locations where *Hylexetastes* specimens sequenced in this study were collected. Different clades were assigned to different taxa based on their respective type localities (Peters 1951). (b) Phylogenetic hypothesis by Bayesian inference of the concatenated mitochondrial (*Cytb* and ND2) and nuclear genes (FGB5, G3PDH, MUSK) for 45 *Hylexetastes* specimens. Numbers associated with each specimen/branch-tip are the same as those listed in Table S1, where detailed voucher and locality information is provided. (c) Multilocus coalescent Bayesian inference of a species tree generated using *BEAST with data from mtDNA (*Cytb* and ND2) and nuclear genes (FGB5, G3PDH, MUSK) for 45 *Hylexetastes* specimens. Node labels indicate posterior probability values. Letters labelling each node indicate estimates of the divergence times from Table 2. Asterisks on nodes indicate clades supported by the species delimitation analysis performed in BPP for all demographic and divergence time models considered. Colours of clades indicate terminal taxa and match the distribution map legend in (a).



respectively, and shallow and deep divergence times: $\tau \sim G$ (2, 2000) and $\tau \sim G$ (1, 10), respectively. The other divergence time parameters were assigned the default Dirichlet prior (Yang & Rannala 2010). An initial run was performed to confirm convergence of the run (model A00) and tested in TRACER 1.6 (Drummond & Rambaut 2007). A heredity file was input to account for the different inheritance patterns in the dataset. Each analysis was run twice to confirm the consistency of the results.

Species tree and divergence times were estimated under a coalescent-based model using the program *BEAST v.1.8.0 (Drummond & Rambaut 2007) and the CIPRES Science Gateway Portal v.3.1 at the San Diego Supercomputer Center (Miller *et al.* 2010; www.phylo.org/portal/). We ran *BEAST twice, with each run consisting of a chain of 10^8 iterations sampled every 10 000 iterations, with 10% burn-in. Two independent approaches were used to date the diversification history of *Hylexetastes* and, for both, coalescent times were estimated using a speciation Yule process as tree prior (Drummond *et al.* 2012), under a relaxed-clock approach with an uncorrelated log-normal distribution, allowing the program to estimate mutation rates for nuclear DNA genes but differing in the calibration scheme for mtDNA loci. The first dating approach, broadly used in avian phylogenetic studies, implemented a mutation rate of 0.01105 (with a standard deviation of 0.0034) substitutions/site/million years (Myr). This is based on an average rate of 2.1% substitutions per million years estimated for the *Cytb* gene (Weir & Schluter 2008). For the second approach, we used a recently proposed calibration for third codon positions of mtDNA protein-coding genes (median substitution rate of 0.0142 substitutions/site/Myr), which considers the correlations between the substitution rate and the body mass of the birds (Nabholz *et al.* 2016). We used the formula in Nabholz *et al.* (2016: 444) to correlate *Hylexetastes* body masses with their proposed median substitution rate for third codon position of mtDNA protein-coding genes (see above), obtaining a custom rate of 0.0309 substitutions/site/Myr. We then applied this rate to third codon positions of *Cytb* and ND2, estimating rates of the nuclear markers. Data on *Hylexetastes* taxa average body masses were obtained from Marantz *et al.* (2003). The same partitions as detailed above were used (Table S3) and mtDNA partitions were set as linked. Convergence of runs was verified in TRACER v.1.6 (Rambaut *et al.* 2007) and tree topologies were summarized in TreeAnnotator v.1.8 to make consensus trees (Drummond *et al.* 2012) and visualized in FigTree 1.4.2 (Rambaut 2006).

Summary statistics

We used the Bayesian algorithm PHASE v.2.1 (Stephens *et al.* 2001, Stephens & Donnelly 2003) implemented in DnaSP v.5.10 (Librado & Rozas 2009) to phase haplotypes of nuclear genes. We used the resulting files to estimate genetic parameters, also in DnaSP, such as nucleotide and haplotype diversity, to perform neutrality tests such as Tajima's D and R_2, and for recombination tests for the nuclear genes (Hudson & Kaplan 1985, Hudson *et al.* 1987, Rozas *et al.* 2001, Librado & Rozas 2009). The program MEGA v.7.0.2 was used to estimate genetic distances for the mitochondrial gene within and between each clade, using 1000 bootstrap replicates (Kumar *et al.* 2016). Finally, differentiation indices (F_{ST} values) were estimated using ARLEQUIN v.3.5 under default settings (Excoffier & Lischer 2010).

Ecological niche-based models

The study area includes the Amazon basin in South America (latitude from 9°N to 17°S and longitude from 77°W to 47°E; Fig. 2a). A total of 246 occurrence records of *Hylexetastes* were collected (Table S1) from museum specimens using Vertnet (2016), from bird vocalization recordings deposited in Xeno-canto (2016) and from several other ornithological museum collections, distributed as follows: *H. s. undulatus* (n = 20); *H. s. stresemanni* (n = 22); *H. s. insignis* (n = 4); *H. p. perrotii* (n = 86); *H. p. uniformis* (n = 88) and *H. p. brigidai* (n = 26). We used geographical references for localities from the Ornithological Gazetteer of Brazil (Paynter & Traylor 1991) to fill in the incomplete occurrence data. The processing of these data was completed using the Geographical Information System QGIS platform v.2.18 (QGIS Development Team 2016). We obtained environmental layers from Worldclim (Hijmans *et al.* 2005) with a spatial resolution of 30 s ($0.93 \times 0.93 = 0.86$ km^2 at the equator) for current conditions (average for 1960–1990). To select

uncorrelated environmental variables for the modelling procedure, we performed a Pearson's correlation analysis in RStudio 3.3.1 (Zar 1999, RStudio Team 2015).

Models were developed with the Maximum Entropy approach using MAXENT v.3.3.3k (Phillips *et al.* 2006, Phillips & Dudik 2008). This modelling technique requires only presence data as input and consistently performs better than other methods (Hernandez *et al.* 2006, Wisz *et al.* 2008, Excoffier & Lischer 2010). A total of 15 model replicates were run, allowing for a random 75% training and 25% testing data partition in each run. Each replicate was analysed using a bootstrap procedure, allowing sampling with replacement. Jackknifing was used to measure the importance of environmental variables in the models. Models were run with auto-features (Phillips *et al.* 2006), and the area under the curve (AUC) of the receiver-operating characteristics (ROC) plot was taken as a measure of individual model fit (Fielding & Bell 1997). We evaluated the model's prediction using the partial ROC approach (Peterson *et al.* 2008) with a proportion omission of 0.05 and 0.15, 50% random points and 500 bootstrap iterations. To estimate the partial ROC we used the niche toolbox site (http://shiny.conabio.gob.mx:3838/nichetoolb2/). To make the binary prediction and obtain the area of occurrence from the area of suitability for *Hylexetastes* species, we used the linear maximum test of sensitivity and specificity, which is considered to have a better performance than other approaches (Liu *et al.* 2005).

Giving the importance of ESUs in conservation biology, we constructed corresponding ENMs for each *Hylexetastes* lineage/ESU recognized in this study (see below). In a low proportion, preliminary ENM tests for each of the six ESUs recovered predicted some occurrence in areas beyond known ranges and demonstrated environmental similarities among the ecological niches of all the *Hylexetastes* species and ESUs. To avoid these ENM overfits, we treated all *Hylexetastes* species as a single ecospecies and estimated a single model for all of them. Finally, we compared the amount of potential distribution area occupied by each *Hylexetastes* ESU with the amount of native forest under protection and affected by deforestation, and other habitat conversions. For this comparison we used raster files for Amazonian land uses at a spatial resolution of 30 m (Almeida *et al.* 2014; http://www.inpe.br/cra/projetos_pesquisas/terraclass2014.php). These files are divided into their respective Landsat 8 satellite orbits (OLI sensor), Lat/Long Projection System and SAD 69 Geodetic Reference System (Almeida *et al.* 2016). Accurate data are only available for the Brazilian Amazon Basin, but at least half of the predicted species ranges are within this region (55–100%; Fig. 3).

RESULTS

Phylogenetic analyses

A total of 3474 base pairs (bp) were sequenced: 1013, 1018, 471, 352 and 575 bp for ND2, *Cytb*, FGB5, G3PDH and MUSK, respectively. The topologies of phylogenetic trees constructed with mitochondrial and nuclear gene sequences (Fig. 2b), or the phylogenetic hypothesis resulting from the concatenation of the mitochondrial dataset (Fig. S1), were congruent with each other, and analyses of these datasets recovered most currently accepted *Hylexetastes* subspecies as monophyletic, the exception being *H. p. uniformis*, with the caveat that only a single sample of *H. s. stresemanni* was included in our analyses. *Hylexetastes p. uniformis* was recovered as paraphyletic with respect to *H. p. brigidai* (Figs 2b and S1); paraphyly of the polytypic *H. perrotii* (*sensu* Marantz *et al.* 2003, BirdLife International 2017, Clements *et al.* 2017, Remsen *et al.* 2017) was also recovered, with the nominate *H. p. perrotii* grouping with strong support with *H. s. stresemanni* + *H. s. insignis* + *H. s. undulatus* rather than with *H. p. uniformis* + *H. p. brigidai* (Fig. 2b). A concatenated phylogenetic analysis using only nuclear sequences recovered three main clades, with *H. p. perrotii* grouping with high support as the sister taxon to another strongly supported clade joining *H. s. stresemanni* + *H. s. insignis* + *H. s. undulatus* and *H. p. uniformis* + *H. p. brigidai* (Fig. S2). In contrast to the trees including mitochondrial data, *H. p. uniformis* and *H. p. brigidai* were not recovered as reciprocally monophyletic by the nuclear tree, although the monophyly of *H. p. brigidai* received high statistical support (Fig. S2). Because of the low number of samples available for *H. s. stresemanni* and *H. s. insignis* (which grouped together as sister taxa in all concatenated analyses), subsequent coalescent multilocus analyses regarded these taxa *a priori* as a single evolutionary unit, to which the name *stresemanni* was applied due to nomenclatural priority (Marantz *et al.* 2003).

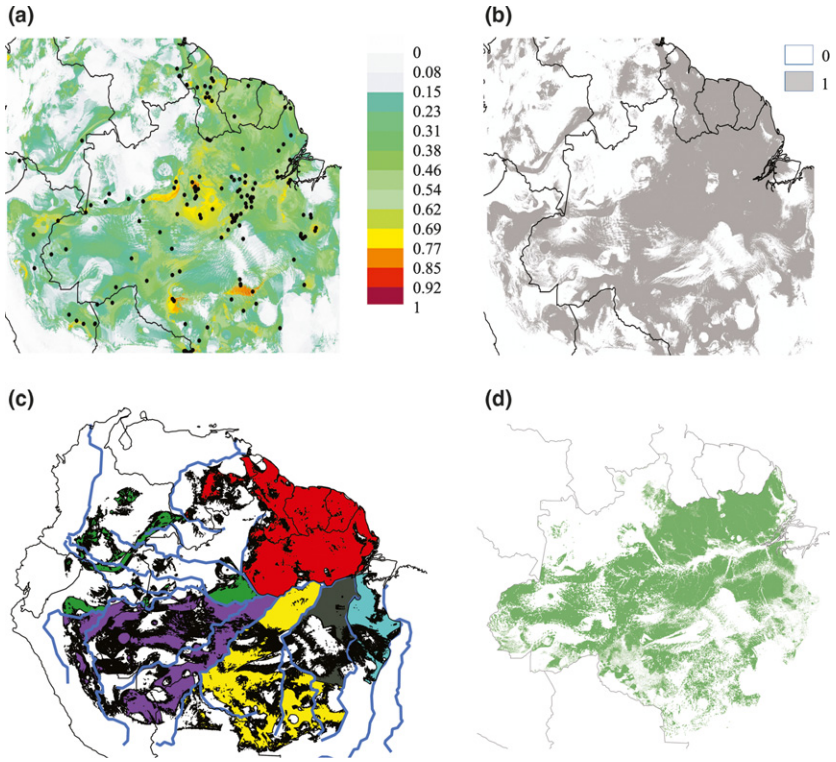


Figure 3. Maps depicting the putative distribution of *Hylexetastes* derived from ecological modelling. (a) Predicted suitability areas for the *Hylexetastes* complex derived from 15 model replicates run in MAXENT. Black points correspond to the occurrence records used. Colours indicate the suitability of areas with warmer colours indicating higher suitability for the complex. (b) Potential distribution of the genus *Hylexetastes* ignoring deforestation. (c) Potential distribution of *Hylexetastes* ESUs: *H. stresemanni stresemanni* (purple), *H. s. undulatus* (purple), *H. p. perrotii* (red), *H. p. uniformis* 1 (yellow), *H. p. uniformis* 2 (grey) and *H. p. brigidai* (light blue). The primary Amazonian rivers are depicted in dark blue. (d) Current distribution of *terra firme* forested areas within the Brazilian Amazon basin.

Two *BEAST species trees, set with distinct calibrations, recovered with high support (posterior probability (PP) > 0.98) most relationships between the six main *Hylexetastes* lineages recovered by the concatenated analyses, although statistical support for the sister relationship of the two *H. p. uniformis* subclades was low (PP = 0.54; Fig. 2c). Both species trees also consistently recovered the polytypic *H. p. perrotii* as paraphyletic with respect to *H. stresemanni* subspecies with high support (Fig. 2c). All combinations of coalescent scenarios tested by species delimitation analyses performed in BPP, irrespective of the demographic and divergence time models considered, were strongly supported (PP = 1). At least six major evolutionary lineages in *Hylexetastes* were identified: *H. p. uniformis* 1; *H. p. uniformis* 2; *H. p. brigidai*; *H. p. perrotii*; *H. s. undulatus*; and *H. s. stresemanni* + *H. s. insignis* (Fig. 2c). Analyses using concatenated and non-concatenated mtDNA loci yielded the same results.

Reconstruction of phylogenies based on the concatenation of the gene sequences and the species tree approaches produced different topologies with respect to the *H. p. uniformis* and *H. p. brigidai* clades. The concatenated analysis of mtDNA recovered one subclade of *H. p. uniformis* as sister to *H. p. brigidai* with strong support, whereas the species tree joined both subclades of *H. p. uniformis* together, with weak support. Therefore, all concatenated and coalescent-based mtDNA and nuclear phylogenetic analyses in this study are concordant in recovering reciprocal monophyly of four main clades or evolutionary lineages, as follows: *H. p. perrotii*, *H. s. stresemanni* (including *H. s. insignis*), *H. s. undulatus* and *H. p. uniformis* (including *H. p. brigidai*; Figs 2, S1 and S2). It should be noted, however, that statistical support for the

Table 1. Uncorrected p-distance (± sd) (A) within and (B) between *Hylexetastes* clades for both mitochondrial genes analysed, *Cytb* (lower diagonal) and ND2 (upper diagonal).

A	*Cytb*	ND2
H. s. undulatus (*n* = 9)	0.004 ± 0.001	0.008 ± 0.002
H. s. insignis (*n* = 2)	0.000 ± 0000	0.000 ± 0.000
H. p. perrotii (*n* = 15)	0.001 ± 0.000	0.003 ± 0.001
H. p. brigidai (*n* = 6)	0.001 ± 0.001	0.002 ± 0.001
H. p. uniformis 1 (*n* = 6)	0.001 ± 0.001	0.001 ± 0.001
H. p. uniformis 2 (*n* = 18)	0.003 ± 0.001	0.005 ± 0.001

B	*H. s. undulatus*	*H. s. insignis*	*H. s. stresemanni*	*H. p. perrotii*	*H. p. brigidai*	*H. p. uniformis* 1	*H. p. uniformis* 2
H. s. undulatus	—	0.013 ± 0.003	0.013 ± 0.005	0.046 ± 0.007	0.053 ± 0.008	0.051 ± 0.007	0.059 ± 0.008
H. s. insignis	0.013 ± 0.004	—	0.002 ± 0.001	0.040 ± 0.006	0.048 ± 0.007	0.046 ± 0.007	0.053 ± 0.008
H. s. stresemanni	0.013 ± 0.004	0.003 ± 0.002	—	0.040 ± 0.006	0.048 ± 0.007	0.046 ± 0.007	0.053 ± 0.008
H. p. perrotii	0.043 ± 0.008	0.043 ± 0.009	0.041 ± 0.008	—	0.049 ± 0.008	0.047 ± 0.007	0.053 ± 0.008
H. p. brigidai	0.056 ± 0.011	0.059 ± 0.011	0.057 ± 0.011	0.060 ± 0.011	—	0.005 ± 0.002	0.016 ± 0.004
H. p. uniformis 1	0.055 ± 0.011	0.059 ± 0.011	0.057 ± 0.011	0.063 ± 0.012	0.011 ± 0.003	—	0.015 ± 0.003
H. p. uniformis 2	0.052 ± 0.010	0.054 ± 0.010	0.052 ± 0.010	0.059 ± 0.011	0.013 ± 0.004	0.009 ± 0.003	—

reciprocal monophyly of *H. s. undulatus* with respect to *H. s. stresemanni* and *H. s. insignis* was low in the nuclear tree (Fig. S2); and the sister relationship between *H. s. stresemanni* and *H. s. insignis* recovered by this phylogeny received marginal support (PP = 0.94).

Genetic diversity levels varied considerably among genes and the *Hylexetastes* mtDNA lineages (Table S4). There were too few samples of *H. s. insignis* (*n* = 2) and *H. s. stresemanni* (*n* = 1) for these estimates. Uncorrected p-distances within *H. stresemanni* subspecies were low, ranging from 0 (*H. s. insignis*) to 0.004 ± 0.001 (*H. s. undulatus*) for *Cytb*, and from 0 (*H. s. insignis*) to 0.008 ± 0.002 (*H. s. undulatus*) for ND2 (Table 1A). For *Hylexetastes* taxa (subspecies), the highest uncorrected p-distance for *Cytb* was between *H. p. uniformis* 1 and *H. p. perrotii* (0.063 ± 0.012), and the lowest uncorrected p-distances were between *H. s. stresemanni* and *H. s. insignis* (0.003 ± 0.002). For ND2 the highest uncorrected p-distance was between *H. s. undulatus* and *H. p. uniformis* 2 (0.059 ± 0.008) and the lowest uncorrected p-distances were between *H. s. stresemanni* and *H. s. insignis* (0.002 ± 0.001; Table 1B). Most of the well-supported clades exhibited relatively high genetic differentiation (F_{ST}) for both the mitochondrial genes (F_{ST} > 0.38; p > 0; Table S5A) and for the nuclear genes (F_{ST} > 0.027; p > 0; Table S5B,C). However, the two subclades of *H. p. uniformis* (1 and 2) exhibited statistically significant genetic differentiation for only one nuclear gene (FGB5; F_{ST} = 0.122; p = 0.012).

Table 2. Divergence time estimates and confidence intervals (95% highest posterior density (HPD)) obtained with *BEAST analyses (see Fig. 2c). Calibration 1 is based on a substitution rate of 0.01105 substitutions/site/lineage/million years for *Cytb* (Weir & Schluter 2008). Calibration 2 is based on a corrected rate accounting for body mass (Nabholz *et al.* 2016). See text for details.

	Calibration 1		Calibration 2	
Node	Mya	95% HPD	Mya	95% HPD
A	3.641	4.479–2.704	1.876	2.518–1.075
B	2.654	3.429–1.761	1.410	1.927–0.808
C	0.753	1.056–0.421	0.432	0.642 0.233
D	0.486	0.759 0.259	0.265	0.422–0.137
E	0.384	0.602–0.150	0.155	0.281–0.054

Although overlapping, time estimates for the diversification within *Hylexetastes* were different for the two calibration approaches used. Avian substitution rate calibration using that employed by Weir and Schluter (2008) provided older divergences than the corrected rates method (Table 2;

Nabholz *et al.* 2016). The oldest divergence corresponds to the split between the *H. p. perrotii* and *H. stresemanni* clade from the *H. p. uniformis* and *H. p. brigidai* clade around 3.64 or 1.88 Myr BP (node A: Fig. 2c). The youngest split corresponds to the split between *H. p. uniformis* clades, around 0.38 or 0.16 Myr BP (node E: Fig. 2c).

Ecological niche-based models

Phylogenetic and species delimitation analyses (Fig. 2b,c) suggest, at a minimum, six ESUs in *Hylexetastes*. The ESUs recovered were coincident with interfluvial areas, indicating *Hylexetastes* taxa are not distributed at random across the Amazon basin. For this reason, rivers were superimposed into the potential distribution of the ecospecies to delimit the ranges of distribution of each *Hylexetastes* ESU (Fig. 3).

Eight different uncorrelated environmental variables were chosen after performing a Pearson's correlation analysis ($r < 0.80$). The ROC plots exhibited high average AUCs with low standard deviations (sd) for both training and test datasets in the model replicates for each species (AUC ≥ 0.817; Table S6). Evaluation of the model predictions using the partial ROC AUC for the two tests gave statistically significant values (1.684 ± 0.03) that support the model selected ($P > 0.05$, Table S6). Further more, the occurrence data adjusted to the predicted areas. We found 'Precipitation of Coldest and Warmest Quarter' to be the climatic variables contributing the most to the ENM for the complex as a whole (Table S7). *Hylexetastes p. perrotii* exhibits the largest potential area of distribution with 1 054 230 km^2, whereas *H. p. brigidai* exhibited the smallest distribution area with only 202 015 km^2 (Fig. 3a, Table 3). The probability of occurrence for each phylogenetically delimited species-level taxon and ESU shows that *H. p. brigidai* and *H. p. uniformis* 1 are coincident with areas in the Brazilian Amazon basin highly impacted by deforestation and habitat degradation. These ESUs are also predicted to occur in regions with the lowest percentage of protected areas (8 and 36%, respectively) where approximately half of the forest has already been lost (only 56 and 75% of the original forest remains, respectively).

DISCUSSION

Phylogenetic relationships and taxonomic implications

As Carstens *et al.* (2013) suggested, researchers investigating species delimitation should analyse their data using a wide range of methods and place their trust in the congruence across the results from different methods. The phylogenetic and species delimitation analyses performed in this study were fully concordant in strongly supporting reciprocal monophyly of at least three primary clades within *Hylexetastes*, which are allopatrically distributed in different interfluves and areas of endemism (AOEs) of the Amazon basin (Marantz *et al.* 2003, Da Silva *et al.* 2005): (1) Bar-billed Woodcreeper *H. stresemanni stresemanni* + *insignis* + *undulatus*; (2) Red-billed Woodcreeper *H. perrotii perrotii*; and (3) Uniform and Brigida's Woodcreepers *H. p. uniformis* 1 + *H. p. uniformis* 2 + *H. p. brigidai*.

Table 3. Potential area of occurrence (km^2) for each *Hylexetastes* ESU compared to land use within the Brazilian Amazon basin.

	H. s. undulatus	*H. s. stresemanni* + *H. s. insignis*	*H. p. perrotii*	*H. p. uniformis 2*	*H. p. uniformis 1*	*H. p. brigidai*
Total potential distribution	811 132	310 312	1 054 230	1 023 440	241 715	202 015
Area within the Brazilian Amazon Basin	545 115	219 988	580 606	1 007 736	241 715	202 015
Forest	477 946 (88%)	87 732 (40%)	488 114 (84%)	433 258 (43%)	181 199 (75%)	112 298 (56%)
Deforestation	289 (0.05%)	0	217 (0.04%)	1374 (0.14%)	508 (0.21%)	313 (0.15%)
Urbanized	501 (0.09%)	247 (0.11%)	370 (0.06%)	1093 (0.10)	235 (0.1%)	360 (0.18%)
Reforestation	0	0	743 (0.13%)	82 (0.01%)	0.001 (0.0%)	88 (0.04%)
Secondary vegetation	7834 (1.43%)	1262 (0.57%)	9400 (1.62%)	35 075 (3.48%)	9441 (3.9%)	13 193 (7%)
Under protection	124 284 (23%)	34 222 (16%)	255 002 (44%)	133 997 (13%)	85 856 (36%)	16 021 (8%)

Divergence time estimates based on the species tree indicate that the first differentiation event in *Hylexetastes* probably occurred during the Pliocene, which is congruent with previous temporal inferences for early differentiation events within other Dendrocolaptidae species complexes (Derryberry *et al.* 2011, Rocha *et al.* 2015). These events are also spatio-temporally congruent with those estimated for other Amazonian endemic bird lineages such as *Psophia* (Psophiidae), which has taxa distributed in each area of endemism of the Amazon basin (Ribas *et al.* 2012).

A clade containing Red-billed Woodcreeper specimens, distributed in northeastern Amazonia (Guiana AOE), is most closely related to a clade of Bar-bellied Woodcreeper specimens rather than other specimens currently assigned to Uniform Woodcreeper and Brigida's Woodcreeper (Marantz *et al.* 2003). Furthermore, Red-billed Woodcreeper exhibit high pairwise genetic distances (≥ 5%) from all the other lineages in the genus. Although some authors (e.g. Halley *et al.* 2017) consider the 'yard-stick approach' (comparison of the genetic distances between clades) to be subjective for the delimitation of allopatric species, the level of differentiation found between Red-billed Woodcreepers and all other *Hylexetastes* lineages is even higher than estimates for other long and well-established Dendrocolaptidae species, such as the Chestnut-rumped Woodcreeper *Xiphorhynchus pardalotus* and the Ocellated Woodcreeper *Xiphorhynchus ocellatus* (which diverge by 3.3%; Sousa-Neves *et al.* 2013). Thus, these results also support Red-billed Woodcreeper as an independent species.

Three clades, one grouping Brigida's Woodcreeper and another grouping two Uniform Woodcreeper clades (*H. p. uniformis* 1 and *H. p. uniformis* 2), are distributed in specific interfluves of the southeastern Amazon basin and central Amazonia south of the Amazon River. These clades exhibit low pairwise genetic distances (< 1.6%) and a recent diversification time (< 0.49 Mya). However, genetic structuring between them is significant. Moreover, besides geographical and genetic differences, subtle morphological differences have also been reported between these clades (Marantz *et al.* 2003, Piacentini *et al.*). For example, Brigida's Woodcreeper differs from Uniform Woodcreeper in the pattern of coloration of the chin, throat and underwing-coverts (Fig. 1; Da Silva *et al.* 1995). Accordingly, BPP analyses suggest that the subclades *H. p. uniformis* 1, *H. p. uniformis* 2 and Brigida's Woodcreeper are three evolutionary distinct lineages. These groups are reciprocally monophyletic when mtDNA is analysed, and exhibit significant genetic structure for one nuclear gene (FGB5; $F_{ST} = 0.122$; $p = 0.012$). However, these lineages have the most recent divergence time within the genus *Hylexetastes* (< 0.48 Mya) and the lowest levels of pairwise genetic differentiation, and lack support for their reciprocal monophyly according to the multilocus coalescent species tree analyses. Furthermore, subtle phenotypic differences between these clades reinforce the notion that they should only be considered ESUs rather than distinct biological species, based on current data (Ryder 1986, Moritz 1994). However, under a lineage-based species concept (De Queiroz 2007), *H. p. uniformis* 1, *H. p. uniformis* 2 and Brigida's Woodcreeper could be considered separate evolutionary species, with the caveat that no available taxon name can be applied to *H. p. uniformis* 2 at this time. However, it is important to note that despite our BPP results having supported *H. p. uniformis* 1, *H. p. uniformis* 2 and Brigida's Woodcreeper as separate 'species', recent data suggest that BPP results might not translate directly into species limits, providing instead only a proxy of phylogeographical structure, which may or may not be accompanied by interspecific differentiation (Sukumaran & Knowles 2017). In this sense, it is critical to contrast BPP results with other sources of evidence related to interspecific limits, such as plumage and vocal characters, which are important traits involved in mate choice among birds in general. Plumage differences between *H. p. uniformis* clades and *H. p. brigidai* are very subtle and alongside their comparatively smaller levels of genetic divergence, these data seem more consistent with recognition of only a single species. Future studies aimed at quantifying levels of gene flow among these lineages with next-generation sequencing might shed more light on their interspecific limits. With respect to vocal characters, our field experience and preliminary exploratory analyses of dozens of personal and web-based recordings (see Xeno-canto recordings in Table S1) seem to indicate that no apparent conspicuous and consistent differences exist among vocalizations of all *Hylexetastes* taxa/ESUs recovered herein (see also Marantz *et al.* 2003); therefore these characters cannot used to delimit species in this genus until further data become available.

Divergence time estimates indicate that the diversification of Bar-bellied Woodcreepers, distributed across the western part of the Amazon basin, is also recent (< 0.75 Mya). The small sampling of specimens in this clade prevents us from rigorously assessing monophyly of its subspecies and thus does not allow for a more detailed taxonomic evaluation. For instance, the major split observed in this clade separated southern (*H. s. undulatus*) from northern (*H. s. stresemanni* + *H. s. insignis*) populations distributed across the upper Amazon (Solimões) River, but only three specimens from two widely scattered localities separated by ca. 500 km of the northern population were sampled, covering a very small part of the range of the northern populations of Bar-bellied Woodcreepers. Moreover, despite BPP indicating that *H. s. undulatus* and *H. s. stresemanni*/*H. s. insignis* are strongly supported independent lineages, these two subclades have a low uncorrected pairwise genetic distance (0.2–1.3%), which, along with their overall morphological similarity, suggest that they may belong to the same biological species, as supported by current taxonomy (Marantz *et al.* 2003). As discussed above for the Brigida's and Uniform Woodcreepers, the BPP results should not necessarily be translated into interspecific limits (Sukumaran & Knowles 2017). On the other hand, our results strongly support the existence of at least two evolutionary lineages in Bar-bellied Woodcreepers (*H. s. undulatus* and *H. s. stresemanni* + *H. s. insignis*), which could also be considered evolutionary species (De Queiroz 2007). We recommend that future studies sample populations of the Bar-bellied Woodcreeper more thoroughly, in terms of both specimens and genetic loci, to better define interspecific limits among its taxa.

We outline a new taxonomic arrangement for *Hylexetastes*, with recognition of at least three independent biological species (De Queiroz 2007): Bar-billed Woodcreeper *H. stresemanni* (including *H. s. stresemanni*, *H. s. insignis* and *H. s. undulatus*); Red-billed Woodcreeper *H. perrotii* (monotypic); and Uniform Woodcreeper *H. uniformis* (including *H. u. uniformis* and *H. u. brigidai*). It is possible that more detailed sampling across the distribution of Uniform Woodcreeper would reveal that the three shallowly diverged and reciprocally monophyletic mitochondrial subclades within this taxon are in fact two or even three separate species. Alternatively, our data also consistently support at least five evolutionary species (*sensu* De Queiroz 2007) in the genus *Hylexetastes*: *H. stresemanni* (including populations attributed to *H. s. insignis*, which may constitute a separate evolutionary species, pending analyses of additional samples), *H. undulatus*, *H. perrotii*, *H. uniformis* (including *H. p. uniformis* 2, which may also constitute a separated, unnamed evolutionary species, pending analyses of a larger number of genetic loci) and *H. brigidai*.

Species distribution and conservation

Effective conservation policies must be based on a variety of data related to a given species and its distribution. For this reason, scientists have employed novel approaches such as ecological niche-based models (ENMs) to predict the effects of anthropogenic alterations of the landscape on the distribution of species. This task requires models with reliable levels of predictive power, as well as minimum risk and low uncertainty (De Barros Ferraz *et al.* 2012, Rangel & Loyola 2012, Hipólito *et al.* 2015).

In this study, the ENM identifies areas of occurrence that follow the general distribution patterns previously documented for each of the three species and six lineages/ESUs of *Hylexetastes* recognized herein (Marantz *et al.* 2003, Piacentini *et al.* 2015). The strong association of these species and ESUs with *terra firme* forest is indicated by the congruence of the areas of potential distribution of each species with forested areas in Amazonia (Fig. 3c,d). Besides climatic factors, historical and ecological factors (such as the presence of barriers to dispersal and interspecific competition, respectively) might also have contributed to defining the distribution patterns of these species, although they were not taken into consideration by this ENM (Franklin 2010, Rangel & Loyola 2012, Ribas *et al.* 2012). The geographical distribution of *Hylexetastes* lineages/ESUs might constitute a case of parapatric ranges of closely related taxa delimited by the major Amazonian rivers (see Ribas *et al.* 2012 and Rocha *et al.* 2015 for other examples). Rivers appear to be effective barriers for lineages of birds restricted to the upland *terra firme* forest, distributed away from rivers and flooded forests (Marantz *et al.* 2003, Da Silva *et al.* 1995). However, Amazonian rivers differ in length, width and underlying geological history, and some, such as the Teles Pires River, may represent comparatively weaker faunal barriers for some species of

birds (Bates *et al.* 2004, Weir *et al.* 2015). This could explain the low and recent genetic divergence between *H. uniformis* lineages (identified in our analyses as *H. p. uniformis* 1 and *H. p. uniformis* 2), which are distributed on opposite banks of the Tapajos River and Teles Pires River headwaters.

Our analyses confirm that the area of occurrence of *H. u. brigidai* is restricted to the Xingu AOE, which is the second most deforested AOE of Amazonia (Da Silva *et al.* 2005, Moura *et al.* 2013, Barlow *et al.* 2016, INPE 2017). Approximately 20% of the forest remaining in the Xingu area is under protection but 27% of forest in this area has already been lost (Da Silva *et al.* 2005). However, only 56% of the predicted area of occurrence of *H. u. brigidai* remains forested, with only 8% of this area currently under protection and more than 7% already affected by deforestation and other habitat conversion. Currently, *H. u. brigidai* is not recognized as a species by BirdLife International or the IUCN, but our results support the need to evaluate the degree of vulnerability of this taxon, which constitutes a distinctive ESU endemic to the Brazilian Amazon Basin. Alternatively, our results also support the current treatment of *H. brigidai* as an independent evolutionary species by some sources (Da Silva *et al.* 1995, Piacentini *et al.* 2015, Gill & Donsker 2017), which highlights the need to evaluate its conservation status separately from other *Hylexetastes* taxa, particularly considering the current degree of forest disturbance in the Xingu area of endemism and the comparatively small range occupied by this taxon (Table 3).

Finally, both *H. u. uniformis* ESUs are confined to the Tapajós and Rondônia AOEs to the west of *H. u. brigidai*, of which at least 9–12% is now deforested (Da Silva *et al.* 2005). About 95% of the potential area of occurrence of *H. u. uniformis* is within the Brazilian Amazon Basin and thus only 63% of its predicted area is likely to be habitable, 22% is under protection and more than 4% is already affected by habitat conversion. Deforestation and expansion of plantations in the Amazon basin are continuously increasing (INPE 2017) and the region presents a high number of bird species and population losses (Ceballos *et al.* 2017). As the distributions of both Uniform Woodcreeper clades overlap with some of the most deforested regions in the Amazon and avian species have a high global extinction rate due to anthropogenic actions (Pimm *et al.* 2014), the conservation status of both lineages/ESUs should be monitored separately and continuously (Ryder 1986, Moritz 1994, Haig *et al.* 2006), maximizing the likelihood that the species and its meta-populations will persist into the future (Hey *et al.* 2003).

CONCLUSIONS

We demonstrate the importance of the integration of several methods to provide an accurate identification of ESUs and species (De Queiroz 2007, Carstens *et al.* 2013) for the woodcreeper genus *Hylexetastes*. The correct delimitation of these units more accurately reflects patterns of biotic diversity and provides critical data for informed conservation planning. Our genetic analyses and ENMs (both potential parapatric geographical distributions and distinct climatic variables influencing these distributions) coincide with previously published morphological descriptions to support the recognition of at least three species and six ESUs within *Hylexetastes* (Da Silva *et al.* 1995, Piacentini *et al.* 2015). The evidence presented herein supports a new taxonomic arrangement for this genus, elevating the Uniform Woodcreeper to full species status, which may or may not include Brigida's Woodcreeper depending on the species concept adopted. Irrespective of the taxonomic rank assigned to the Brigida's Woodcreeper (which is dependent on adopted species concepts), our analyses demonstrated that this lineage represents a distinct ESU under the highest level of threat in the entire genus, highlighting the need for continuous conservation actions towards this taxon. Likewise, the same applies to both ESUs recognized in the Uniform Woodcreeper, whose distributions also overlap with other severely deforested sectors of Amazonia within the entire range of the *Hylexetastes* genus.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Summary of records of *Hylexetastes* specimens used in this study for genetic and ecological niche modelling analyses.

Table S2. List of primers and general PCR conditions used for each gene amplified and sequenced.

Table S3. Best partitions and models of nucleotide substitution for mitochondrial and nuclear genes based on the Bayesian information criterion (BIC) and analysis in PartitionFinder.

Table S4. Summary statistics of the genes sequenced for all *Hylexetastes* taxa.

Table S5. Genetic structure (F_{ST}) between *Hylexetastes* clades.

Table S6. Average and standard deviation of training and test AUC of each Maxent model for *Hylexetastes*.

Table S7. Contribution of each environmental variable to the distribution models for the *Hylexetastes* complex.

Figure S1. Phylogenetic tree of the genus *Hylexetastes* estimated using Bayesian inference of concatenated mtDNA genes (C*ytb* and ND2) for 57 terminal taxa.

Figure S2. Phylogenetic tree of the genus *Hylexetastes* obtained using Bayesian inference of three concatenated nuclear genes (FGB5, G3PDH and MUSK) for 45 terminal taxa.