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Biogeography and spatio-temporal diversification of *Selenidera* and *Andigena* Toucans (Aves: Ramphastidae)

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ABSTRACT

Andean uplift, Plio-Pleistocene climatic fluctuation, and river dynamics in the Amazon basin have all been implicated in the diversification of the South American avifauna. We reconstructed phylogenetic relationships in the genus *Selenidera*, which has served as a classic case of putative refugial speciation, and the closely related genus *Andigena*, to better understand the processes driving their diversification. Using mitochondrial and nuclear DNA sequences, we constructed a phylogeny to estimate the pattern and timing of divergence within and between seven lowland *Selenidera* toucanets and the five species of *Andigena* mountain-toucans, which together form a single clade. All phylogenetic analyses supported the monophyly of the montane genus *Andigena*, but indicated that the genus *Selenidera* is likely paraphyletic with respect to *Andigena*. Our time tree analysis is consistent with the orogenic uplift of the northern Andean range having initiated the divergence between *Selenidera* and *Andigena*, and that the movement and fragmentation of montane habitats in response to Pleistocene climatic oscillations likely influenced diversification within *Andigena*. Estimated divergence times for lowland Amazonian *Selenidera* did not support the Last Glacial Maximum (LGM) refuge hypothesis as an important biogeographic factor for the diversification of lineages studied here. The timing of divergence within *Selenidera* is consistent with the hypothesis that geographic isolation of areas of endemism generated by Amazonian river dynamics during the Plio-Pleistocene contributed to *Selenidera* speciation and current species distributions.

1. Introduction

Analyses of DNA sequence data have demonstrated that the mechanisms responsible for the immense biodiversity found in Neotropical humid forests are complex, and may include orogeny, glacial cycling, river formation, and dispersal, depending on the focal taxonomic group (e.g. Burney and Brumfield, 2009; Fuchs et al., 2011; Ribas et al., 2007; Weir and Price, 2011). Studies of the distributions of South American birds have identified regional areas of endemism for many different taxa (Bates et al., 1998; Cracraft, 1985; Fig. 1a). Multiple biogeographic models have been implicated in the formation of these endemic areas, beginning with Haffer (1969), who hypothesized that these distribution patterns were caused by allopatric speciation due to the isolation of widespread populations in Plio-Pleistocene forest refugia. Another model of vicariant speciation suggests that major rivers formed during the Plio-Pleistocene induced speciation by similarly dividing formerly connected populations into areas of endemism (e.g. Patel et al., 2011; Ribas et al., 2012; Wallace, 1889). Previous studies have used species distributions (Bates et al., 1998; Borges, 2007) and morphological phylogenies (Cracraft and Prum, 1988; Prum, 1988) to define areas of endemism, reconstruct area relationships and discuss the process of formation of these areas (e.g. Bates, 2001). Molecular phylogenies and timing analyses also have also been used to test hypotheses of vicariant speciation and dispersal in Andean lineages (Gutiérrez-Pinto et al., 2012; Miller et al., 2007; Quintero et al., 2012). In the past decade, molecular phylogenetic data and analyses have provided the most powerful comparative data to assess the relative importance of competing ecological and evolutionary factors in shaping Neotropical diversity (e.g. Armenta et al., 2005; Burney and Brumfield, 2009; Chaves et al., 2011; Eberhard and Bermingham, 2005; Marks et al., 2002; Patel et al., 2011; Ribas et al., 2012; Weckstein, 2005; Weir and Price, 2011).

We gathered and analyzed molecular phylogenetic data to both test relationships and estimate the timing of diversification events in two genera of toucans, one lowland (*Selenidera*; toucanets) and one highland (*Andigena*; mountain-toucans). *Selenidera* species

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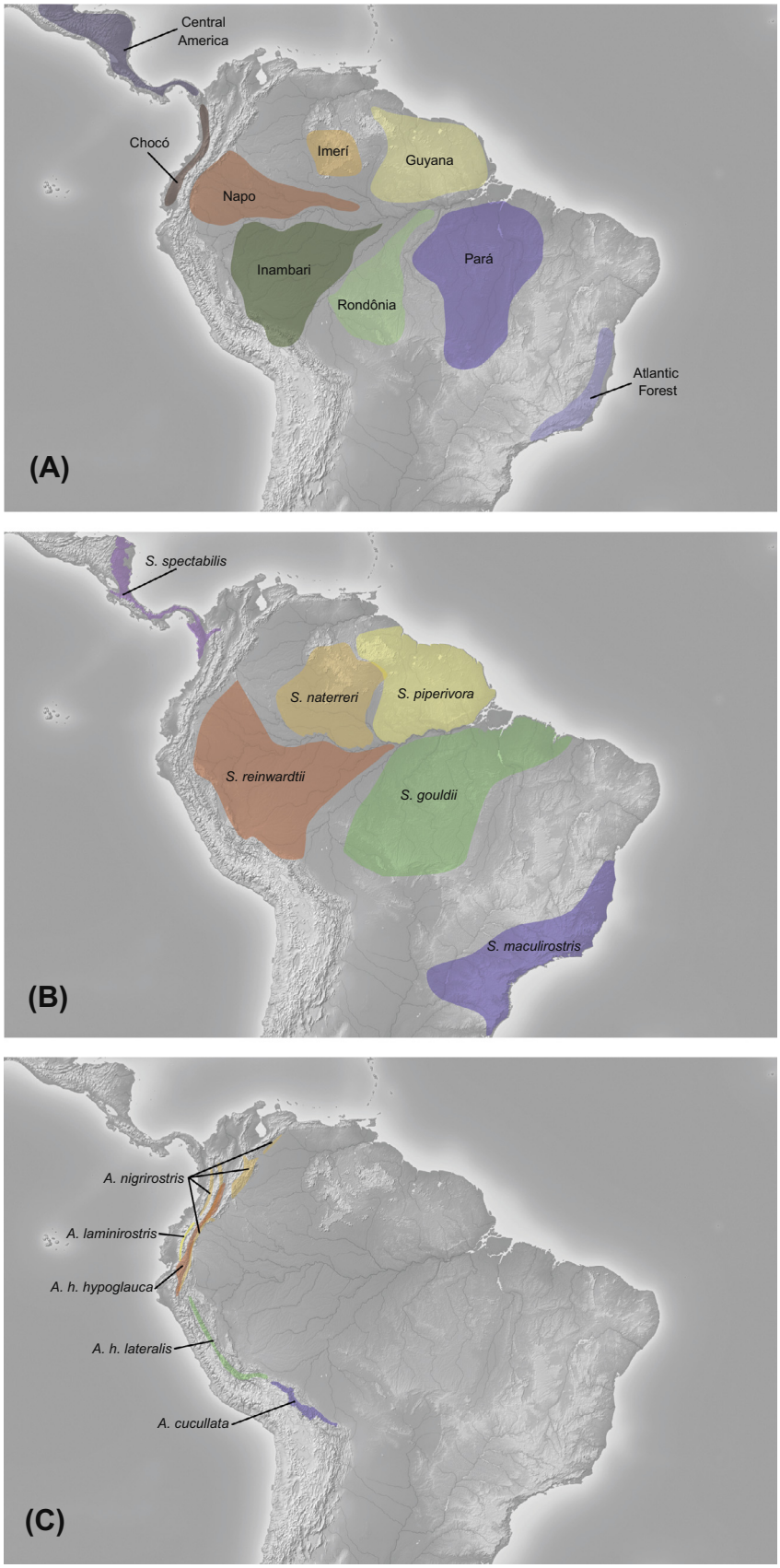

Fig. 1. Approximate distributions of (A) Amazonia areas of endemism, (B) *Selenidera* species, and (C) *Andigena* species.

are allopatrically distributed from the lowlands of Honduras south to Argentina and have been a model group for the study of lowland Neotropical diversification (Cracraft and Prum, 1988; Haffer, 1974; Prum, 1988). As *Selenidera* species are distributed according to

Table 1
Study taxa and localities.

	Species	Common name	Locality	Source	ID number	Genbank accession number			
						Cyt b	ND2	COI	βFib7
Ingroup									
1	*Selenidera spectabilis*	Yellow-eared Toucanet	Panama: Darién	LSUMNS	B2120	KF424576	KF424567	KF424558	KF424587
2	*Selenidera piperivora*	Guianan Toucanet	Brazil: Pará	LSUMNS	B35643	KF424577	KF424568	KF424559	KF424588
3	*Selenidera reinwardtii langsdorffii*	Gold-collared Toucanet	Peru: Loreto	LSUMNS	B27756	AY959829[a]	AY959856[a]	AY959802[a]	HQ424122[b]
4	*Selenidera reinwardtii reinwardtii*	Gold-collared Toucanet	Ecuador	LSUMNS	B4164	KF424578	NA	NA	NA
5	*Selenidera nattereri*	Tawny-tufted Toucanet	Brazil: Amazonas	LSUMNS	B25399	KF424579	KF424569	KF424560	KF424589
6	*Selenidera gouldii*	Gould's Toucanet	Brazil: Mato Grosso	LSUMNS	B35413	KF424580	KF424570	KF424561	KF424590
7	*Selenidera gouldii*	Gould's Toucanet	Brazil: Rondonia	FMNH	FMNH389772	KF424581	KF424571	KF424562	KF424591
8	*Andigena hypoglauca hypoglauca*	Grey-breasted Mountain Toucan	Peru: Cajamarca	LSUMNS	B32020	KF424582	KF424572	KF424563	KF424592
9	*Andigena hypoglauca lateralis*	Grey-breasted Mountain Toucan	Peru: Pasco	LSUMNS	B8309	KF424583	KF424573	KF424564	KF424593
10	*Andigena laminirostris*	Plate-billed Mountain Toucan	Ecuador: Pichincha	LSUMNS	B7777	KF424584	KF424577	KF424565	KF424595
11	*Andigena nigrirostris spilorhynchus*	Black-billed Mountain Toucan	Peru: Cajamarca	LSUMNS	B32513	KF424585	KF424575	KF424566	KF424595
12	*Andigena cucullata*	Hooded Mountain Toucan	Bolivia: La Paz	LSUMNS	B1273	KF424586	AY959855[a]	AY959801[a]	KF424596
13	*Selenidera maculirostris*	Spot-billed Toucanet	Captive	FMNH		AF100552[c]	NA	NA	NA
Outgroup									
14	*Aulacorhynchus prasinus atrogularis*	Emerald Toucanet	Peru: Madre de Dios	LSUMNS	B21201	GQ457999	HQ424067	GQ457981	HQ424095
15	*Aulacorhynchus prasinus caruleogularis*	Emerald Toucanet	Panama: Chiriqui	LSUMNS	B26403	HQ424041	HQ424091	GQ457982	HQ424096
16	*Semnornis frantzii*	Prong-billed Barbet	Costa Rica: Heredia	LSUMNS	B16019	GQ458000	GQ458014	GQ457983	HQ424124
17	*Semnornis ramphastinus*	Toucan Barbet	Ecuador: Pichincha	LSUMNS	B7771	GQ458001	GQ458015	GQ457984	HQ424125
18	*Ramphastos vitellinus ariel*	Channel-billed Toucan	Brazil: Sao Paulo	LSUMNS	B35555	AY959837	AY959864	AY959810	AY279259
19	*Pteroglossus bailloni*	Saffron Toucanet	Paráguay: Caazapa	LSUMNS	B25891	AY959826	AY959853	AY959799	AY279262
20	*Pterglossus inscriptus*	Lettered Aracari	Bolivia: Pando	LSUMNS	B8819	AY959827	AY959854	AY959800	AY279261

[a] Weckstein (2005).
[b] Patel et al. (2011).
[c] Nahum et al. (2003).

Table 2
Characteristics of mitochondrial and nuclear DNA gene sequences.

Gene	Total (bp)	Constant (%)	Variable uninformative (%)	Parsimony informative (%)
Cytochrome *b*	1048	62.5	9.8	27.7
COI	379	71.5	7.7	20.8
ND2	1041	55.2	12.5	32.2
βFib7	747	88.8	11.2	6.4
Total	3215	67.3	10.7	23.4

well-established areas of Neotropical endemism (Atlantic forest, Pará, Rondônia, Inambari, Guyana, Imerí, Napo, and the Central America/Chocó region; Fig. 1b), investigating the phylogenetic relationships and divergence times within *Selenidera* serves as a good proxy for reconstructing the evolutionary history of these regions. Conversely, *Andigena* mountain-toucans (Fig. 1c) appear to have diversified entirely within the Andes. Here, we test whether diversification within *Andigena* was produced by vicariant Andean uplift events, as has been suggested for other avian taxa (e.g. Bonaccorso et al., 2011; Chaves et al., 2011; Weir and Price, 2011; Quintero et al., 2012), or whether diversification occurred *in situ* following Andean uplift.

We collected DNA sequence data for three mitochondrial genes and one nuclear intron to reconstruct the phylogenetic relationships within and between these genera, and then compared the molecular phylogenetic hypothesis for *Selenidera* with previously published hypotheses (Haffer, 1974) and morphological phylogenies (Cracraft and Prum, 1988). We compared the area relationships indicated by our molecular phylogeny with those published by Haffer (1974), Prum (1988), Silva and Oren (1996), and Bates et al. (1998), and compared the timing of diversification in this group to other recent studies of similarly distributed lineages (e.g. Patel et al., 2011; Ribas et al., 2012).

2. Material and methods

2.1. Taxonomic sampling

Ingroup sampling was nearly complete and included all *Selenidera* and *Andigena* species level taxa (Clements, 2007). We also included nearly all recognized *Selenidera* and *Andigena* subspecies and were missing only two subspecies, *A. n. nigrirostris* and *A. n. occidentalis*,

because tissues were unavailable for these taxa. We used seven outgroup taxa, including representatives of all other ramphastid genera. Two sister species pairs in the genera *Semnornis* and *Aulacorhynchus* were included to provide two outgroup calibration points for dating divergence times in the phylogeny (Table 1).

2.2. DNA extraction, amplification, and sequencing

We extracted DNA from frozen tissues for 20 taxa (13 ingroup and seven outgroup) using the DNeasy extraction kit (Qiagen, Valencia, California). We sequenced a total of 3,215 base pairs from three mitochondrial genes: cytochrome oxidase I (COI) (379 bp), cytochrome *b* (Cyt*b*) (1048 bp), and NADH dehydrogenase subunit 2 (ND2) (1041 bp), and one nuclear intron, the β-fibrinogen Intron 7 (βfib7) (747 bp) (Table 2). Individual regions were amplified via the polymerase chain reaction. Primers and thermal cycling conditions were the same as those used by Patel et al. (2011), with the exception of modified internal primers designed to more specifically amplify ND2 in *Selenidera* and *Andigena* (H5776TOUC22: 5′-GGTGGGAGATGGARGAGAAGGC-3′ and H6307: 5′-CTCTTATTTA AGGCTTTGAAGGCCTYCGG-3′). PCR products were purified using a standard gelase protocol (Epicentre Technologies, Madison, Wisconsin). Purified DNA was cycle-sequenced using ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin-Elmer); sequencing parameters followed manufacturer's recommendations. Samples were sequenced with an ABI 3730 Automated DNA sequencer (Applied Biosystems, Foster City, California). Double-stranded sequences were reconciled and aligned using Sequencher v.4.8 (GeneCodes, Ann Arbor, Michigan). Protein-coding partitions were translated into amino acids to search for stop codons and to confirm the functionality of the amplified gene. All new sequences were deposited in GenBank (accession numbers, pending acceptance).

2.3. Phylogenetic analysis

We created a sequence alignment consisting of 3215 base pairs from three mitochondrial genes (COI, Cyt*b*, ND2) and one nuclear gene (βfib7). For one taxon, *S. r. reinwardtii*, we sequenced only Cyt*b* and for another, *S. maculirostris*, the only sequence available was GenBank accession number AF100552 (also Cyt*b*) from Nahum et al. (2003). To test for incongruence between COI, Cyt*b*, ND2, and βfib7 data partitions we performed three partition homogeneity tests (ILD test; Farris et al., 1995) as implemented in PAUP* v4.0b10 (Swofford, 2003), one among the three mtDNA genes, one between the combined mtDNA genes and βfib7, and one between all four genes, using 1000 partition homogeneity replicates for each test. We also used PAUP* v4.0b10 to calculate uncorrected p-distances. We constructed phylogenies using maximum parsimony (MP) as implemented in PAUP* v4.0b10 (Swofford, 2003), maximum likelihood (ML) as implemented in Garli 2.0 (Zwickl, 2006), and Bayesian inference (BI) as implemented in BEAST 1.7.2 (Drummond and Rambaut, 2007).

For MP analyses, all characters were treated as unordered and equally weighted. We performed a heuristic search with 100 random addition replicates using tree bisection reconnection (TBR) branch-swapping. We bootstrapped these data using 1000 heuristic search replicates with 10 random additions per replicate and TBR branch-swapping (Felsenstein, 1985). To assess the relative contribution of the βfib7 data to the combined phylogenetic estimate we also conducted MP and MP bootstrap analyses (100 heuristic search replicates with 1 random addition replicate, TBR branch-swapping and max trees set at 100 per replicate) on this partition of the data alone.

For ML and BI analyses we used jModeltest v0.1.1 (Posada 2008) to determine the best-fit models of molecular evolution, according to the BIC criterion, for a total of 22 alignment subsets including: the whole alignment (1), each of the four genes (4), each codon position across the mtDNA alignment (3), each codon position by gene (9), codon first plus second positions (by gene and the whole mtDNA) (4), and the whole mtDNA subset (1). We assumed the sample size (n) as being the number of sites of each of the subset alignments.

Given the best-fit model for each of those subsets, we ran Garli v2.0 (default options) with seven different partitioning schemes: the whole alignment (1 partition), by genome type (2 partitions: mtDNA and βFib7), by gene (4 partitions: COI, Cyt*b*, ND2, and βFib7), by codon position (4 partitions: mtDNA 1st codon position, 2nd codon position, 3rd codon position, and βFib7), by gene and codon position (10 partitions: COI 1st codon position, COI 2nd codon position, COI 3rd codon position; Cyt*b* 1st codon position, Cyt*b* 2nd codon position, Cyt*b* 3rd codon position, ND2 1st codon position, ND2 2nd codon position, ND2 3rd codon position, and βFib7), by codon 1st + 2nd vs. 3rd position (3 partitions: mtDNA 1st and 2nd codon positions, mtDNA 3rd codon positions, and βFib7), and by gene and codon 1st + 2nd vs. 3rd position (7 partitions: COI 1st and 2nd codon positions, COI 3rd codon position; Cyt*b* 1st and 2nd codon positions, Cyt*b* 3rd codon position, ND2 1st and 2nd codon positions, ND2 3rd codon position, and βFib7). Then we used BIC (calculated in an Excel spreadsheet to account for the correct number of parameters on each partitioning scheme) to choose the best-fit partitioning scheme. We ran five independent replicates of the ML search in Garli 2.0 (Zwickl, 2006) for each partitioning scheme, each with a different starting point and chose the tree with the lowest log-likelihood score as the best ML tree, with the values of parameters that best fit the data being estimated during the analysis. ML bootstraps were calculated in Garli using 1000 pseudoreplicates assuming only the best-fit partitioning model.

For the BI we used the same partitioning scheme chosen for ML above. The clock.rate parameter was fixed as 1.0 across partitions (meaning relative rates across partitions have mean equal to 1.0), so that branch lengths are measured in units of substitutions per site. Two runs were performed in BEAST for 50,000,000 generations, or until each parameter in both runs exceeded an effective sample size (ESS) of 200. The results were compared across runs using Tracer v1.5 (Rambaut and Drummond, 2007), to check for convergence of the MCMC chains.

2.4. Geologic and timing analyses

We used a Bayesian MCMC approach to estimate divergence times using BEAST v1.7.4 (Drummond and Rambaut, 2007) by combining information from calibrating splits in the tree and rates of molecular evolution using a concatenated data approach. We calibrated the age of splits between two *Semnornis* species (*S. frantzii* and *S. ramphastinus*) and representatives from two *Aulacorhynchus prasinus* clades from highlands north and south of the Isthmus of Panama (*A. p. atrogularis* and *A. p. caeruleogularis*). We assume that establishment of taxa north and south of the isthmus could not have occurred before the uplift of the Talamanca highlands of Costa Rica and western Panama, and use this uplift at about 4.5 Mya (Abratis and Worner, 2001; Grafe et al., 2002) as a maximum age for divergence of the two outgroup calibration points. One taxon from each of these outgroup pairs used for calibration is endemic to the South American highlands, whereas the other is from the Central American highlands. Thus, we were able to use the uplift of the Central American Talamanca Highlands as a soft maximum age of the split for both the *Semnornis* and *Aulacorhynchus* taxa. The 95% soft maximum of 4.5 Mya was implemented using an exponential distribution, and fixing at 0.0 Mya as a hard minimum. This distribution also incorporates

the timing reported for isthmus formation, another important biogeographic event in the region. In concert with this geological calibration we also applied a rate calibration based on Weir and Schlueter's (2008) analysis of empirical data from birds (see below).

The tree prior was a Birth–Death process (Gernhard, 2008) with default values. The data were partitioned according to the best-fit ML scheme as explained above. Model parameters for each partition were estimated during the BEAST run using a Gamma (0.05, 10) distribution for the relative rate parameters, an Exponential (0.5) for the alpha parameter of a discrete gamma distribution of rates (with four categories) across sites, and a Uniform [0.0, 1.0] distribution for the proportion of invariant sites. We used independent reversible models (the ones chosen by jModelTest, as explained above) for each partition (for a total of four partitions) and different uncorrelated lognormal distribution of rates (Drummond et al., 2006) to estimate absolute evolutionary rates and divergence times. To do that, first we fixed a broad prior for the ucld.mean (0.0105; Weir and Schluter, 2008) and ucld.stdev (0.8326, in log scale) parameters across partitions, which corresponds to a 95% interval of [0.3, 7.6] s/s/Mya (therefore permitting rates 7× lower and 3× higher than the average rate for mtDNA in Neotropical birds, 2.1 s/s/Mya; Weir and Schluter, 2008). Then we set the estimation of relative rates across partitions by fixing their weighted average to 1.0. After the run was completed, we multiplied the mean value of each of those relative rates by the mean of the "meanRate" parameter obtained in the run, and set those values obtained respectively as the ucld.mean parameters for each of the partitions in a subsequent run. Therefore this second run had four lognormal distributions of rates, one per partition, rather than the single lognormal distribution across all partitions applied during the first run. In this second run, we fixed ucld.stdev = 0.2 (which has an almost symmetric lognormal shape) for all partitions. We performed two of these runs to estimate divergence dates, with both including one cold chain plus three hot chains, for a total of 50,000,000 generations each, starting from different random points, and compared the results to check for convergence of the chains.

Divergence dates were obtained by initially removing the burnin of the tree files visually with the aid of Tracer v1.5 (Rambaut and Drummond, 2007), and then the final tree with dates was summarized using TreeAnnotator v1.7.4 (Drummond and Rambaut, 2007), using the "maximum clade credibility tree" option (which is the product of all clade posterior probabilities), and fixing node heights using the "mean heights" option. Divergence time bars were obtained automatically in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) from the output using the 95% highest posterior density (HPD) of the ages for each node. For each BEAST run, we also independently ran a "prior run" (without the DNA sequence alignment), to test whether the data was informative for estimating divergence dates (i.e., whether or not the posterior including data had narrower intervals and/or different mean values than the posterior without the data; Drummond et al., 2006).

We reconstructed the biogeographic transitions between lowland and highland areas using both MP and ML ancestral character state reconstruction methods implemented in Mesquite (version 2.75; Maddison and Maddison, 2011). We scored extant taxa as 0 for lowland and 1 for highland and used unordered MP, and also a one rate Mk1 model under a ML framework.

3. Results

3.1. Sequence attributes

The combined mtDNA and nuclear sequence data included 2,163 constant, 300 variable, and 752 parsimony informative characters. For ingroup taxa, uncorrected sequence divergence ranged from 0.3–10.1% for Cyt*b*, 0.0–9.2% for COI, 0.3–11.4% for ND2, and 0.0–2.8% for βFib7. Comparison across all four genes revealed no identical sequences between individual specimens, and thus all were included in the phylogenetic analyses. The results of all three ILD tests were non-significant (three mtDNA genes: $P = 0.99$; combined mtDNA genes vs. βfib7: $P = 0.56$; all four genes: $P = 0.98$) so we could not falsify the hypothesis that the four partitions share the same underlying phylogeny; therefore, concatenation of the data partitions for all of the subsequent analyses was justified.

3.2. Phylogenetic results

The maximum parsimony heuristic search found one most parsimonious tree (TL = 2237, CI = 0.589, RI = 0.600), in which *S. spectabilis* is sister to a clade of *Andigena* and *S. piperivora* is sister to all other *Selenidera* plus *Andigena*, rendering the genus *Selenidera* paraphyletic (tree not shown). However, the placement of *S. piperivora* is not well supported by bootstrapping, and there was marginal MP bootstrap support for the sister relationship between *S. spectabilis* and *Andigena* (62%).

The best-fit partitioning scheme determined by BIC for use in the ML, BI, and dating analyses was by codon (Table 3), with four partitions (mtDNA 1st codon position, 2nd codon position, 3rd codon position, and βfib7). We applied the following models (acronyms according to jModelTest): mtDNA 1st codon position: TPM2uf + G; mtDNA 2nd codon position: TPM1uf + G; mtDNA 3rd codon position: TIM1 + I + G; and βfib7: HKY + G, with the model for each of those data subsets determined previously by jModeltest v0.1.1.

The MP, ML, and BI topologies were nearly identical and differed in only two respects. First, the MP/BI topology and the ML topology differ in the relative placement of *A. nigrirostris* and *A. laminirostris*. In the MP/BI topology, *A. nigrirostris* and *A. laminirostris* are weakly supported as sisters (MP = 58%, BI = 0.59; Fig. 2), whereas in the ML topology *A. nigrirostris* is sister to *A. hypoglauca* and *A. cucullata* and *A. laminirostris* is sister to all *Andigena*. However, the relative position of *A. nigrirostris* with respect to *A. laminirostris* is not strongly supported by any of the analyses. Second, the MP, ML, and BI topologies differ in the placement of *S. piperivora* with respect to other members of *Selenidera* and *Andigena*. In this case, the MP topology has *S. piperivora* sister to *Andigena* plus the rest of *Selenidera*, but this is not supported by bootstrapping. ML and BI topologies have *S. piperivora* sister to all *Selenidera* to the exclusion of *S. spectabilis*, but with weak support (ML = 51%, BI = 0.84; Fig. 2).

Our analyses suggest that *Selenidera* is paraphyletic, with *S. spectabilis* sister to *Andigena*, and this is moderately to strongly supported (MP = 62%, ML = 62%, BI = 0.96; Fig. 2). The placement of *S. spectabilis* using the concatenated dataset is consistent with analyses run on the mtDNA-only data set. However, separate analysis of the nuclear βFib7 data provided little resolution with respect to this relationship. All phylogenetic reconstructions placed

Table 3
The Bayesian Information Criterion (BIC) calculated for each partitioning scheme. The best model for each data subset separately was determined by jModelTest (also using BIC).

Partition scheme	lnL	K[a]	N[b]	BIC
By codon	−13616.47	66	3215	27765.94
By gene and codon	−13532.91	101	3215	27881.45
By codon [1 + 2]	−13703.77	60	3215	27892.07
By gene and codon [1 + 2]	−13639.22	87	3215	27981.02
By genome type	−14605.65	52	3215	29631.22
By gene	−14546.98	67	3215	29635.03
All concatenated	−14850.63	45	3215	30064.66

[a] K = number of free parameters in the partitioned model.
[b] N = sample size (here assumed as total alignment size).

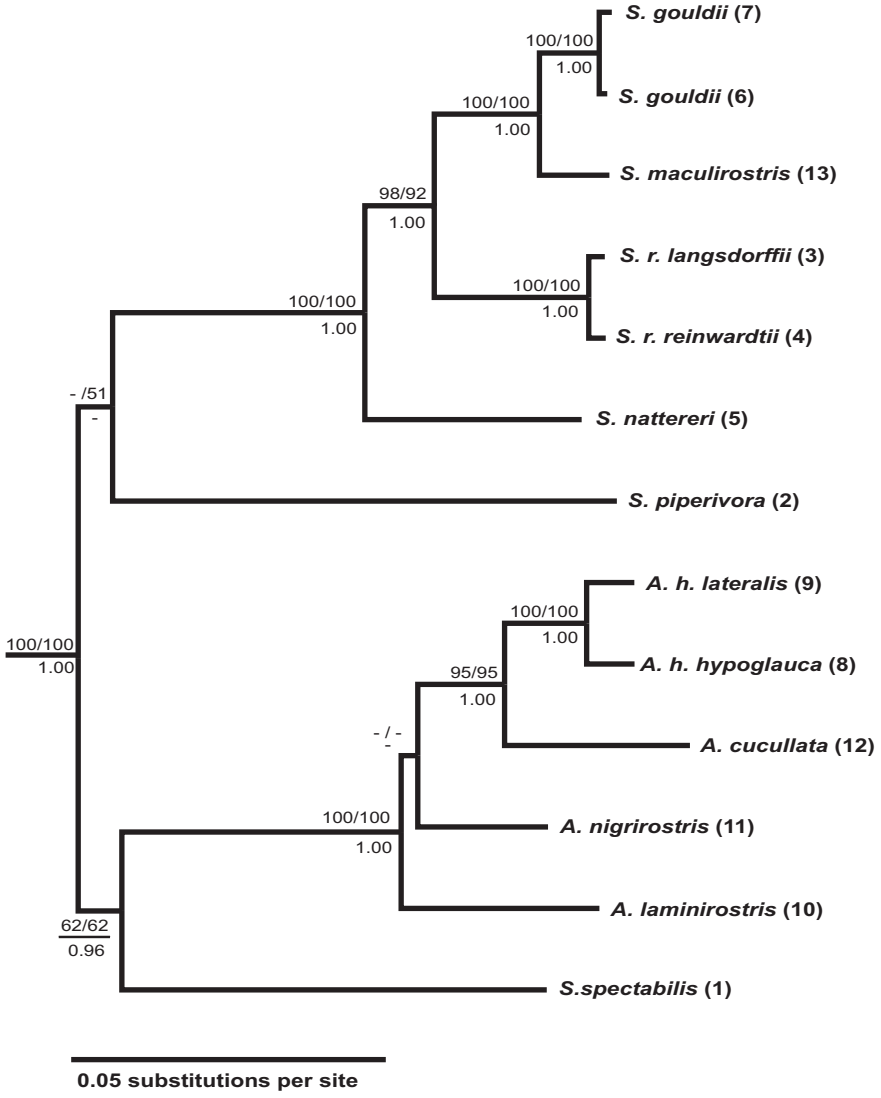

Fig. 2. Maximum likelihood phylogram. Values above nodes are maximum parsimony/maximum likelihood bootstrap values and those below nodes are Bayesian posterior probabilities.

S. nattereri as sister to a strongly supported clade (ML = 100%, MP = 100%, BI = 1.00) consisting of the following well-supported relationships: ((*S. r. reinwardtii* + *S. r. langsdorffii*) + (*S. gouldii* + *S. maculirostris*)).

Within the *Andigena* clade, *A. h. hypoglauca*, *A. h. lateralis*, and *A. cucullata*, the southern-most species, form a well supported clade (MP = 95%, ML = 95%, BI = 1.00) with the following relationships (*A. cucullata* + (*A. h. hypoglauca*, *A. h. lateralis*)). Separate analyses of the βfib7 data via MP (not shown) suggest that the phylogenetic relationships reconstructed here are mostly driven by the mtDNA data. In general MP bootstraps generated by the βfib7 MP analysis were below 50% for most of the ingroup. However, monophyly of *Andigena* was weakly supported by 55% MP bootstrap support and the remaining nodes within the *Andigena* clade were also weakly supported.

3.3. Timing and character analyses

All the prior-only runs of the BEAST analysis (i.e., without the alignment) had much broader date intervals than the BEAST analysis using the DNA sequence data alignment, and in most cases the mean divergence dates were different between the BEAST analyses with and without DNA sequence data (not shown). Thus, this comparison indicates that our DNA sequence data set is informative for estimating divergence dates (Drummond et al., 2006). In all cases, duplicate BEAST runs converged on very similar values, and thus for each type of analysis we log-combined the two tree files using LogCombiner v1.7.2 (Drummond and Rambaut, 2007). For all BEAST runs all ESSs were >200 and all parameters had reached stationarity.

The BEAST analysis (Fig. 3) indicates that the basal split between *Selenidera* lineages (excluding *S. spectabilis*), occurred between 7.3 and 10.1 Mya, whereas the basal split between *Andigena* species occurred between 3.2 and 4.6 Mya. The split between *cis*-Andean *Selenidera* and trans-Andean S. *spectabilis*/*Andigena* occurred between 8.1 and 10.8 Mya, and the split between Amazonian *S. gouldii* and Atlantic Forest *S. maculirostris* occurred between 0.7 and 1.8 Mya. Both MP and ML (model = Mk1) reconstructions of lowland/highland character states indicate a single transition from a lowland ancestral area to montane regions. On the time tree this transition occurred on the branch that dates between 3.2 and 9.9 Mya (Fig. 3).

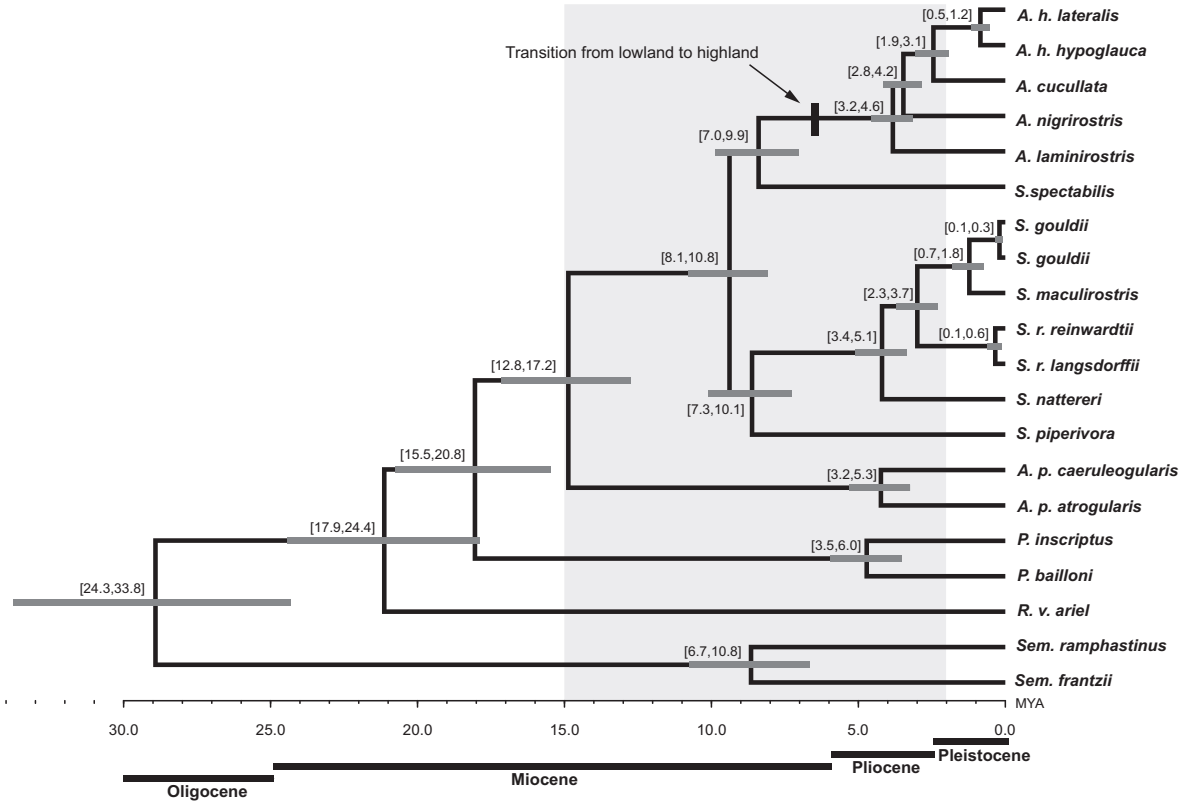

Fig. 3. BEAST chronogram. Horizontal gray bars represent 95% confidence intervals on the median estimates of the timing of maximum divergence events. Vertical gray shading indicates period during which divergences could be attributable to Andean uplift.

4. Discussion

4.1. Phylogenetic relationships

The toucan genera *Selenidera* and *Andigena* have long been considered prime examples of diversification in both the Andes (*Andigena*) and the Neotropical lowlands (*Selenidera*, Haffer, 1974). Our results suggest that *S. spectabilis,* the only species of these two genera found in the lowlands west of the Andes, is sister to the Andean genus *Andigena*, and therefore *Selenidera* is paraphyletic. Within *Selenidera*, our molecular phylogeny differed substantially from the morphological phylogeny published by Cracraft and Prum (1988; Fig. 4). We found a strongly supported sister relationship (ML = 100%, MP = 100%, BI = 1.00) between *S. nattereri* and the well supported clade consisting of *S. gouldii*, *S. maculirostris*, *S. r. reinwardtii,* and *S. r. langsdorffii* (ML = 98%, MP = 92%, BI = 1.00), whereas Cracraft and Prum (1988) placed *S. nattereri* as sister to a clade consisting of *S. r. reinwardtii, S. r. langsdorffii*, and *S. spectabilis* (Fig. 4b). Cracraft and Prum's (1988) placement of *S. piperivora* as most closely related to *S. gouldii* and *S. maculirostris* is also not supported by the molecular phylogenetic hypotheses presented here.

Diversification dates for the *Selenidera* and *Andigena* clades as a whole ranged from 0.1 to 10.8 Mya, upper Pleistocene to late Miocene. As indicated above, *S. spectabilis*, which is found in the lowlands west of the Andes from Colombia to Honduras, is sister to the Andean *Andigena*, rather than to the remaining *Selenidera,* which are all *cis*-Andean lowland species. The position of *S. piperivora*, a species distributed across the Guianan Shield, is either sister to *Selenidera* (excluding *S. spectabilis*) or sister to all other *Selenidera* and *Andigena*. Thus, the oldest splits in the *Selenidera*/*Andigena* tree appear concentrated in northern South America, including the splits between *S. spectabilis* from west of the Andes and the Andean *Andigena* clade, the split between (*S. spectabilis* + *Andigena*) from other *Selenidera*, and the split between northeastern Amazonia (Guianan shield *S. piperivora*) and the rest of Amazonian *Selenidera*.

Timing of diversification within the *Selenidera* and *Andigena* lineages mostly occurred after the completion of the last pulse of Andean uplift which had completed by 6–10 Mya (Graham, 2009; Gregory-Wodzicki, 2000), and before the last glacial maximum (LGM) of the Pleistocene, which took place 19,000–26,500 ya (Clark et al., 2009). The separation between the exclusively *cis*-Andean lowland clade (*Selenidera* spp., excluding *S. spectabilis*) and the montane-lowland clade (*Andigena* spp., including lowland *S. spectabilis*) occurred in the upper Miocene (10.8–8.1 Mya). The transition from lowland to highland occurred between 3.2 and 9.9 Mya (also upper Miocene) and appears to be the result of divergence from a *trans*-Andean lowland lineage. This is an unusual pattern that differs from hypothesized patterns of avian colonization of the Andes postulated by Chapman (1917).

4.2. Comparison to other studies

Selenidera has long been recognized for its strong concordance with major lowland areas of endemism (Haffer, 1969), thus it is informative to compare the *Selenidera* time tree with divergence dates estimated from molecular data for other lowland groups. *Selenidera* exhibits similar Amazonia/Atlantic Forest divergence times to *Ramphastos* toucans (Patané et al., 2009), *Pionus* parrots (Ribas et al., 2007), and some lineages of *Mionectes* flycatchers (Miller et al., 2008; Table 4). Older divergence times of ~2–3.6 Mya are estimated for *Pteroglossus* araçaris (Patel et al., 2011), *Brotogeris* parakeets (Ribas et al., 2009), *Xiphorhynchus*

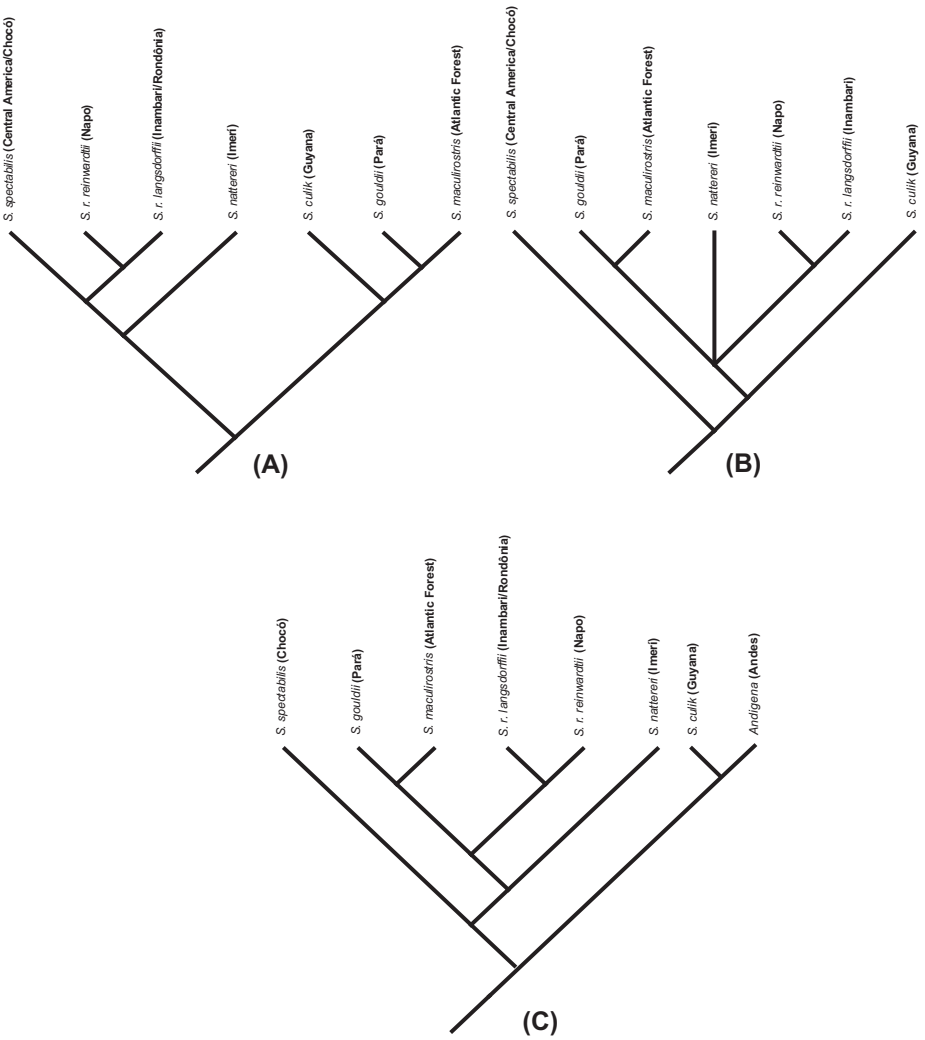

Fig. 4. A comparison the molecular phylogeny of *Selenidera* and implied area relationships with previously published *Selenidera* phylogenetic tree topologies and area relationships: (A) dendrogram of *Selenidera* (Haffer, 1974), (B) morphological phylogeny of *Selenidera* (Cracraft and Prum, 1988), and (C) molecular phylogeny of *Selenidera* (this study).

woodcreepers (Cabanne et al., 2008), and some *Mionectes* flycatchers (Miller et al., 2008), with even older splits of 6.6 Mya estimated for *Pionopsitta* parakeets (Eberhard and Bermingham, 2005). These comparative data are consistent with the hypothesis that there have been multiple periods of connectivity between Amazonia and Atlantic Forest lowland regions (DeOliveira et al., 1999; Patel et al., 2011; Willis, 1992). For *cis*/*trans*-Andean splits, all of these avian taxa, with the addition of *Dendrocincla* woodcreepers (Weir and Price, 2011), exhibit strikingly recent divergence times compared to *Selenidera*. This suggests that there may have been multiple events leading to *cis*/*trans*-Andean divergences as well.

The estimate of basal Amazonian divergence for *Selenidera* (7.3–10.1 Mya) also differs when compared to other avian lineages. For example, Ribas et al. (2012) estimated a basal divergence of ~3 Mya for the endemic Amazonian genus *Psophia* (trumpeters) and Patel et al. (2011) estimated a basal divergence of 3.05–4.76 Mya for *Pteroglossus* araçaris. These differences probably reflect both differences in history, differences in dispersal ability (Burney and Brumfield, 2009), and possibly other differences such as lineage-specific rates of evolution.

Similar to molecular studies of other trans-Amazonian taxa, our results support earlier splits in *Selenidera* in northwestern Amazonia (across the Guianan shield/western sedimentary basins suture zone, sensu Aleixo and Rossetti, 2007; and the Negro River), followed by splits across the Solimões (upper Amazon) River, with the more recent divergences across the Madeira and main rivers on the Brazilian shield (Fernandes et al., 2013; d'Horta et al., 2013; Patané et al., 2009). However, this pattern differs from that in other trans-Amazonia lineages (*Psophia* spp. and *Sclerurus rufigularis*), where the earliest split separated populations from both sides of the Amazon-Solimões River, with populations in northwestern Amazonia and the Brazilian shield diverging afterwards (d'Horta et al., 2013; Ribas et al., 2012). For *Selenidera*, the Guianan species *S. piperivora* diverged from the remaining lowland *Selenidera* (excluding *S. spectabilis*) in roughly the same time period as *Andigena* diverged from Central American *S. spectabilis*. After the basal split of the Guianan shield *S. piperivora,* a "counter-clockwise" history of divergences across the headwaters and tributaries of the upper Amazon is apparent, with the youngest splits occurring in southeast Amazonia (on the Brazilian shield) and then between southeast Amazonia and the Atlantic Forest for those lineages occurring in both biomes (Fernandes et al., 2013; d'Horta et al., 2013; Patané et al., 2009).

These comparisons highlight that although some co-distributed Amazonian taxa share the same divergence patterns and temporal histories, others do not. Indeed, based on current estimates, there

Table 4
Comparative timing of divergence in some neotropical avian lineages (Mya).

	Amazonia/Atlantic forest	*cis*/*trans* -Andean
Selenidera (Ramphastidae)	0.7–1.8	8.1–10.1
Pteroglossus (Ramphastidae)[a]	2.18–3.59	2.05–3.28
Ramphastos (Ramphastidae)[b]	0.77–1.40	2.60–4.15
		0.99–1.75
		0.33–0.81
		0.20–1.40
Brotogeris (Psittacidae)[c]	3.0–3.6	~3.0
Pionopsitta (Psittacidae)[d]	6.6	4.2
Pionus (Psittacidae)[e]	0.15–1.41	0.15–1.41
Dendrocincla (Dendrocolaptidae)[f]	–	~3.26
	–	~2.1
	–	~0.9
Xiphorhynchus (Dendrocolaptidae)[g]	~3.0	–
Mionectes (Tyrannidae)[h]	~2.3	~1.9
		~1.5
	~0.9	~0.3

[a] Patel et al. 2011.
[b] Patané et al. 2009.
[c] Ribas et al. 2009.
[d] Eberhard and Bermingham 2005.
[e] Ribas et al. 2007.
[f] Weir and Price 2011.
[g] Cabanne et al. 2008.
[h] Miller et al. 2008.

are striking differences between two co-distributed clades in the same family – *Pteroglossus* and *Selenidera* – that diversified at different times across many seemingly shared biogeographic regions and barriers. For example, the split of the Guianan shield *S. piperivora* from its sister taxa is much older (7.3–10.1 Mya) than the split between the Guianan shield *P. viridis* and its sister taxa (~1 Mya).

Comparison of speciation patterns within *Andigena* to other montane avian taxa supports the hypothesis that dispersal throughout the Andes followed by later *in situ* isolation has likely driven diversification within *Andigena*. Many studies have emphasized the important role of dry intermontane valleys and other low elevation barriers in promoting diversification and maintaining isolation between avian populations distributed across the Andes (e.g. Bonaccorso, 2009; Gutiérrez-Pinto et al., 2012; d'Horta et al., 2013; Vuilleumier, 1969). In particular, the North Peruvian Low (NPL)/Marañon Valley has been implicated as an important biogeographic barrier and thus driver of speciation in the northern Andes (Gutiérrez-Pinto et al., 2012; Miller et al., 2007; Parker et al., 1985; Quintero et al., 2012; Weir, 2009). Here we show that *Andigena hypoglauca*, the only *Andigena* species to extend across the Marañon, exhibits modest intraspecific divergence across this barrier. Uncorrected pairwise distances for *A. h. hypoglauca* and *A. h. lateralis* across the Marañon are low (Cyt*b* = 0.012, ND2 = 0.018) relative to some other studies (Weir, 2009; Winger, pers. comm.). Phenotypic differences across the NPL between these subspecies are slight as well, and may correspond to differences in iris and facial skin coloration originally used to define these taxa (Haffer, 1974). These data suggest that the divergence between *Andigena hypoglauca* subspecies across the Marañon may have been more recent than in some other Andean bird groups that show higher mtDNA sequence divergence across this barrier (e.g. Bonaccorso, 2009; Quintero et al., 2012; Weir, 2009).

4.2.1. Drivers of diversification within montane Andigena

Our timing and phylogenetic analyses support an emerging consensus that Andean uplift helped jump-start diversification of Andean *Andigena* taxa, but did not directly cause the speciation of extant montane avian species-level taxa (Chaves et al., 2011; Hoorn et al., 2010; Quintero et al., 2012; Ribas et al., 2007; Weir and Price, 2011). Our time tree (Fig. 3) is consistent with either the hypothesis that the basal divergence between the *S. spectabilis*/*Andigena* clade and all other *Selenidera* resulted from vicariance driven by Andean uplift (*sensu* Ribas et al., 2007) or via dispersal into the uplifting Andes. More importantly, the timing is inconsistent with dispersal into the Andes after the completion of uplift. Divergence of *Andigena* from *S. spectabilis* occurred between 3.2 and 9.9 Mya, a time frame during which the northern Andes of Ecuador and Colombia were still forming (Coltorti and Ollier, 2000; Hoorn et al., 2010; Steinmann et al., 1999). However, the timing of the splitting off of *Andigena* could be interpreted in two ways. First, if it is a dispersal-driven speciation event, then the timing of the dispersal event would be at the node and would be predicted to have occurred between 7.0 and 9.9 Mya. However, if it is a vicariance-driven speciation event, then the transition could have occurred anywhere along the branch, between 3.2 and 9.9 Mya.

Divergence between *A. laminirostris* and the rest of the *Andigena* species between 3.2 and 4.6 Mya was followed by expansion to the southern-most range of the Peruvian and Bolivian Andes. Subsequent diversification of *Andigena* species occurred between 0.5 and 4.6 Mya, and thus was probably driven by a combination of dispersal events and habitat fragmentation caused by elevational shifts during Plio-Pleistocene climatic oscillations, rather than orogeny. The most recent divergences between *A. cucullata* and the central Andean taxa *A. h. hypoglauca* and *A. h. lateralis* occurred during the late Pliocene and early Pleistocene, between 1.9 and 3.1 Mya. *A. h. hypoglauca* and *A. h. lateralis*, found north and south of the Marañon River respectively, have uncorrected mtDNA pairwise distances of 1.2% (Cyt*b*) and 1.8% (ND2). Thus, recent divergence times and relatively low uncorrected mitochondrial p-distances support the hypothesis that diversification within the *Andigena* clade was post Andean uplift and thus likely driven by dispersal during cool periods that shifted the elevation of montane habitats lower, connecting previously isolated montane regions across the Marañon, with subsequent differentiation occurring during warm periods that isolated populations on montane "islands" (Vuilleumier, 1969).

4.2.2. Drivers of diversification within lowland Selenidera

Previous studies of lowland Neotropical birds have often posited that shared patterns of regional endemism suggest a common history. For instance, Haffer hypothesized that climatic fluctuations in the Neotropics during the Plio-Pleistocene period reduced large tracts of forest into isolated pockets, or refugia (Haffer, 1969, 1974, 1997). The phylogenetic data and analyses presented here augment our knowledge about one such lineage, *Selenidera,* which has long been recognized as being differentiated at the species level in nearly all major lowland areas of endemism. However, the area relationships implied by our molecular phylogenetic data are different than those indicated by previously published morphological phylogenies and arrangements for the genus (Cracraft and Prum, 1988; Haffer, 1974; Prum, 1988). These relationships also contrast with results of some parsimony analyses of endemism (Bates et al., 1998; Silva and Oren, 1996), and molecular-based studies of other avian taxa (e.g. Armenta et al., 2005; Marks et al., 2002; Ribas et al., 2012). In particular, diversification within Amazonian *Selenidera* lineages is consistent with the pattern recovered for *Ramphastos vitellinus* (Patané et al., 2009), *Glyphorynchus spirurus* (Fernandes et al., 2013; Fig. 4), *Dendrocolaptes certhia* (Batista et al., 2013), *Lepidocolaptes albolineatus* (Rodrigues et al., 2013) and *Sclerurus caudacutus* (d'Horta et al., 2013), whereby taxa from the Guianan areas are the most divergent Amazonian lineage, and those from the Imerí are sister to lineages from the remaining Amazonian areas.

The distributions of lowland *Selenidera* species match closely to proposed areas of Pleistocene refugia. However, until now there has been no published molecular phylogenetic hypothesis for the genus, and current descriptions of intrageneric relationships rely on other characteristics. Haffer (1974) proposed a relationship among toucans based on morphological features that shows few similarities to our molecular phylogeny (Fig. 4). In Haffer's dendrogram, there is a polytomy between the clade containing *S. gouldii* and *S. maculirostris*, *S. nattereri*, and the clade containing *S. r. reinwardtii* and *S. r. langsdorffii*. Cracraft and Prum (1988) analyzed 21 morphological characters for the genus *Selenidera* and produced a phylogeny that more closely resembles our reconstruction. The relationships as described by Cracraft and Prum (1988), (*S. spectabilis* (((*S.maculirostris*, *S. gouldii*) (*S. r. langsdorffii*, *S. r. reinwardtii*), *S. nattereri*), *S. piperivora*)), are mostly supported by the molecular data presented here. However, Cracraft and Prum (1988) assumed that *Selenidera* and *Andigena* were reciprocally monophyletic, whereas our molecular data support the placement of *S. spectabilis* as sister to *Andigena*.

Our time tree analyses suggest that basal divergences within *Selenidera* predate the LGM, indicating that if the current distributions of species within *Selenidera* are the result of separation into refugia, these refugia must have been formed and maintained over the course of many glacial cycles throughout the Pliocene and Pleistocene without necessarily leading to speciation in more recent climatic cycles. Alternatively, and possibly in addition to the effects of forest refuge formation, major rivers that form most boundaries between areas of endemism could serve as barriers to gene flow, and thus lead to speciation. The timing of divergence (see below) between *S. gouldii* (Pará), *S. gouldii* (Rondônia), *S. maculirostris* (Atlantic Forest), *S. r. reinwardtii* (Napo), *S. r. langsdorffii* (Inambari), and *S. nattereri* (Imerí) in their respective areas of endemism is congruent with known vicariance events associated with fluvial formations and shifts (Ribas et al., 2012).

4.3. Taxonomic implications

Our molecular results present a potential quandary for delimiting genera. Because of the apparent paraphyly of *Selenidera*, one could argue that all members of both genera be placed in *Selenidera*, which has nomenclatural priority. The type for *Selenidera* is *S. gouldii* (Gould, 1837). If it was certain that *S. piperivora* is sister to the rest of *Selenidera* (minus *S. spectabilis*), an alternative would be to erect a new genus for *S. spectabilis* to recognize its unique phylogenetic position and the respective reciprocal monophyly of *Andigena* and the *Selenidera* (minus *S. spectabilis*) clades. Finally, a third alternative would be the placement of *S. spectabilis* in the genus *Andigena*, a sister relationship with moderate to high statistical support values in our dataset. However, we feel that additional data are needed to clarify the position of *S. piperivora* with more confidence, and hence the basal relationships in the *Selenidera - Andigena* clade, before a new generic arrangement can be proposed.

5. Conclusion

This phylogenetic analysis provides information on relationships for the last remaining group in *Ramphastidae* to be studied using DNA sequence data. For these lineages, we provide an assessment of the timing and patterns of diversification into the Andes and across the Neotropical lowlands. At a deeper phylogenetic level, the transition from lowland to highland distributions in the *Selenidera*/*Andigena* clade occurred during the Pliocene to Late Miocene, consistent with either the hypothesis of vicariance due to the final uplift of the Central Andes or dispersal driven speciation into the uplifting Central Andes. These data suggest that, as has been shown in other studies of Andean lineages, that orogeny probably played an important role in "jumpstarting" divergence, and that later dispersal followed by climatic oscillations and subsequent altitudinal shifts in habitat and fragmentation promoted further isolation followed by divergence. The radiation within *Selenidera*, which has long been considered a classic example of lowland diversification, proves to have surprising results at the base of the tree with a "counter-clockwise" (i.e., northwestern–southeastern) pattern of diversification across the *cis*-Andean lowlands.

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