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CURSO DE DOUTORADO EM ZOOLOGIA

Sistemática molecular, biogeografia e taxonomia do gênero *Megascops* Kaup, 1848 (Aves, Strigidae)

SIDNEI DE MELO DANTAS

Tese de doutorado apresentada ao Programa de Pós-graduação em Zoologia, Curso de Doutorado, do Museu Paraense Emílio Goeldi e Universidade Federal do Pará como requisito para obtenção do grau de Doutor em Zoologia.

Orientador: Dr. Alexandre Aleixo

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Sumário

RESUMO GERAL

O gênero *Megascops* é o maior gênero de corujas (Strigidae) endêmico do Novo Mundo, com cerca de 20 espécies distribuídas do Canadá à Argentina, atingindo o ápice de sua diversidade nos Andes e na América Central. A diagnose dos táxons do gênero é em geral bastante difícil devido a uma grande similaridade morfológica entre a maioria dos mesmos, e ao polimorfismo presente em praticamente todas as populações. Estudos filogenéticos sobre o gênero incluíram poucos táxons e genes, e as relações de parentesco entre as espécies e subespécies de *Megascops* é muito pouco compreendida. Os táxons florestais amazônicos, *M. watsonii watsonii* e *M.w. usta*, separados pelo Rio Amazonas, e a espécie da Mata Atlântica *M. atricapilla*, por exemplo, são consideradas como proximamente relacionadas, mas estudos anteriores sugeriram que essas espécies são parapátricas em relação umas às outras. Dentro da distribuição de *M.w. usta*, há variações vocais geograficamente distribuídas, que sugerem a presença de espécies crípticas, e estudos mais aprofundados são necessários para que se compreenda melhor a situação taxonômica desse grupo. No presente trabalho, nós propomos uma filogenia para o gênero *Megascops* baseada em dados moleculares (Primeiro capítulo), e conduzimos um estudo de biogeografia e taxonomia dos táxons *M. watsonii watsonii, M.w. usta* e *M. atricapilla* baseado em dados moleculares, morfológicos e vocais (Segundo capítulo). Os dados moleculares em ambos os capítulos foram obtidos pelo sequenciamento de três genes mitocondriais (citocromo b, ND2 e CO1) e três nucleares (b-fibrinogênio 5, CHD e MUSK) para todas as amostras. Os dados moleculares foram analisados através de análises de Máxima Verossimilhança, Inferência Bayesiana e Árvore de Espécies. Análises de Relógio Molecular e de S-DIVA foram rodadas para inferir o tempo de divergência e as áreas ancestrais para todos os táxons analisados do gênero *Megascops*. Em todas as análises, *Megascops* é parafilético se incluirmos no gênero a espécie *M. nudipes*, que se agrupou próxima ao grupo externo, *Otus flammeolus*. As demais espécies se separam em três grandes clados: Um composto por *M. choliba, M. koepckeae, M. albogularis, M. clarkii* e *M. trichopsis*, um segundo composto por espécies andinas, e um terceiro com as espécies restantes. Muitos dos ramos receberam baixo apoio estatístico nas análises, o que torna a relação entre muitas espécies indeterminada, dentro dos grandes clados. As espécies *M. watsonii* e *M. atricapilla* são parafiléticas, e valores de divergência entre populações disjuntas de algumas espécies (*M. vermiculatus*, *M.ingens*, *M. trichopsis*) se aproximam dos de alguns pares de espécies inequívocas, sugerindo uma diversidade dentro do gênero

maior do que a atualmente reconhecida. A especiação dentro do gênero (excetuando-se *M. nudipes*) aconteceu nos últimos oito milhões de anos, e o gênero provavelmente se originou nos Andes, posteriormente colonizando as Américas. *Megascops nudipes* deve ser retirada do gênero *Megascops*. Mais estudos são necessários para avaliar a real situação taxonômica das espécies do gênero *Megascops*, especialmente as que apresentam subespécies, como *M. vermiculatus* e *M. ingens*. O soerguimento dos Andes pode ter desempenhado um papel extremamente importante na diversificação do gênero.

Para os táxons *Megascops watsonii watsonii, M.w. usta* e *M. atricapilla*, foram sequenciados os mesmos genes, para 49 amostras de tecido muscular. Os dados foram analisados através de análises de Máxima Verossimilhança, Inferência Bayesiana, Árvore de Espécies e Relógio Molecular. Foram feitas medições morfométricas em 251 peles, e medidos parâmetros vocais de 85 gravações, cobrindo todas as regiões zoogeográficas habitadas por esses táxons. Os dados morfométricos e vocais foram analisados através de Análises de Função Discriminante, um método de diagnose de pares de espécies através de dados vocais também foi aplicado. Os dados moleculares se dividiram em seis clados bem apoiados, cuja distribuição não está de acordo com a taxonomia atual desse grupo de espécies. Um dos clados está restrito a poucos fragmentos de floresta atlântica muito pequenos e isolados, no leste do Brasil. Se reconhecido como uma espécie válida, quase certamente será o táxon em maior perigo de extinção dentro do gênero *Megascops*. As análises dos dados morfométricos não detectaram diferenças significativas entre os clados, e a grande variação no padrão de plumagem dentro das populações torna quase impossível fazer generalizações. Porém, alguns morfos tendem a ser mais comuns em algumas populações do que em outras. As análises vocais apontaram diferenças entre alguns dos clados, mas há uma considerável sobreposição entre alguns deles, e há variação vocal dentro de algumas populações, especialmente no clado de maior distribuição. A especiação desse grupo aconteceu durante o Pleistoceno-Plioceno, e os fatores mais importantes a definir a atual distribuição dos clados parecem ser a formação do atual sistema hídrico da Amazônia, uma posterior expansão da distribuição de algumas populações, e a separação da Amazônia e da Mata Atlântica, com uma divisão posterior dessa última pela formação do Rio São Francisco. Recomendamos a redefinição dos táxons *M.w. watsonii, M.w. usta* e *M. atricapilla* e o reconhecimento de outras três populações filogenéticas, duas das quais não possuem nome disponível.

1. INTRODUÇÃO GERAL

1.1. A filogeografia como uma ferramenta para compreender os processos de especiação no Neotrópico

O continente americano e, em especial o Neotrópico, abriga a mais diversa avifauna do planeta. Muito se tem especulado sobre as causas dessa extraordinária diversidade, que aumenta em riqueza das regiões temperadas até atingir seu ápice nos Andes e nas florestas tropicais sul-americanas. Dentre os diversos fatores que têm sido apontados como promotores dessa riqueza destacam-se o soerguimento dos Andes (Sedano e Burns, 2009), a formação de refúgios na Amazônia durante épocas mais secas (Haffer, 1974), e a formação do sistema atual de rios Amazônicos (Ribas *et al*., 2012). O isolamento de várias unidades geográficas e a formação de diferentes habitats decorrentes desses processos promoveram a especiação de aves no Neotrópico através de vicariância, especiação ecológica, dentre outros processos evolutivos (Weir e Price, 2011).

A grande diversidade de aves neotropicais resulta da atuação de vários desses processos, bem como das características dos grupos de espécies, como capacidade de dispersão e territorialidade, entre outros fatores. Testar essas hipóteses históricas diretamente não é possível, portanto uma das soluções é verificar como a história evolutiva de diversos grupos de espécies acompanha as idades propostas para os grandes eventos geológicos formadores das modernas paisagens e biomas do continente americano. Tais estudos têm incluído grupos de aves diversos como Dendrocolaptidae (Aleixo, 2002; Cabanne *et al*., 2008, Milá *et al*., 2009; Weir e Price, 2011; Fernandes *et al*., 2013), Trochilidae (Chaves *et al*., 2011), Thraupidae (Sedano e Burns, 2009), Psophidae (Ribas *et al*., 2012), Ramphastidae (Patané *et al*., 2009; Patel *et al*., 2011; Lutz *et al*., no prelo), dentre outros. A reconstrução dessas histórias tem sido feitas com metodologias modernas de datação de relógios moleculares, e tem-se verificado coincidências entre a diversificação de muitos grupos e a formação de barreiras, como os Andes e os rios Amazônicos (Chaves *et al*., 2011; Ribas *et al*., 2011; Sedano e Burns, 2009). Com base nesses estudos, tem se proposto hipóteses complementares sobre os processos de evolução da biota das Américas. Um exemplo disso é o recente estudo sobre diversificação do gênero endêmico da Amazônia *Psophia* (Ribas *et al*., 2012), em que

cada clado é separado por um dos grandes rios da região. Nesse estudo, foi possível propor uma ordem para a formação dos rios, baseado no tempo e seqüência de formação de espécies no gênero. Obviamente, cada grupo faunístico apresenta uma história evolutiva diferente. As aves do gênero *Psophia*, por serem praticamente terrícolas e restritas a florestas de terra firme, apresentam uma capacidade de dispersão relativamente limitada, sendo mais facilmente passíveis de responderem aos rios como barreiras (Ribas *et al*., 2012).

Estudos em larga escala de grupos com uma riqueza grande de táxons, que ocupem várias regiões biogeográficas, são ideais para compreender os processos que levaram à diversificação da avifauna no Neotrópico. Um dentre os diversos grupos de aves que se encaixam nesse padrão é o gênero *Megascops* Kaup, 1848, o qual é o maior gênero de corujas em número de espécies e endêmico das Américas. Compreende cerca de 21 espécies e 63 táxons (modificado de Marks *et al*., 1999) distribuídos do sul do Canadá à Argentina, em ambientes diversos como florestas, savanas e manguezais. A riqueza do gênero é maior em regiões montanhosas tropicais e sub-tropicais das Américas: na América do Norte encontram-se três espécies de *Megascops*; na América Central, sete; nos Andes, nove, e nas terras baixas da América do Sul, quatro (Marks *et al*., 1999).

Originalmente criado como um subgênero de *Scops* para designar os táxons *M. indica*, *M. atricapilla*, *M. brasiliana*, *M. asio* e *M. albopunctata*, em 1910 foi reclassificado como um sub-gênero do gênero cosmopolita *Otus* Pennant, 1769, na 3ª Edição da "check-list" da "American Ornithologist's Union" (AOU). *Megascops* foi elevado novamente a gênero pleno em 2003, com base em estudos envolvendo dados moleculares e vocais (van der Weyden, 1975; Marshall e King, 1988; Wink e Heidrich, 1999; Wink *et al.*, 2000, 2009; Fuchs *et al.*, 2008), que não apoiaram sua união a *Otus*, que passou a ser um gênero quase exclusivo do Velho Mundo, com apenas uma espécie americana: *O. flammeolus*. *Megascops* é aparentemente mais próximo filogeneticamente de gêneros como *Bubo*, *Strix* e *Pulsatrix* (Wink *et al*., 2009). Verificou-se que *O. flammeolus* também não se agrupa com as *Otus* do Velho Mundo, sendo aparentemente basal em *Megascops* (Proudfoot *et al*., 2007).

Por ser um grupo de aves estritamente noturnas, e por muitas populações terem distribuições restritas e/ou viverem em locais pouco explorados cientificamente, é um grupo ainda pouco estudado (Freile e Castro, 2013). Várias espécies e subespécies foram descritas apenas recentemente (Weske e Terborgh, 1981; Hekstra, 1982; Fitzpatick e O´Neill, 1986), e atualmente há táxons em vias de descrição (Baiker, 2011).

Uma breve descrição da distribuição das diversas espécies do gênero encontra-se abaixo. A taxonomia segue Marks *et al*. (1999):

- *Megascops kennicottii* (Elliot, 1867): Descrita inicialmente dentro do gênero *Scops*. Distribui-se do sul do Alasca ao México, passando pela costa oeste do Canadá e EUA continental.
- *Megascops seductus* (Moore, 1941): Descrita inicialmente dentro de *Otus*, é considerada por alguns autores como parte de uma superespécie que inclui *M. kennicottii, M. cooperi* e *M. asio*. É também por vezes tratada como raça de *M. asio* ou *M. kennicottii*, sendo a população da localidade de Colima às vezes tratada como uma raça à parte (*colimensis*). É endêmica do sudoeste do México.
- *Megascops cooperi* (Ridgway, 1878): Inicialmente descrita dentro de *Scops*, geralmente é tratada como subespécie de *M. kennicottii* ou *M. asio*. Distribui-se do sul do México ao noroeste da Costa Rica acompanhando a costa pacífica.
- *Megascops asio* (Linnaeus, 1758): Descrita inicialmente no gênero *Strix*, foi considerada no início como coespecífica a *M. kennicottii*, podendo cruzar com esta em áreas de simpatria (Marks *et al*., 1999). Distribui-se do sul do Canadá ao nordeste do México, passando pelo centro-leste dos EUA.
- *Megascops trichopsis* (Wagler, 1832): Descrita inicialmente no gênero *Scops*. Suas relações de parentesco são incertas (Marks *et al.*, 1999), e distribui-se do sudeste do Arizona ao Norte da Nicarágua.
- *Megascops choliba* (Vieillot, 1817): Descrita inicialmente no gênero *Strix*. Evidências obtidas até o momento apontam que essa espécie não tem parentesco próximo dentro do gênero *Megascops* (Marks *et al.*, 1999)**,** embora *M. koepckeae* e *M. roboratus* já tenham sido consideradas raças dessa espécie. Distribui-se da Costa Rica à Argentina.
- *Megascops koepckeae* (Hekstra, 1982): Originalmente descrita como subespécie de *M. choliba*. É encontrado do noroeste do Peru, aparentemente até o departamento de La Paz na Bolívia, mas sua distribuição é mal conhecida.
- *Megascops roboratus* (Bangs e Noble, 1918): Inicialmente descrita dentro de *Otus*. Distribui-se do sudoeste do Equador ao noroeste do Peru.
- *Megascops clarkii* (Kelso e Kelso, 1935): Inicialmente descrita dentro de *Otus*. Distribui-se da Costa Rica ao extremo noroeste da Colômbia.
- *Megascops barbarus* (Sclater e Salvin, 1868): Inicialmente descrita dentro de *Scops*. Distribui-se das montanhas do sul do México ao norte da Guatemala.

- *Megascops ingens* (Salvin, 1897): Inicialmente descrita dentro de *Scops*. Habita o norte da Colômbia ao centro-oeste da Bolívia.
- *Megascops colombianus* (Traylor, 1952): Inicialmente descrita dentro de *Otus*. Às vezes tratada como subespécie de *M. ingens* (Marks *et al.*, 1999). Distribui-se nos Andes, do centro-oeste da Colômbia ao noroeste do Equador.
- *Megascops petersoni* (Fitzpatrick e O'Neill, 1986): Inicialmente descrita dentro de *Otus*. Pode formar uma superespécie com *M. marshalli*, e ambas foram previamente agrupadas sob um nome atualmente inválido: *M. huberi* (Marks *et al.*, 1999). Habita a cordilheira de Cutucú, do sudeste do Equador ao noroeste do Peru.
- *Megascops marshalli* (Weske e Terborgh, 1981): Inicialmente descrita dentro de *Otus*. Sua distribuição vai da cordilheira Yanachanga, no centro-sul do Peru, à cordilheira Vilcabamba, em Cuzco.
- *Megascops watsonii* (Cassin, 1848): Descrita como *Ephialtes watsonii*, é bastante próxima de *M. atricapillus* (Heidrich *et al.*, 1995). É dividida em duas subespécies, M.w.*watsonii* e M.w. *usta*, com base em diferenças vocais e morfológicas, embora as morfológicas pareçam ser mínimas. Também há variação regional no canto de *usta*: as populações ao leste do rio Madeira são vocalmente muito mais semelhantes às de *watsonii*, do que às demais de *usta* a oeste do Madeira (obs. pess.). Habita toda a bacia amazônica.
- *Megascops guatemalae* (Sharpe, 1875): Descrita no gênero *Scops*. Pode formar uma superespécie com *M. vermiculatus, M. hoyi, M. atricapillus* e *M. sanctaecatarinae.* Distribui-se do noroeste do México ao norte da Nicarágua.
- *Megascops vermiculatus* Ridgway, 1887: Descrita no gênero *Scops*. Pode formar uma superespécie com *M. guatemalae, M. hoyi, M. atricapillus* e *M. sanctaecatarinae.*Distribui-se em populações disjuntas do nordeste da Costa Rica ao norte da Bolívia.
- *Megascops hoyi* (König e Straneck, 1989): Descrito inicialmente dentro de *Otus*, e considerado como parte da espécie biológica *M. atricapilla*, mas geneticamente distinta dessa espécie (Heidrich *et al.*, 1995). Habita florestas montanhosas do sul da Bolívia ao noroeste da Argentina.
- *Megascops atricapilla* (Temminck, 1822): Descrita no gênero *Strix*, é agrupada por alguns autores junto com *M. hoyi* e *M. sanctaecatarinae*, mas todas essas formas são geneticamente distintas (Heidrich *et al.*, 1995). Habita o sul da Bahia e Goiás até Santa Catarina, sudeste do Paraguai e nordeste da Argentina. Uma população recentemente

descoberta em florestas de altitude de Alagoas foi atribuída a essa espécie (Roda e Pereira, 2006), mas seu status taxonômico ainda é incerto.

- *Megascops sanctaecatarinae* (Salvin, 1897): Descrita no gênero *Scops*, foi separada de *M. atricapillus* com base em morfologia, vocalizações e genética (Heidrich *et al.*, 1995). A taxonomia de algumas populações do sul do Brasil e de Missiones, na Argentina, precisa ser esclarecida. Distribui-se da região sul do Brasil, ao nordeste da Argentina e Uruguai.
- *Megascops nudipes* (Daudin, 1800): Descrita no gênero *Strix*. Habita Porto Rico e diversas outras ilhas próximas.
- *Megascops albogularis* Cassin, 1849: Descrita no gênero *Syrnium.* Posicionada por alguns autores no gênero monotípico *Macabra*, com base na ausência das "orelhas" de penas, mas o padrão da vocalização condiz com o observado em outras espécies de *Megascops* (van der Weyden, 1975). Distribui-se do noroeste da Venezuela até o centro da Bolívia.

1.2. O gênero *Megascops*: problemáticas taxonômicas

A grande maioria dos táxons desse gênero possui a plumagem muito semelhante entre si, ao mesmo tempo em que ela geralmente varia dentro de uma mesma população, o que torna a diagnose e a compreensão dos limites entre táxons problemáticos em muitos casos (Marks *et al.*, 1999; Proudfoot *et al.*, 2007; Weske e Terborgh, 1981; Fitzpatrick e O'Neill, 1986; Sick, 1997; Wink e Heidrich, 2000). Proudfoot *et al.*, (2007) concluíram que diversas subespécies de *Megascops asio* e *M. kennicottii*, ambas da América do Norte, reconhecidas com base em caracteres de plumagem, não eram apoiadas pela análise molecular.

Há poucos estudos recentes sobre a filogenia do gênero (Heidrich *et al.*, 1995; Wink e Heidrich, 1999, 2000; Wink *et al.*, 2009; Proudfoot *et al.*, 2007). Esses estudos incluíram poucas espécies do gênero, e nenhum fez diagnósticos de táxons unindo caracteres moleculares, vocais e morfológicos. Isso dificulta inclusive o diagnóstico do status de conservação para o grupo, na medida em que há uma grande incerteza quanto à delimitação das espécies, tornando inadequado o seu uso como unidades de análise em planejamentos estratégicos da conservação (i.e., seleção de áreas e espécies prioritárias para a preservação). Estudos aprofundados sobre vocalização e genética se fazem necessários para uma melhor compreensão da real diversidade do gênero *Megascops*

(Sick, 1997; Heidrich *et al.*, 1995; Proudfoot *et al.*, 2007). O caso de uma das espécies do gênero, *Megascops guatemalae*, ilustra bem a incerteza quanto à taxonomia do gênero, uma vez que esta pode ser ou considerada uma espécie única com distribuição disjunta desde o sul do México até o norte da Bolívia, ou ser dividida em duas, três ou quatro espécies (Marks *et al.*, 1999).

Uma situação de semelhante incerteza taxonômica ronda a espécie florestal da planície amazônica, *Megascops watsonii*, dividida nas subespécies *M. watsonii watsonii e M.w. usta* (Marks *et al.*, 1999). Há controvérsias na literatura sobre os caracteres que distinguem *M.w. watsonii* de *M.w. usta*: Marks *et al.* (1999) afirmam que *M.w. watsonii* é maior que *M.w. usta*, enquanto König e Weick (2008) afirmam o contrário. Outras características, como diferenças na coloração geral – *M.w. usta* seria em geral mais avermelhada que *M. watsonii* – e a vocalização – *M. watsonii* emite um canto "rápido", de cerca de sete notas por segundo, enquanto que *M.w. usta* emite um canto "lento", de cerca de duas notas por segundo - são consideradas como insuficientes ou não-conclusivas para a separação das duas formas (Remsen *et al.*, 2013). Existem diferenças regionais de vocalização tanto em *M.w. watsonii*, que ocorre ao norte dos Rios Amazonas e Solimões, como em *M.w. usta*, que a substitui ao sul dos mesmos. A vocalização principal (longsong) das populações de *M.w. usta* a leste do Rio Madeira, por exemplo, se assemelha mais à respectiva vocalização de *M.w. watsonii*, que à da população de *M.w. usta* a oeste do Madeira (obs. pess.). A falta de coletas na maior parte da área de distribuição desses táxons, que estão entre as mais amplas do gênero, dificulta a tomada de decisões taxonômicas sobre os mesmos.

Por outro lado, *M. atricapilla*, espécie florestal da Mata Atlântica, pode ser mais relacionada à *M.w. usta*, do que esta a *M.w. watsonii* (Marks *et al.*, 1999). Existem populações isoladas e recentemente descobertas no Estado brasileiro de Alagoas, ao Norte do rio São Francisco, de taxonomia ainda duvidosa, atribuídas a esse táxon (Roda e Pereira, 2006). Caso seja considerado um táxon diferenciado, essa população pode ser considerada como extremamente ameaçada, por ter uma distribuição muito pequena (uma das menores, se não a menor, dentre todas as espécies de *Megascops*). Os poucos estudos existentes sobre a filogenia do grupo utilizaram poucas amostras dessas espécies em suas análises, tornando difícil tirar conclusões sobre seus limites específicos. A compreensão e discussão sobre esses limites podem ter profundas implicações sobre a conservação de espécies (Zink e McKitrick, 1995; Zink, 2004; de Queiroz, 2005; Aleixo, 2007; Silveira e Olmos, 2007).

1.3. A Problemática do conceito de espécies

A dificuldade de delimitação de táxons no gênero *Megascops* traz a tona um problema bastante atual na taxonomia: Como delimitar espécies e/ou subespécies? O clássico Conceito Biológico de Espécies (CBE) estabelece que espécies plenas são unidades taxonômicas isoladas reprodutivamente de outras unidades. Na prática, há muitos casos nos quais é impossível testar esse conceito, como nos casos de espécies alopátricas. Isso levou à proposição de conceitos alternativos de espécies, que levassem em consideração a complexidade da distribuição e interações entre diferentes populações. Um desses conceitos é o Conceito Filogenético de Espécies (CFE), no qual espécies são definidas como o menor grupo diagnóstico de indivíduos onde exista um padrão de ancestralidade e descendência, que em conjunto passam a constituir unidades diagnósticas basais (Cracraft, 1983). Na prática, isso significa que uma população pode ser considerada como uma espécie plena se apresentar pelo menos um caráter que a distinga de outras, seja esse morfológico, vocal ou genético. Esse conceito recebeu críticas, pois não define as espécies com base em uma filogenia, e sim em caracteres descontínuos, definindo assim entidades sem uma base necessariamente evolutiva. Também foi criticado pelo fato de o nível de diagnose entre populações ser arbitrário, e altamente influenciado pelo tamanho da amostragem das populações.

Uma proposição para unir esses e outros conceitos de espécie foi advogada por de Queiroz (1998), sob o nome de Conceito Filético Geral de Espécies, ou CFGE (Aleixo, 2007). De Queiroz enfatizou que a grande diferença entre o CBE e o CFE é a importância dada ao grau de diferenciação entre as populações: O CBE considera duas populações divididas em espécies quando alcançam isolamento reprodutivo, enquanto que populações que ainda não alcançaram esse momento, mas apresentam diferenças morfológicas entre si, são consideradas como subespécies – um conceito ainda mais arbitrário que o conceito de espécies. Já o CFE considera essas populações minimamente diferenciadas como espécies. O CFGE propõe que uma população seja considerada como espécie quando for comprovado seu monofiletismo em relação a outras populações, através de um estudo conjunto de caracteres, como os genéticos, morfológicos e vocais. A aplicação do CFGE pode ter profundas implicações na taxonomia de grupos como os Strigidae, cujos táxons foram descritos em grande parte

levando-se em conta preponderantemente características de plumagem, como na taxonomia ornitológica de um modo geral até o final do século 20.

Por esses motivos, propõe-se no presente estudo uma revisão sistemática do gênero *Megascops*, com base em dados moleculares e uma revisão taxonômica das espécies *M.w. watsonii, M.w. usta* e *M. atricapilla*, com base em dados genéticos, morfológicos e vocais. O estudo está dividido em dois capítulos: no primeiro, uma hipótese sobre a filogenia do gênero *Megascops* com base em dados moleculares é apresentada e discutida. No segundo, é abordada a biogeografia histórica e revisada a taxonomia das espécies *M. watsonii* e *M. atricapilla*, as duas primeira habitando a Amazônia, e a terceira, a Mata Atlântica.

2. OBJETIVOS

2.1. Objetivo Geral

Entender as relações filogenéticas entre as espécies do gênero *Megascops* Kaup, 1848, com ênfase nas espécies de ampla distribuição na Amazônia e Mata Atlântica.

2.2. Objetivos específicos

- Revisar a sistemática do do gênero *Megascops* com base numa hipótese filogenética que inclua a maioria das espécies do gênero.
- Estabelecer os limites inter-específicos entre táxons dos complexos *Megascops watsonii* e *M. atricapilla*, propondo mudanças quando necessário com base no CFGE.
- Diagnosticar, redescrever e, quando necessário, descrever novos táxons dos complexos *Megascops watsonii* e *M. atricapilla*, com base numa combinação de caracteres moleculares, morfológicos e vocais.

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3. CAPÍTULO 1.

MOLECULAR PHYLOGENY OF THE NEW WORLD SCREECH-OWLS (*MEGASCOPS*: AVES, STRIGIDAE): BIOGEOGRAPHIC AND TAXONOMIC IMPLICATIONS

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Abstract

The genus *Megascops* is the largest owl genus endemic to the New World, comprising about 20 species distributed throughout the Americas, and reaching its highest richness in the Andes and Central America. Species and subspecies diagnoses are in general difficult due to strong similarities among taxa and to significant plumage polymorphism in most of them. Previous phylogenetic studies on the genus have included few taxa, and relationships among species and subspecies remain largely unknown. We estimated a phylogeny for the genus *Megascops* species covering all known species, except one (*M. seductus*), based on three mitochondrial (cytb, ND2 and CO1) and three nuclear genes (BF5, CHD and MUSK). Phylogenies were built with Maximum Likelihood, Bayesian Inference, and multilocus coalescent species tree analyses. A chronology, inferred from the species tree and a Statistical Dispersal Vicariance Analysis (S-DIVA), was used to reconstruct the spatio-temporal context of *Megascops* diversification.

According to all trees obtained, *Megascops* is paraphyletic if *M. nudipes* is kept in the genus, since this species appear to be sister to *Otus flammeolus*, which, in turn, cannot be placed in the genus *Otus* as previously demonstrated. The remaining species of *Megascops* clustered in one big clade with three major subdivisions: one including *M. choliba, M. koepckeae, M. albogularis, M. clarkii* and *M. trichopsis*; a second endemic to the Andes (*M. ingens, M. colombianus, M. marshalli, M. petersoni* and *M. hoyi*), and a third containing the remaining species (*M. guatemalae, M. vermiculatus, M. asio, M. kennicottii, M. barbarus, M. cooperi, M. roboratus, M. watsonii, M. atricapilla,* and *M. sanctaecatarinae*). Two species (*Megascops watsonii* and *M. atricapilla*) were recovered as paraphyletic, with the allopatric populations of some species (*M. watsonii, M. vermiculatus, M. ingens,* and *M. trichopsis*) being highly divergent and approaching or even surpassing levels of genetic differentiation found between well-established species-pairs. Speciation within the genus (excepting *M. nudipes*) is estimated as having taken place during the last 8 million years, with the genus more probably appearing in the Andes, and later spreading over much of the Americas. The Andean uplift may have played a key role in the diversification of *Megascops*, with successive episodes of dispersal followed by vicariance isolating and diversifying populations in other mountain ranges of the Neotropics and the lowlands, where rivers and ecotones currently delimit the ranges of many distinct evolutionary lineages.
Key Words: *Megascops*, phylogeny, biogeography.

1. Introduction

Molecular data have shed light on a series of difficult problems involving Neotropical bird taxonomy, and have provided new insights, and sometimes changed drastically the knowledge about species, genera and family relationships (Aleixo, 2002; Weir and Price, 2011; Ribas *et al.*, 2012; Navarro-Sigüenza *et al.*, 2008; Klicka *et al.*, 2007; Sedano and Burns, 2009; Benz and Robbins, 2011; Derryberry *et al.*, 2010; Han *et al.*, 2010). Molecular data have been undoubtedly useful in cases involving cryptic or similar-plumaged taxa, in which it is very difficult to make inferences based on morphology alone.

The American Screech-owl genus *Megascops*, recently split from *Otus* based on vocal and molecular evidence (van der Weyden,1975; Marshall and King, 1988; Wink *et al.*,

1999; Wink and Heidrich, 2000; Fuchs *et al.*, 2008) currently comprises 21 species and 63 taxa (Marks *et al*., 1999). Recognition of species limits and inferences about relationships is notoriously difficult in this genus, due to great plumage similarity among different taxa, and significant individual variation within most of them (Weske and Terborgh, 1981; Fitzpatrick and O'Neill, 1986; Sick, 1997; Wink and Heidrich, 2000). For instance, several subspecies described by Hekstra (1982) based on plumage are not currently accepted (Marks *et al.*, 1999). *Megascops albogularis*, one of the most distinct species in the genus, has sometimes been placed in the monotypic genus *Macabra* because of its overall appearance being so different from the other screech-owls, but molecular and vocal evidence indicate it is indeed part of *Megascops*, apparently closest to *M. choliba* (Wink *et al.*, 2009).

Previous phylogenic hypotheses exist for *Megascops* (Heidrich *et al.*, 1995; Wink and Heidrich, 2000; Proudfoot *et al.*, 2007; Wink *et al.*, 2009), but none has succeed in covering a significant part of the genus, or solve most of the internal relationships, since usually only partial sequences of one or two genes were employed in the analyses. Many species in the genus have several subspecies, with many of them previously claimed to be independent species level taxa based on morphological and vocal evidence, but coverage of subspecies by the previous molecular phylogenetic studies was scanty. As an illustration of this problem, *Megascops guatemalae* is either treated as one species with allopatric populations distributed from Mexico to the Bolivian Andes, or as two, three or four species (Marks *et al.*, 1999). Here, we present the most complete phylogenetic study on the genus *Megascops* to date, based on a broad taxon sampling regime which covered almost all species currently recognized. The proposed phylogeny is used to discuss the taxonomy and the historical diversification of *Megascops* in the Americas, with a particular focus in the Neotropics, the genus' stronghold.

2. Material and methods

2.1 DNA extraction, amplification and sequencing

We included in the analyses tissue samples from 51 individuals of 28 taxa (Appendix 1) in *Megascops* belonging to all species recognized by Marks *et al.* (1999), except one (*M. seductus*). DNA was extracted using DNeasy tissue extraction kit (Qiagen, Valencia, California) or with a phenol-chlorophorm protocol. We used sequences of six

different genes from four loci, one mitochondrial (cytochrome-b, NADH dehydrogenase subunits 2 and Cytochrome c oxidase subunit 1), and three nuclear (Intron 5 of the nuclear b-fibrinogen gene, CHD and MUSK). Primers used for each gene are listed in table 1. For amplification and sequencing of NADH dehydrogenase subunits 2 (ND2), two internal primers designed in this study were also used: L5758Mega (5'RRTGRGARATDGATGARAAGGC3') and H5776Mega (5'GGNTGRATRGGCYTRAACCARAC3').

Table 1: Primers used in the study.

gene	**primer**	**reference**
cytochrome-b (cytb, 1035 bps)	L14841	Kocher et al., 1989
	H16065	Helm-Bychowski and Cracraft, 1993
NADH dehydrogenase subunits 2 (ND2, 1040 bps)	L5215	Hackett, 1996
	H6313	Sorenson et al., 1999
	L5758Mega	This study
	H5776Mega	This study
Cytochrome c oxidase subunit 1 (CO1, 379 bps)	L6625, H7005	Hafner *et al*., 1994
Intron 5 of the nuclear b-fibrinogen gene (BF5, 560 bps)	FIB5L and FIB5H	Driskell and Christidis, 2004
CHD (349 bps)	CHD-18F, CHD-18R	Jacobsen *et al.*, 2010
MUSK (605 bps)	MUSK-F, MUSK-R	Kimball *et al.*, 2009

Fragments were PCR-amplified using standard conditions in thermocyclers: denaturation at 94°C, annealing between 46 and 56°C, and extension at 72°C, for 30 or 35 cycles. For the nuclear genes, the annealing temperature was incrementally decreased from 58°C for five cycles to 54 °C for five cycles and 50°C for 30 cycles. The PCR products were run on agarose gel to verify whether amplification was successful and of sufficient quantity to be sequenced. Most products were cleaned using Exonuclease and Shrimp Alkaline Phosphatase (ExoSap) enzymatic reactions (United States Biochemical), or using PEG 8000 20% NaCl 2,5 M. Some products that showed non-specific bands were cleaned by cutting a single band of PCR product from a low melt agarose gel and cleaning with GELase (Epicentre Technologies, Madison, WI) following the manufacturer's recommended protocol. The products of heterogeneous nuclear genes were cloned using Cloning PCR-Invitrogen kit, and the PCR product was recovered by direct amplification. Amplifications were cycle-sequenced using a BigDye 3.1 Terminator kit (BigDye, Applied Biosystems, Foster City, CA) and the same primers used for amplification. Cycle sequencing reactions were cleaned with ethanol EDTA precipitation, and resuspended in Hi-Di formamide. Sequences were then

visualized through an ABI 3730 automated sequencer and aligned and reconciled using the computer program Sequencher 3.1.1 (Gene Codes Corp, Ann Arbor, MI).

2.2. Phylogenetic Analysis

Pairwise uncorrected genetic distances between lineages were calculated with MEGA 5 (Tamura *et al.*, 2011), using only the concatenated mitochondrial data.

We aligned sequences of all sampled *Megascops* individuals on Sequencher or in Bioedit v. 7.1.3 (Tom Hall, Ibis Biosciences) programs, and concatenated then in one single dataset using the Java exe command CONCAT. Saturation in sequence bases was evaluated with DAMBE (Xia and Xie, 2001). We used *Otus flammeolus*, considered the sister group of *Megascops* (Proudfoot *et al.*, 2007), *Glaucidium peruanum* and *Otus megalotis* as outgroups. Phylogeny was reconstructed using Maximum Likelihood (ML) as implemented in PAUP 4.0d* and RAxML softwares, as well as concatenated Bayesian Inferences (BI) as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), and by a multilocus coalescent Species Tree (ST) analysis, as implemented in *BEAST v. 1.4 (Drummond and Rambaut, 2007). The best model of sequence evolution for each gene was obtained with MrModeltest 2.3 (Nylander, 2004), based on Akaike´s information criterion (AIC), and then applied to BI and ST analysis. We tested different partitioning schemes in BI, as follows: one partition (all data combined); two partitions (mitochondrial genes combined and nuclear combined); four (mitochondrial genes separated and nuclear genes combined; mitochondrial genes combined and nuclear genes separated); six (all genes separated). The best partitioning scheme selected was that will all mitochondrial genes separated and all nuclear genes combined. Two parallel runs, with four Markov chains and 10 million generations each, were carried out, sampling the chains every 500 generations.

We used the resulting 20,000 parameter point-estimates minus the burn-in generations (5,000) to create a 50% majority-rule consensus tree and to calculate Bayesian posterior probabilities (PP) to assess nodal support. Using TRACER, version 1.5 (Drummond and Rambaut, 2007), we determined that the chosen burn-in setting (10%) was sufficient for the log likelihood values of parallel runs to reach stationarity, with all parameters meeting benchmark effective sample-size values (>200).

A multilocus coalescent Species Tree Analysis was run in *BEAST. A file was created using Beauti (Drummond and Rambaut, 2007), and then run in *BEAST. Fourteen runs

of 100 million generations, sampling every 10,000 generations, were performed, in order to obtain the recomended ESS values (200 or higher).

2.3. Diversification timing and ancestral area reconstruction analyses

A multilocus coalescent molecular clock analysis was performed in *BEAST, assuming a Relaxed Uncorrelated Lognormal Clock Model (Drummond *et al.*, 2006) for all genes, using the best-fit substitution models selected previously for each locus. We used a Yule speciation process for the tree prior, and as time calibration a cytb 0.0105 substitution rate per million years (Weir and Schluter, 2008). MCMC chains were run for 10 million generations sampling every 1000 generations. TRACER v. 1.5 was used to visualize the posterior distributions for every parameter. A Statistical Dispersal-Vicariance Analysis (S-DIVA) was performed in RASP program (Yu *et al.*, 2010) to infer ancestral areas reconstructions for the genus. We divided the Americas into four main areas in this analysis: A) North America; B) trans-Andean South America and Central America; C) Andes; and D) cis-andean South America. We assigned each species to one or more areas, according to their distributions as in Marks *et al.* (1999).

3. Results

3.1. Sequences divergence

Transition versus transversion plots did not indicate saturation among ingroup taxa. Uncorrected mitochondrial sequence divergence levels among all *Megascops* taxa analyzed ranged from 0.7% (between two *M. watsonii usta* clades) to around 18% (*Otus flammeolus* and *M. nudipes* to the other species). The lowest divergence level between two different species was 2% (*M. w. usta* and *M. atricapilla*). The divergence levels among some populations of *M. vermiculatus, M. trichopsis, M. ingens* and *M. watsonii* are close to or above this minimum value.

3.2. Mitochondrial DNA

Megascops is paraphyletic genus if we include *M. nudipes* in it, which turned out to be sister to *Otus flammeolus*, although with low support according to ML (Fig. 1). In any event, ML and BI nodal support values for the placement of *M. nudipes* outside the main *Megascops* clade is high (Fig. 1). The remaining species (the "true" *Megascops*) are divided into three major clades, all with significant nodal support according to both

BI and ML analyses (Fig. 1). The first one is constituted by *M. choliba* and *M. koepckeae* grouped as sisters to *M. albogularis*, all these being mainly South American (SA) species (*M. choliba* is distributed as far north as Costa Rica); and this clade in turn is sister to *M. trichopsis* and *M. clarkii*, two mainly Central American (CA) taxa. *Megascops choliba duidae*, a Tepui subspecies with a very different plumage when compared to other *M. choliba* subspecies, is nested well inside this species´ clade (samples LSU 7413 and LSU 7420 in Fig. 1). The two *M. trichopsis* samples, from different subspecies (FMNH 394215: *M.t. trichopsis* and KUHNM 4931: *M. t. mesoamericanus*) diverged by 3.7% (uncorrected p distance), hence equaling the lowest level of divergence between different species according to the current taxonomy (*M. ingens* and *M. colombianus*; Marks *et al.*, 1999).

One of the other two clades contains the South American (SA) species *M. ingens, M. colombianus, M. petersoni, M. hoyi*, and *M. marshalli*. With the exception of the basal relationships in the *M. petersoni/hoyi/marshalli* clade, all nodal support values in this second major clade were moderate to high (Fig. 1). The *M. ingens* samples were divided into two groups, an Ecuadorian and a Peruvian one (Fig. 1). Even though they belong to the same subspecies (*ingens*), they differed genetically by 3.5% (uncorrected p distance).

The third clade contains the remaining SA, Central American (CA), and North American (NA) species. Basal relationships in this third clade received overall low statistical support, with the phylogenetic position of *M. asio, M. kennicottii* and *M. cooperi* with respect to one another essentially unresolved. Similarly, the placement of the well-supported *M. guatemalae* superspecies (*M. guatemalae* and *M. vermiculatus*) within this third major clade is also unresolved (Fig. 1). In contrast, internal relationships in this clade are all well supported, with "true" *M. guatemalae* (two samples from Mexico diverging from each other by a 2% uncorrected distance) being monophyletic with respect to the *M. vermiculatus* group. Within the latter species, the Tepuian (*roraimae*) and Andean (*napensis*) populations are sisters, with the Panamanian sample (*vermiculatus*) coming out as basal. Pairwise uncorrected genetic distances between *M. guatemalae* and *M. vermiculatus* samples ranged from 9.0% to 10.1%, with distances within *M. vermiculatus* ranging from 3.9% (*roraimae-napensis*) to 7.3% (*vermiculatus-napensis*). The remaining species include those in the well supported South American clade grouping *M. sanctacatarinae*, (Krabbe *et al.*, in prep.), *M. roboratus, M. watsonii,* and *M. atricapilla* (Fig. 1), and an individual from a population

from the Santa Marta Mountains (INC 38770) reported as *M. choliba* by Todd and Carriker (1922), but which didn´t fit with this or any of the recognized species in this study. This individual will be referred as "unnamed taxon" from now on. Within this clade, *M. watsonii* and *M. atricapilla* are paraphyletic species with respect to each other (Fig. 1). *Megascops roboratus*, an open habitat to deciduous forest species from western Ecuador and Peru, is the sister species to the *M. watsonii*/*atricapilla* clade, with the unnamed population from the Santa Marta mountains coming out as sister to this entire group, although with low statistical support (only a cytb sequence was available for this population). Finally, *Megascops sanctaecatarinae*, sometimes placed as sister to *M. atricapilla*, came out as sister to the entire exclusively South American clade cited above (Fig. 1).

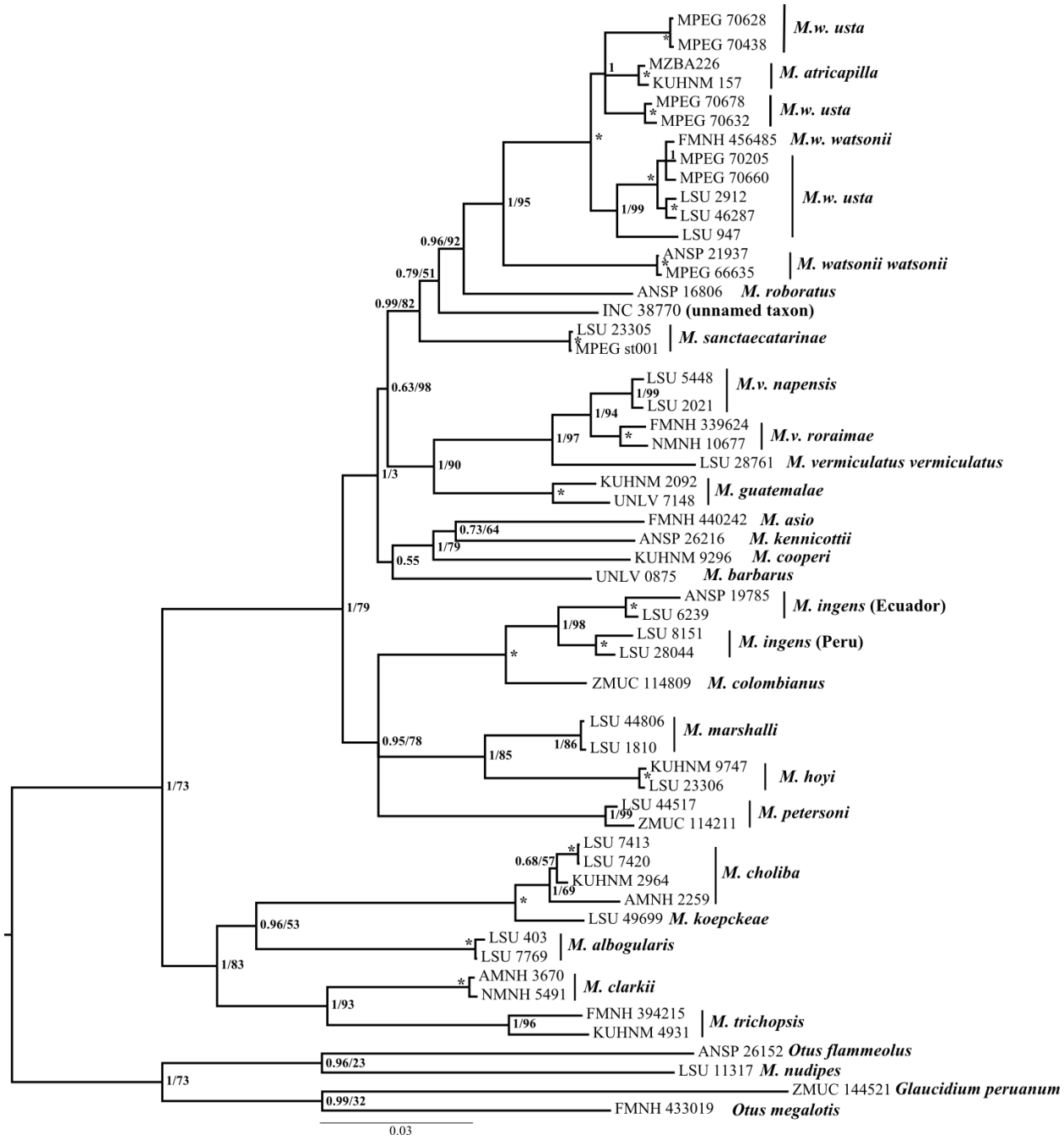

Figure 1. BI and ML phylogeny estimates based on the mitochondrial genes sequenced (cytb, ND2, CO1). Node numbers are BI/ML posterior probability and bootstrap support values, respectively. Asterisks associated with nodes indicate maximum support according to both BI posterior probability and ML bootstrap values. Nodes with a single value indicate that it was present either in BI or ML analysis.

3.3. Nuclear DNA

Most branches had low support values according to both ML and BI analyses based on the nuclear datas*et al*one (Fig. 2). Some clades recovered by these analyses agreed with

those obtaining using the mitochondrial dataset, such as the one uniting *Otus flammeolus* and *Megascops nudipes* (well supported according to ML), as well as those clades containing *M. albogularis, M. choliba* and *M. koepckeae*, and one containing the *M. vermiculatus* subspecies. Also, the nuclear data grouped the samples of *M. watsonii* and *M. atricapilla* in a single clade, as did the mitochondrial dataset. But even in these cases, statistical support for these relationships were low. When analyzed separately, the nuclear genes also yielded unresolved trees, especially in the case of CHD and MUSK (not shown). BF5 generally yielded higher support values for some nodes, particularly those containing *M. choliba/albogularis/koepckeae/trichopsis* (thus agreeing with mitochondrial results; not shown), and another containing *M. cooperi/ asio/ kenicotti/ sanctaecatarinae/ barbarus/ guatemalae* /*watsonii* /*colombianus*, which differed significantly from the trees based on the mitochondrial genes (Fig. 1).

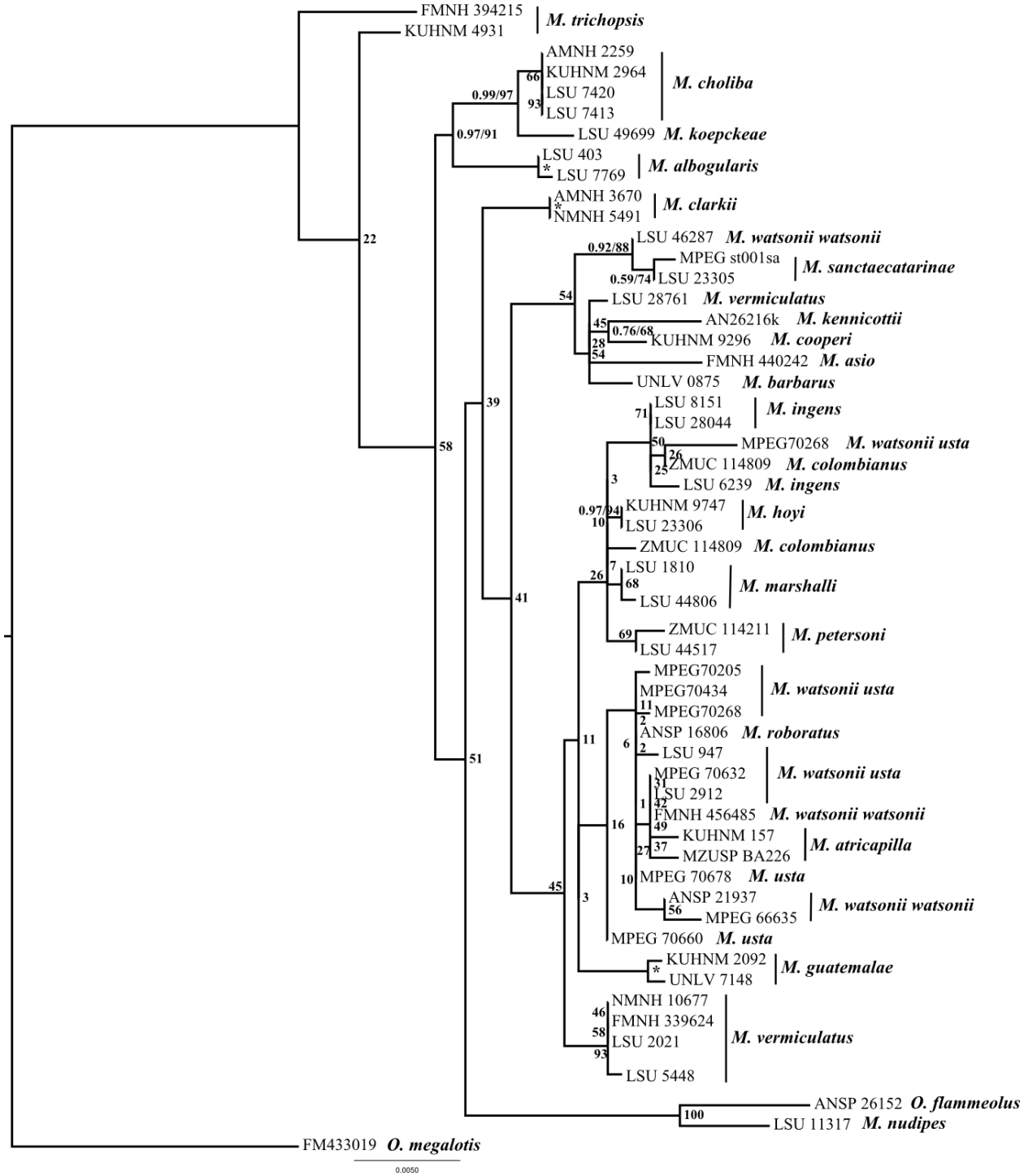

Figure 2. BI and ML phylogeny estimates based on the nuclear genes sequenced (BF5, CHD and MUSK). Node numbers are BI/ML posterior probability and bootstrap support values, respectively. Many branches are too short to be shown while others were not present on BI analysis.

3.4. Concatenated phylogeny estimates and species tree results

A four-partition model (by mitochondrial gene, with nuclear genes concatenated) was selected as the best partition scheme for the concatenated mitochondrial and nuclear genes in BI and ML phylogeny estimates (not shown). An important difference between the concatenated ML and BI trees with respect to the mitochondrial one is a clade uniting *M. sanctaecatarinae* (a southern SA taxon) with *M. asio, M. kennicotti, M. cooperi* and *M. barbarus* (four NA and CA species), in a moderate to well supported clade (BI posterior probablity= 85%; ML bootstrap =100%) . Other than that, the topology and nodal support values agreed in general with the mitochondrial results (Fig. 1).

Four species (*M. asio, M. kenicotti, M. roboratus* and the outgroup *Otus flammeolus*) were not included in the Species Tree (ST) analysis due to lack of sequences for some genes. Even after combining 14 runs of 100 million generations each, some ESS values were below the recommended threshold (200). Nonetheless, the overall typology of the ST tree converged on those estimated by ML and BI based on the concatenated and mitochondrial datasets, although with smaller support values for some of the nodes. *Megascops sanctaecatarinae,* instead of being clustered with *M. cooperi* and *M. barbarus,* grouped as sister to the *M. watsonii/atricapilla* clade, although with low support. *Megascops barbarus* and *M. cooperi* grouped together with moderately high support values, and together were sister to a clade including *M. watsonii, M. atricapilla, M. sanctaecatarinae,* and *M. guatemalae/ vermiculatus*.

3.5. Molecular dating and ancestral area reconstructions

The split between the "true" *Megascops* and the outgroup (*Megascops nudipes*), took place around 17 million years ago (mya), during the Miocene (Fig. 3). Most of speciation within the "true" *Megascops* is inferred as occurring within the last eight million years, when the first split within this clade took place (95% HPD: 4,99-11.19 mya). Pairwise divergence times between currently accepted species reciprocally monophyletic species ranged from 0,74 (0,24-1,34 mya; *choliba-koepckeae*) to 6 (3.4-9.1 mya; *albogularis* to *choliba/koepckeae*) mya.

According to the S-DIVA analysis, *Megascops* most probably originated in the Andes (Fig. 4), further spreading over CA, NA and Cis-Andean SA, which was colonized twice by Andean ancestors. One colonization event occupied open areas (*M. choliba*), while the other occupied forest habitats in the Amazonian and Atlantic forest lowlands

as well as southern Cis-Andean mountain ranges (*M. watsonii*, *M.w. usta*, *M. atricapilla*, and *M. sanctaecatarinae*). Central America was colonized three times, from Andean ancestors, according to S-DIVA analysis, while North America was colonized once (or twice, if we consider *M. trichopsis*, which ranges into the southwestern USA). South America was recolonized from CA once, by the ancestor of the lineage leading to *M. vermiculatus*.

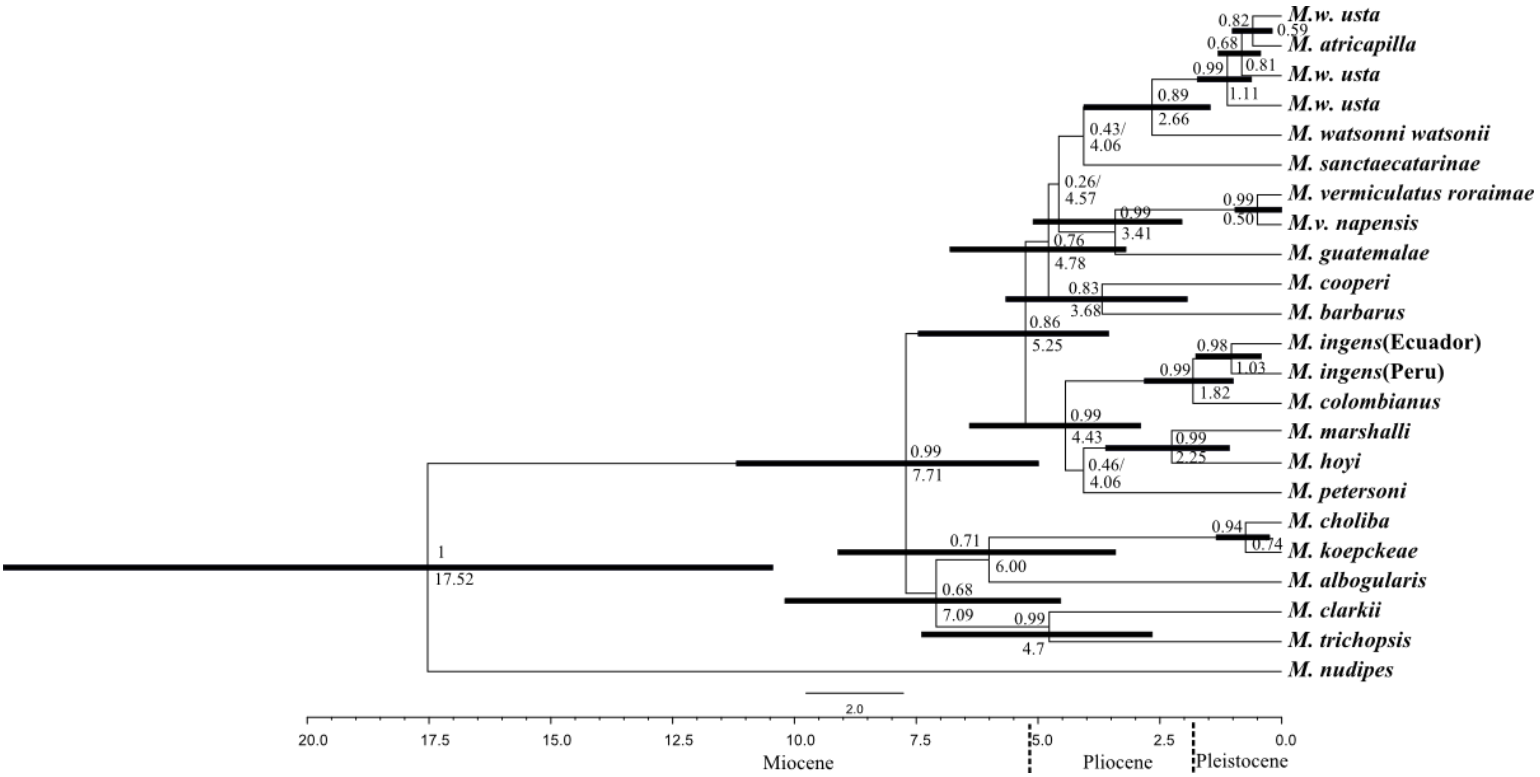

Figure 3. Multilocus coalescent species tree and the resulting dating analysis. Black bars denote 95% age estimates credible intervals for the respective nodes, with numbers above and below black bars indicating posterior probability values and node ages.

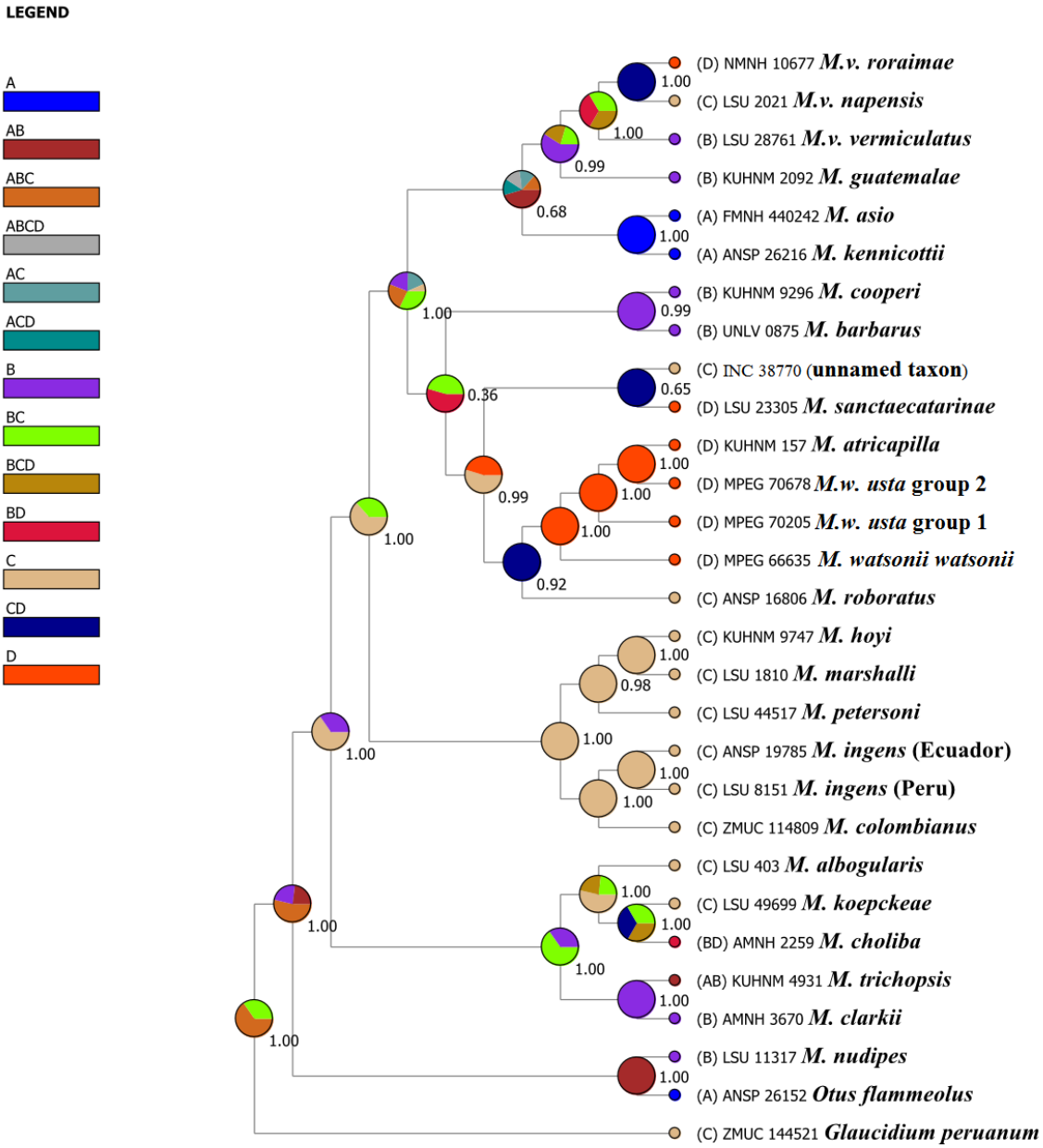

Figure 4. Ancestral area state reconstructions in *Megascops* by a RASP Statistical Dispersal-Vicariance Analysis (S-DIVA), based on concatenated mitochondrial and nuclear genes data. Letters in legend and before sample identifications refer to the area assigned to them: A = North America; B = Trans-Andean South America and Central America; C = Andes; D= Cis-Andean South America. Numbers refer to S-DIVA support values.

4. Discussion

4.1. Systematics and taxonomy of the genus Megascops

Our study provides a comprehensive phylogeny for all the species of the genus *Megascops*, except *M. seductus*, a species with a very small range in southwestern Mexico that is sometimes treated as a subspecies of either *M. asio* or *M. kennicottii*, and placed in the same superspecies as the last two species in addition to *M. cooperi* (Marks *et al.,* 1999). Our mitochondrial tree recovered a well-supported node uniting *M. asio*/*kennicottii/cooperi* in a single clade (Fig. 1), hence supporting the treatment of these taxa as members of a superspecies. Therefore, since particularly in this instance the traditional taxonomy based on morphological characters and the molecular data agree, it is likely that the only species missing from our sample would cluster either within or nearby the *M. asio*/*kennicottii/cooperi* clade.

Megascops nudipes (Puerto Rican Screech-owl) clearly does not belong in the genus, being instead recovered with high statistical nodal support by both mitochondrial and nuclear datasets as the sister species of *Otus flammeolus* (Figs. 1, 2, and 4), a western NA species with at least some populations wintering in CA (Marks *et al.*, 1999). The *M. nudipes*/*Otus flammeolus* clade in turn does not cluster within the Old World Screech-owls group (the true *Otus*), with the one species sequenced (the Asian *O. megalotis*) grouping with *Glauciiudm* rather than the new world *O. flammeolus*. This result mirrors those of Proudfoot *et al.,* (2007) and Wink *et al.*, (2009), whereby *O. flammeolus* was recovered as the sister group to *Megascops* with high support rather than with the old world *Otus*. This finding prompted Wink *et al.*, (2009) to resurrect the genus *Psiloscops* for allocating *O. flammeolus*, a taxonomic treatment also supported by the present dataset. If future studies with a broader taxonomic sampling of the new world owls also recover *O. flammeolus* and *M. nudipes* as sister species, the latter should also be assigned to the genus *Psiloscopus*, or even to a separate genus. However, given our limited generic sampling, we recommend that *M. nudipes* is be kept in *Megascops* until more reliable information concerning its phylogenetic position becomes available.

Even though our phylogenetic trees resolved relationships among many species and clades, some large groups remained with their phylogenetic positions undetermined. For instance, the *M. guatemalae/vermiculatus* clade was not placed with strong statistical support as sister to any particular lineage within the large clade uniting *M. barbarus/ cooperi/ kennicottii/ asio/ guatemalae/ vermiculatus/ sanctaecatarinae/ roboratus/*

watsonii/ atricapilla (Fig. 1). Similar results were found concerning the basal relationships in the otherwise well-supported *M. petersoni/ hoyi/ marshalli/ colombianus/ ingens* clade (Fig. 1). The phylogenetic position of *M. sanctaecatarinae* changed according to the mitochondrial (Fig. 1) and nuclear (Fig. 2) datasets, and was apparently highly influenced by BF5 sequences alone; the multi-locus coalescent species tree recovered *M. sanctaecatarinae* in the same position as that recovered by the mitochondrial trees (Figs. 1 and 3) i.e., sister to the *M. watsonii/ atricapilla/ roboratus/* Santa Marta clade, but with low statistical support. Hence, the phylogenetic placement of *M. sanctaecatarinae* in the genus *Megascops* is still unresolved, reflecting previous assessments based on morphological and vocal evidence (Marks *et al.,* 1999).

Our molecular data confirmed some presumed relationships based on plumage and vocalizations, such as that between *M. koepckeae* and *M. choliba*, as the first one was described as a subspecies of the latter (Hekstra, 1982) but later elevated to full species status (Fjeldså *et al.,* 2012). According to the current taxonomy, the only paraphyletic species recovered by the molecular data was *M. watsonii*, with two populations from eastern Amazonia grouping with high support in a clade with *M. atricapilla*. These results also indicate that *M. watsonii* includes several species level lineages nested within it, but only three of them with already available names (*usta*, *watsonii* and *ater*; Peters 1940; Hekstra, 1982). Detailed analyses dealing with the phylogeography and species limits in the *M. watsonii / atricapilla* clade will be published elsewhere (Dantas *et al.*, in prep). Some other species, although monophyletic, showed considerable genetic variation among populations, indicating long-term isolation, and probably separate species level status. For instance, the molecular data favor not only the split between *M. guatemalae* and *M. vermiculatus* (as adopted by Marks *et al.,* 1999 but not by Remsen *et al*., 2013), but also that *M. vermiculatus* includes three main reciprocally monophyletic and statistically well-supported groups, each corresponding to a recognized subspecies. Average pairwise p-uncorrected genetic distances among these clades (6%) are higher than that between two currently recognized species based on morphological evidence (Marks *et al.,* 1999): *M. atricapilla* and its closest *M. watsonii* populations (2%). We suggest *M.v. vermiculatus, M.v. roraimae and M.v. napensis* should be attributed full species status, as already suggested by some authors (König and Weick, 2008), based on morphological and vocal differences between them. Two Eastern Andean slope populations of *M. ingens* from Ecuador and Peru, regarded as belonging to the same taxon (*M. ingens ingens*) diverged by an average 3.5% p-

uncorrected distance. The central Mexican (*trichopsis*) and El-Salvadorian (*mesoamericanus*) samples of *M. trichopsis* diverge by an average 4.1% p-uncorrected distance. These results may be an indication of genetic differentiation within the species, although they are not sufficient to advocate their split as separate taxa. Molecular studies with better sampling regimes and preferably complemented with morphological as well as vocal characters are desired before more species are split off *M. ingens* and *M. trichopsis*. The sample from Santa Marta mts. (INC 38770) comes from a population identified as *M. choliba* by Todd and Carriker (1922), although the authors noted that it's very distinct from that species, and could represent an unknown taxon. The sample did not fall within the clade of *M. choliba* or any other *Megascops* clade, and its lowest uncorrected distance to other groups in this study (6.1%, between it and *M.w. usta*) is higher than that between two recognized species. These results support the recognition of this population as a distinctive species.

4.2. Historical biogeography

According to the dating analyses, the first split within *Megascops* took place in the Miocene, ca. 8 mya, separating the widespread *M. choliba/ koepckeae/ albogularis/ clarkii/ trichopsis* clade from the remaining species (Figs. 3 and 4). The ancestral area state reconstructions support an Andean / trans-Andean ancestral distribution for the ancestor of all "true" *Megascops* species (Fig. 4), which is consistent with most endemic species in the genus (nine) being distributed there. *Megascops* diversification in the Andes took place during the last 7 million years, with most species originating since the Pliocene, i.e., in the last 4.5 million years. Most of them inhabit the Central and Southern Andes, with only three species found north of Ecuador. The uplift of the Andes is accepted to have had a very important role in the diversification of the Neotropical avifauna, by producing a series of isolated areas / habitats, where populations could colonize and evolve independently (Sedano and Burns, 2009; Chaves *et al.*, 2010; Weir and Price, 2011). The Andean uplift occurred from south to north, with the ages associated with nodes of the more central and southern species older than those of the northernmost ones, as observed in a clade that contains only Andean species (*M. ingens, M. colombianus, M. petersoni, M. clarkii*, and *M. hoyi*). In a similar way, Bonaccorso *et al.* (2011) found some southern clades of *Aulacorhynchus* toucanets to be basal to more northern clades, in the Andes. On the other hand, *M. albogularis*, occurring in the central / northern Andes, has an ancient origin (ca. 6 mya), with *M.*

koepckeae and *M.vermiculatus napensis,* both from the central Andes, having a relatively recent origin. This can be observed in other avian taxa, like the superspecies *Drymophila caudata* (Isler *et al.*, 2012), which diversified first in the northern Andes. Orogeny of the Andes seems to have played a striking role in the diversification of *Megascops,* and this process seems to have occurred in many geographic directions.

Around 7 mya, the trans-Andean / North-American clade *M. clarkii/trichopsis* split from the widespread *M. choliba/koepckeae/albogularis* clade, with the ancestor to all these species estimated as having been distributed most likely in the Andes and Trans-Andean South America (Fig. 4). According to ancestral area reconstructions, Central America was colonized at least three times independently by *Megascops,* while North America at least twice (Fig. 4). The timing of these events span the last 7 mya, hence, before the estimated time for the end of the Andes uplift (Gregory-Wodzicki, 2000) and after the estimated time for the buildup of the Panama isthmus (Farris *et al.*, 2011). Most CA *Megascops* species occupy montane habitats, and are estimated as having evolved from Andean ancestors, making the crossing of the northern Andes more feasible for these lineages. Even after the end of the uplift of the northern Andes, some SA groups crossed into CA, such as capuchin monkeys (Alfaro *et al.*, 2012) and *Dendrocincla* woodcreepers (Weir and Price, 2011), so permeability must not have been so limited at that area. Closure of the Panama isthmus has been estimated to 3.5 mya (Coates and Obando, 1996), but recent theories have suggested this event to have occurred 12-20 mya (Farris *et al.*, 2011). Under this assumption, many CA avian colonization events, like the ones by core tanagers (Sedano and Burns, 2009), doves (Johnson and Weckstein, 2011), low-dispersal species as *Sclerurus* (d´Horta *et al.*, 2013) and *Megascops* all took place after the completion of the isthmus.

Cis-Andean South America was colonized at least three times, probably by Andean ancestors in all instances, during the last 5 million years (Fig. 4). Only one *Megascops* lineage diversified intensively in the Cis-Andean lowlands (*M. watsonii / atricapilla*). The first splitting event (dates to about 2.6 mya) isolated Guianan shield populations of *M. watsonii* from the remaining ones in the *M. watsonii / atricapilla* clade and could be related to the formation of the Amazon and Negro rivers in the Amazonian lowlands. This is consistent with the timing suggested for the origin of the modern trans-continental Amazon river (ca. 2.5 mya; Ribas *et al.*, 2012, d´Horta *et al.*, 2013). Populations outside the Guianan Shield later got divided in three Amazonian clades, separated to a some degree (two of them may come in contact) by rivers Tapajós and

Tocantins, in southeast Brazilian Amazon basin, and one Atlantic Forest ones. These population split events, plus the separation of East Amazonian and Atlantic Forest populations are inferred to have occurred in the last one to two million years, a similar time range of the split of east amazonian populations of *Psophia* (Ribas *et al.*, 2011) and *Sclerurus* (d´Horta *et al.*, 2013). The relations and biogeographical history of these Amazonian and Atlantic Forest species complexes will be detailed in chapter 02 of this thesis.

The present study clarifies the taxonomic situation of some species groups, like *M. guatemalae* and *M. vermiculatus*. Also, suggests a scenary where there is still a great amount of work to be done on phylogenetics and taxonomy of the genus *Megascops*. Better sampling of other species with some degree of differentiation within them, like *M. ingens* and *M. trichopsis*, will clear the taxonomic situation of these and other taxa. Better sampling will also be crucial to understand the phylogenetic position of many species, especially between the North and Central American species group *M. asio, M. kennicottii*, *M. barbarus* and *M. cooperi*. Our study was a first step on recovering the phylogeographical story of *Megascops*, and future studies on this group might help us to fully comprehend the diversification of bird species in the Neotropics.

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Appendix 1. Tissue samples.

Acronym for the source institutions: AMNH = American Museum of Natural History; ANSP = Academy of Natural Sciences of Drexel University; FMNH = Field Museum of Natural History; KUNHM = Kansas University Natural History Museum; LSUMZ = Louisiana State University Museum of Natural Science; MPEG = Museu Paraense Emilio Goeldi; MZUSP = Museu de Zoologia da Universidade de São Paulo; NMNH = Smithsonian Institution National Museum of Natural History; UNLV = University of Nevada, Las Vegas; ZMUC = Zoological Museum – University of Copenhagen.

Source	Tissue number	Taxon	Locality
LSUMNS	7769	*Megascops albogularis aequatorialis*	Ecuador; Azuay Province.
LSUMNS	403	*Megascops albogularis remotus*	Peru; Piura Department.
FMNH	440242	*Megascops asio naevius*	USA, Illinois, Cook County, Lemont.
MZUSP	BA226	*Megascops atricapilla*	Brazil; RPPN Serra Bonita, BA.
KUNHM	157	*Megascops atricapilla*	Paraguay; Concepción, San Luis National Park
UNLV	JK08-75	*Megascops barbarus*	Mexico; Chiapas, San Cristobal.
AMNH	2259	*Megascops choliba suturutus*	Bolivia; Santa Cruz, Prov. Cordillera.
KUNHM	2964	*Megascops choliba choliba*	Paraguay
LSUMNS	7420	*Megascops choliba duidae*	Venezuela; Amazonas territory.
LSUMNS	7413	*Megascops choliba duidae*	Venezuela; Amazonas territory.
AMNH	3670	*Megascops clarkii*	Costa Rica; Cartago.
NMNH	5491	*Megascops clarkii*	Panama; Chiriqui.
ZMUC	114809	*Megascops colombianus*	Ecuador; Masquipucuna, Pichincha.
KUNHM	2092	*Megascops guatemalae thompsoni*	Mexico, Campeche, Silvituc
UNLV	7148	*Megascops guatemalae guatemalae*	Mexico; Oaxaca.
LSUMNS	28761	*Megascops vermiculatus vermiculatus*	Panama; Colón Province; Achiote Road.
NMNH	10677	*Megascops vermiculatus roraimae*	Guyana; Acari Mountains.
FMNH	339624	*Megascops vermiculatus roraimae*	Venezuela; Bolivar, Santa Elena Highway.
LSUMNS	2021	*Megascops vermiculatus napensis*	Peru; Pasco Department.
LSUMNS	5448	*Megascops vermiculatus napensis*	Peru; San Martin Department.
KUNHM	9747	*Megascops hoyi*	Argentina.
LSUMNS	23306	*Megascops hoyi*	Argentina; Misiones Province.
ANSP	19785	*Megascops ingens ingens*	Ecuador; Zamora-Chinchipe.
LSUMNS	6239	*Megascops ingens ingens*	Ecuador; Morona-Santiago Province.
LSUMNS	8151	*Megascops ingens ingens*	Peru; Pasco Department.
LSUMNS	28044	*Megascops ingens ingens*	Peru; Loreto Department.
ANSP	26216	*Megascops kennicottii yumanensis*	USA; New Mexico.
LSUMNS	49699	*Megascops koepckae*	Peru; La Libertad Department.
LSUMNS	44806	*Megascops marshallii*	Peru; Pasco Department.
LSUMNS	1810	*Megascops marshallii*	Peru; Pasco Department.

Source	Tissue number	Taxon	Locality
LSUMNS	11317	*Megascops nudipes nudipes*	Puerto Rico; San German.
ZMUC	114211	*Megascops petersoni*	Ecuador; Zamora-Chinchipe.
LSUMNS	44517	*Megascops petersoni*	Peru; San Martin Department.
ANSP	16806	*Megascops roboratuspacificus*	Ecuador; Loja.
LSUMNS	23305	*Megascops sanctaecatarinae*	Argentina; Misiones Province.
MPEG	st001	*Megascops sanctaecatarinae*	Brazil; Chapecó, SC.
KUNHM	4931	*Megascops trichopsis mesoamericanus*	El Salvador; Morazan.
FMNH	394215	*Megascops trichopsis trichopsis*	Mexico; km 14 de la carretera Ocuilan-Cuernavaca.
FMNH	456485	*Megascops watsonii watsonii*	Brazil; Japurá, AM.
ANSP	21937	*Megascops watsonii watsonii*	Guyana, Potaro-Siparuni
MPEG	66635	*Megascops watsonii watsonii*	Brazil; Óbidos, PA.
LSUMNS	2912	*Megascops watsonii usta*	Peru: Loreto Dept.
LSUMNS	947	*Megascops watsonii usta*	Bolivia; La Paz.
LSUMNS	46287	*Megascops watsonii usta*	Peru; San Martin.
MPEG	70660	*Megascops watsonii usta*	Brazil; Porto Velho, RO.
MPEG	70678	*Megascops watsonii usta*	Brazil; Belterra, PA.
MPEG	70205	*Megascops watsonii usta*	Brazil; Querência, MT.
MPEG	70632	*Megascops watsonii usta*	Brazil; Parauapebas, PA.
MPEG	70268	*Megascops watsonii usta*	Brazil; Benevides, PA.
MPEG	70434	*Megascops watsonii usta*	Brazil; Benevides, PA.
INC	38770	Unnamed taxon	Colombia; Sierra Nevada de Santa Marta.
ZMUC	144521	*Glaucidium peruanum*	Apurimac, Peru.
ANSP	26152	*Otus flammeolus*	USA; New Mexico.
FMNH	433019	*Otus megalotis*	Phillipines; Luzon.

Gene	Primer	Reference
cytochrome-b (cytb, 1035 bps)	L14841	Kocher et al., 1989
	H16065	Helm-Bychowski and Cracraft, 1993
NADH dehydrogenase subunits 2 (ND2, 1040 bps)	L5215	Hackett, 1996
	H6313	Sorenson et al., 1999
	L5758Mega	This study
	H5776Mega	This study
Cytochrome c oxidase subunit 1 (CO1, 379 bps)	L6625, H7005	Hafner *et al*., 1994
Intron 5 of the nuclear b-fibrinogen gene (BF5, 560 bps)	FIB5L and FIB5H	Driskell and Christidis, 2004
CHD (349 bps)	CHD-18F, CHD-18R	Jacobsen *et al.*, 2010
MUSK (605 bps)	MUSK-F, MUSK-R	Kimball *et al.*, 2009

4. CAPÍTULO 2.

MULTI-CHARACTER TAXONOMIC REVIEW AND SYSTEMATICS OF THE TAWNY-BELLIED / BLACK-CAPPED SCREECH-OWL COMPLEX (*Megascops watsonii/ atricapilla*)

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Abstract

Megascops is the richest owl genus in the New World, with more than 20 species. Phylogenetic relationships in this group are notoriously difficult to establish due to much plumage similarity among species and considerable polymorphism within species. Previous systematic studies suggested that the widespread lowland Amazonian *M. watsonii* might include more than one species, and that the Atlantic Forest endemic *M. atricapilla* is closely related to the previous group, but these relationships are as yet poorly understood. A recent phylogeny one of the genus *Megascops* showed that *M. watsonii* is paraphyletic with respect to *M. atricapilla* and that genetic divergences among some populations of *M. watsonii* equal or surpass the degree of differentiation between some populations of *M. watsonii* and *M. atricapilla*. To shed light on the taxonomic status of these species and populations, we conducted a multi-character study based on molecular, morphological, and vocal characters. We sequenced three mitochondrial (cytb, CO1 and ND2) and three nuclear genes (BF5, CHD and MUSK)

for 49 tissue samples, covering most of *M. watsonii* and *M. atricapilla* ranges, and used these sequences to estimate phylogenies under alternative Bayesian, Maximum Likelihood, and a multilocus coalescent species tree approach. We took morphometric measurements from 251 specimens, and measured vocal parameters from 83 recordings from 83 individuals, distributed throughout the ranges of *M. watsonii* and *M. atricapilla*. We used Discriminant Function Analysis (DFA) to analyze both morphometric and vocal data, and a pairwise diagnostic test to evaluate the significance of vocal differences between distinct genetic lineages. Phylogenetic analyses consistently recovered six statistically well-supported clades whose relationships are not entirely in agreement with currently recognized species limits in *M. watsonii* and *M. atricapilla*. Morphometric analyses did not detect significant differences among clades, with broad individual plumage variation (usually two or more color morphs, among other plumage details highly variable) within clades also preventing consistent morphological diagnoses. In contrast, vocal analyses detected significant differentiation among some clades but considerable overlap among others, with some populations (particularly the most widespread one) exhibiting significant regional variation. The combined results allow for a redefinition of species limits in both *M. watsonii* and *M. atricapilla*, with the recognition of three additional species, two of them not yet named. Most cladogensis in the *M. watsonii* / *M. atricapilla* complex is estimated as having taken place during the Plio-Pleistocene, with the development of the modern Amazonian and São Francisco drainages and the expansion / retraction of forest biomes during interglacial / glacial periods as likely events to have accounted for this relatively recent burst of diversification.

Key words: Taxonomy, systematics, *Megascops watsonii, Megascops atricapilla*.

1. Introduction

The genus *Megascops* Kaup, 1848 (Strigidae), with about 21 recognized species distributed throughout the Americas (Marks et al., 1999), is the largest exclusively Neotropical owl genus. In 1910, it was lumped as a subgenus of *Otus* Pennant, 1769, and later separated based on vocal and genetic differences (van der Weyden,1975; Marshall and King, 1988; Wink and Heidrich, 1999; Wink and Heidrich, 2000; Fuchs *et al.*, 2008). *Megascops* is a genus that comprises small to medium-sized owls inhabiting a large variety of terrestrial habitats, reaching its maximum species diversity in

mountainous areas, such as the Andes and Central America (Marks et al., 1999). Historically, phylogenetic relationships in this group are notoriously difficult to establish due to much plumage similarity among species and considerable polymorphism within species. Cryptic coloration and conserved appearance are common features in nocturnal birds such as owls and nightjars (Han et al., 2010), and traditional taxonomy (based mainly on morphological characters) may be deficient in bringing a more accurate knowledge of relationships and taxonomy of these groups. The complex variation in plumage and vocal characters, not always correlated with speciation (Roulin *et al.*, 2011), urges for an independent genetically based appraisal of the taxonomic status of many species and populations in the genus *Megascops*.

In *Megascops*, some currently recognized species such as *M. watsonii* (Cassin, 1848) have uncertain limits, with some of its populations grouped under *M. w. usta* (Sclater, 1858) being historically treated alternatively as species or subspecies (Marks et al., 1999). Nonetheless, the current treatment (Remsen et al., 2013) does not consider *usta* a separate species, mainly because of "inadequate geographic sampling and analysis" of previous studies investigating evolutionary relationships in *Megascops* (König et al., 1999, Wink et al., 2008). The treatment of *M. w. usta* as an independent species from *M. watsonii* has been suggested on basis of plumage and vocal differences, with *usta* possessing a more reddish (rather than grayish) overall plumage and a slower-paced song (König et al., 1999; Marks *et al*., 1999; König and Weick, 2008) . However, the high degree of plumage and vocal variation in any given population of *M. watsonii* challenges these generalizations, with both reddish and grayish color morphs and faster-paced songs present in populations of the proposed *M. usta* split (SMD pers. obs.). There is contradiction in the literature concerning the morphological diagnoses in this group, with one source (Marks et al,. 1999) stating that *M. w. usta* is slightly smaller than *M. w. watsonii*, and other (König and Weick 2008) positing the opposite. Vocal differences between bird populations are sometimes regarded as indicative of speciation (Isler *et al*., 1998; Remsen, 2005), but no detailed vocal analyses are available for most *Megascops* species, including *M. watsonii* and *M. atricapilla*, for which the only vocal study available (König et al., 1999) was regarded as insufficient to provide a robust framework for splitting populations in these taxa (Remsen et al., 2013). From a molecular standpoint, recent studies (Heidrich et al., 1995; Dantas et al., in prep.) have recovered *M. watsonii* populations as paraphyletic with respect to *M. atricapilla* (Black-Capped Screech-Owl) of the Atlantic Forest in eastern South America, hence in

accordance with a previous treatment of *M. watsonii* / *atricapilla* as a single species (Hekstra 1982). Therefore, depending on the taxonomic source, between one and three different species are recognized in *M. watsonii* / *atricapilla* underscoring the difficulty in establishing interspecific limits in this group.

It has been suggested that a recently discovered population of *M. atricapilla* in the northernmost portion of the Atlantic Forest in Brazil represents a distinct species on the basis on its isolation and differences in morphology and vocalizations (Roda and Pereira, 2006), but this has not been evaluated by any taxonomic study to date (Roda and Pereira, 2006). If this recently discovered population of *M. atricapilla* truly represents a distinct evolutionary lineage, it would be in extreme danger of extinction due to severe habitat fragmentation within its tiny range. To assess this and other uncertainties concerning the systematics and taxonomy of *M. watsonii* and *M. atricapilla* populations, we conducted a multi-character study to provide a framework for guiding taxonomic decisions concerning the ranking of these taxa.

2. Materials and methods

2.1. DNA extraction, amplification and sequencing

We obtained tissue samples from 49 individuals of *Megascops watsonii* and *M. atricapilla* (Fig. 1; Appendix 1), in addition to species belonging to the outgroup (*M. choliba, M. roboratus* and *M. sanctaecatarinae*), which were chosen based on previous taxonomic studies and a nearly complete molecular phylogeny for *Megascops* (Dantas et al., in prep.). DNA was extracted using DNeasy tissue extraction kit (Qiagen, Valencia, California) or with a phenol-chlorophorm protocol. We used sequences of six different genes from four loci, one mitochondrial (cytochrome-b, NADH dehydrogenase subunits 2 and Cytochrome c oxidase subunit 1), and three nuclear (Intron 5 of the nuclear b-fibrinogen gene, CHD and MUSK). The primers used for sequencing are listed in table 1.

Table 1: Primers used in the study.

gene	**primer**	**reference**
cytochrome-b (cytb, 1035 bps)	L14841	Kocher et al., 1989
	H16065	Helm-Bychowski and Cracraft, 1993
NADH dehydrogenase subunits 2 (ND2, 1040 bps)	L5215	Hackett, 1996
	H6313	Sorenson et al., 1999
	L5758Mega	This study

	H5776Mega	This study
Cytochrome c oxidase subunit 1 (CO1, 379 bps)	L6625, H7005	Hafner *et al*., 1994
Intron 5 of the nuclear b-fibrinogen gene (BF5, 560 bps)	FIB5L and FIB5H	Driskell and Christidis, 2004
CHD (349 bps)	CHD-18F, CHD-18R	Jacobsen *et al.*, 2010
MUSK (605 bps)	MUSK-F, MUSK-R	Kimball *et al.*, 2009

Fragments were PCR amplified using standard conditions in thermocyclers: denaturation at 94°C, annealing between 46 and 56°C, and extension at 72°C, for 30 or 35 cycles. For the nuclear genes, the annealing temperature was incrementally decreased from 58°C for five cycles to 54 °C for five cycles and 50°C for 30 cycles. The PCR products were run on agarose gel to verify whether amplification was successful and of sufficient quantity to be sequenced. Most products were cleaned using Exonuclease and Shrimp Alkaline Phosphatase (ExoSap) enzymatic reactions (United States Biochemical), or using PEG 8000 20% NaCl 2,5 M. Some products that showed non-specific bands were cleaned by cutting a single band of PCR product from a low melt agarose gel and cleaning with GELase (Epicentre Technologies, Madison, WI) following the manufacturer's recommended protocol. The products of heterogeneous nuclear genes were cloned using Cloning PCR-Invitrogen kit, and the PCR product was recovered by direct amplification. Amplifications were cycle-sequenced using a BigDye 3.1 Terminator kit (BigDye, Applied Biosystems, Foster City, CA) and the same primers used for amplification. Cycle sequencing reactions were cleaned with ethanol EDTA precipitation, and resuspended in Hi-Di formamide. Sequences were then read by an ABI 3730 automated sequencer and aligned and reconciled using the computer program Sequencher 3.1.1 (Gene Codes Corp, Ann Arbor, MI).

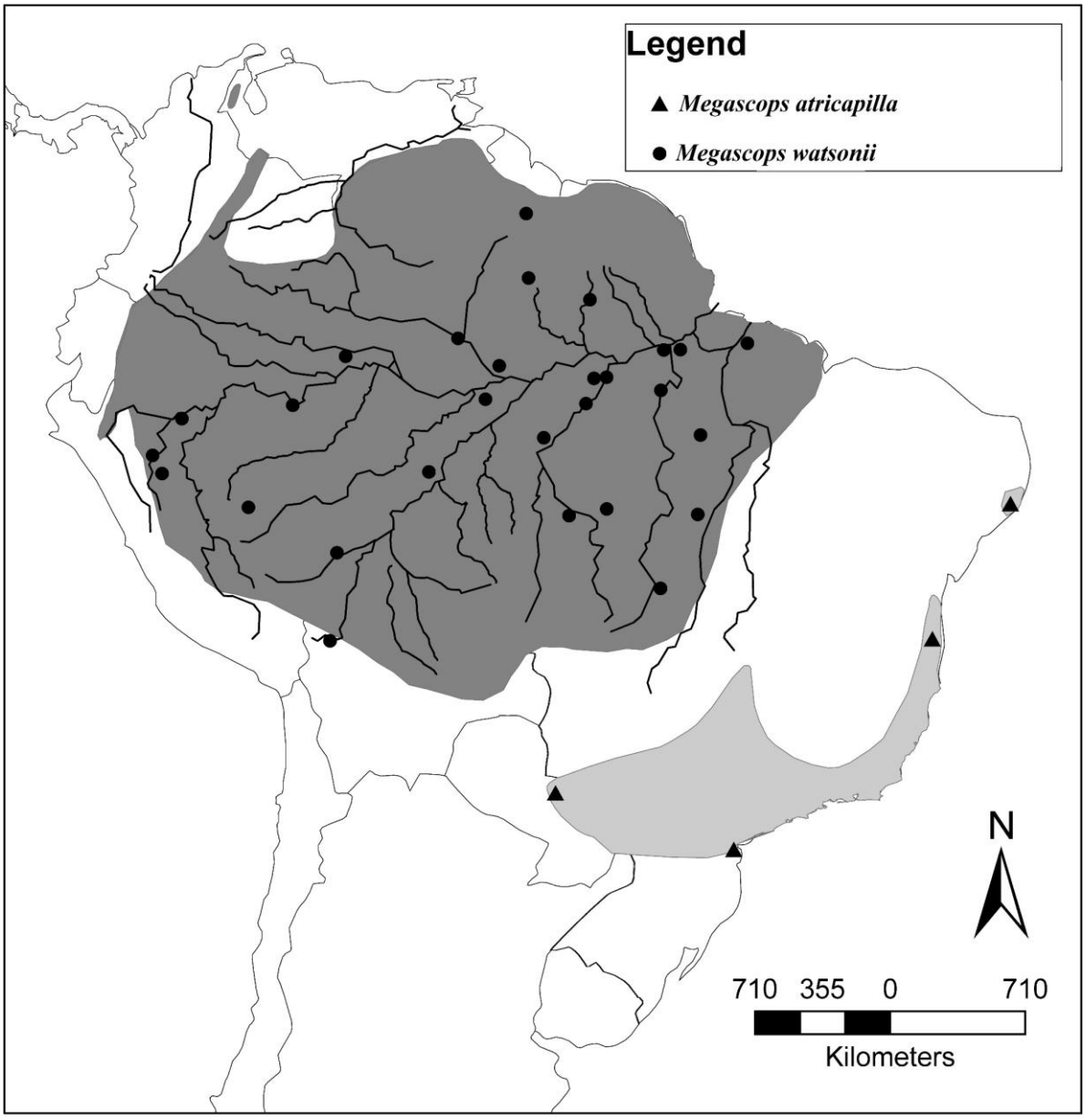

Figure 1: Geographic distribution of tissue samples of *Megascops watsonii* and *M. atricapilla* examined and included in the analyses. Shaded areas correspond to the allopatric distributions of *M. watsonii* and *M. atricapilla* as in InfoNatura (2007).

2.2. Phylogenetic Analyses

We aligned sequences of all sampled *Megascops watsonii* / *atricapilla* individuals and outgroups on Sequencher or in Bioedit v. 7.1.3 (Tom Hall, Ibis Biosciences) programs, and concatenated then in one single dataset using Java exe command CONCAT. Phylogeny was reconstructed using Maximum Likelihood (ML) as implemented in PAUP 4.0d* and RAxML softwares, as well as concatenated Bayesian Inferences (BI) as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), and by a

multilocus coalescent Species Tree (ST) analysis, as implemented in *BEAST v. 1.4 (Drummond and Rambaut, 2007). The best model of sequence evolution for each gene was obtained with MrModeltest 2.3 (Nylander, 2004), based on Akaike´s information criterion (AIC), and then applied to BI and ST analysis. We tested different partitioning schemes in BI, as follows: one partition (all data combined); two partitions (mitochondrial genes combined and nuclear combined); four (mitochondrial genes separated and nuclear genes combined; mitochondrial genes combined and nuclear genes separated); six (all genes separated). The best partitioning scheme selected was that will all mitochondrial genes separated and all nuclear genes combined. Two parallel runs, with four Markov chains and 10 million generations each, were carried out, sampling the chains every 500 generations.

We used the resulting 10,000 parameter point-estimates minus the burn-in generations (1,000) to create a 50% majority-rule consensus tree and to calculate Bayesian posterior probabilities (PP) to assess nodal support. Using TRACER, version 1.5 (Drummond and Rambaut 2007), we determined that the chosen burn-in setting (10%) was sufficient for the log likelihood values of parallel runs to reach stationarity, with all parameters meeting benchmark effective sample-size values (>200).

A multilocus coalescent Species Tree Analysis was run in *BEAST. A file was created using Beauti (Drummond and Rambaut, 2007), and then run in *BEAST. Fourteen runs of 100 million generations, sampling every 10,000 generations, were performed.

Pairwise uncorrected genetic distances between lineages were calculated with MEGA 5 (Tamura *et al.*, 2011), using only the mitochondrial data.

2.3. Molecular clock analysis

A multilocus coalescent molecular clock analysis was performed in *BEAST, assuming a Relaxed Uncorrelated Lognormal Clock Model (Drummond, 2006) for all genes, using the best-fit substitution models selected previously for each locus. We used a Yule speciation process for the tree prior, and as time calibration a cytb 0.0105 substitution rate per million years (Weir and Schluter, 2008). MCMC chains were run for 10 million generations sampling every 1000 generations. TRACER v. 1.5 was used to visualize the posterior distributions for every parameter.

2.4. Morphological Analyses

We examined 251 study skins from throughout the ranges of *Megascops watsonii* and *M. atricapilla* housed in several collections (Figure 2; Appendix 2). We measured for each specimen seven morphological characters with an electronic caliper (to the nearest 0.01 mm): wing, tail, tarsus length, bill length from the tip to the distal point of the nostrils, bill depth and width at the distal point of the nostril, and average width of ventral stripes. We did not include weight data in the morphological analyses due to the lack of this information for most specimens, however, we performed a regression analysis on the available weight data for *M. atricapilla*. We used Smithe (1975) to describe and study plumage coloration, which was scored qualitatively to each exemplar. We classified each individual in a color morph (red, dark, brown, gray, cinnamon) based mainly in its general back, chest and belly colors, using the codes (numbers) of the color guide.

A Discriminant Function Analysis (DFA) was performed based on the measurements obtained for all specimens examined, which were classified *a priori* in natural groups (clades) according to the molecular phylogeny. Because only a smaller portion of specimens included in the morphological analyses were also sequenced for their DNA, and hence used in the molecular analyses, most of the measured specimens (study skins) were assigned to groups based on their congruent distributions within the ranges of the identified molecular clades (see results below). Analyses were not performed separately for each sex because most specimens studied were either males or not-sexed. Analyses were performed in SYSTAT, version 12, for Windows (Systat Software, San Jose, California). In all tests, statistical significance was accepted at $P \leq 0.05$.

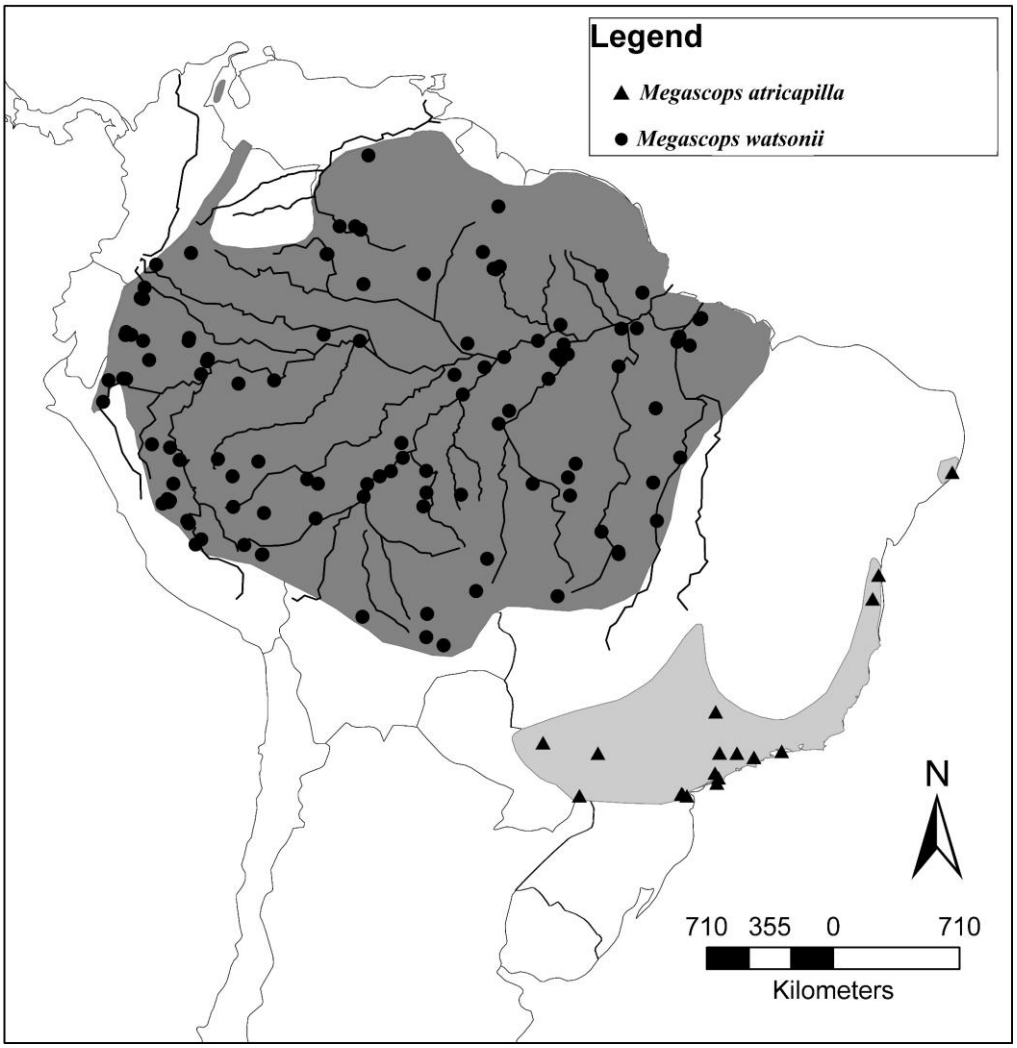

Figure 2: Geographic distribution of specimens of *Megascops watsonii* and *M. atricapilla* examined and included in the morphological analyses. Shaded areas correspond to the allopatric distributions of *M. watsonii* and *M. atricapilla* as in InfoNatura (2007).

2.5. Vocal Analyses

We analyzed 83 recordings belonging to *M. watsonii* and *M. atricapilla* (Appendix 3), covering most of the distribution of these taxa (Figure 3). Whenever possible, we analyzed only one recording per locality, to guarantee sampling independency. When not, we considered samples from the same place to be independent from each other. Recordings were obtained by us directly in the field or provided by colleagues and online sound archives, such as xeno-canto (www.xeno-canto.org) and the Macaulay Library (http://macaulaylibrary.org/). Only one sound bout (sequence) per recording was used in the analyses. Two types of vocalizations were analyzed, and are referred

here as “longsongs” and “shortsongs”. Longsongs are usually longer than shortsongs (but total length of both is variable in any given population), consisting of series of similar notes uttered at an even pace throughout the whole sequence; longsongs usually start softly and then become gradually louder, until reaching a more or less defined “plateau” of volume. Shortsongs are usually shorter than longsongs, and have two distinct parts: 1) slower-paced; which suddenly turns into 2) faster-paced, gradually slowing down towards the end. Vocalizations were classified into longsongs and shortsongs aurally and by visual inspections of spectrograms, and analyzed separately in this study.

The following vocal characters were measured: length of each note and the interval between them in seconds; pace (number of notes. sec^{-1}) of the entire song; peak frequency (in Hz) and Maximum Power (in dB) of each note. In longsongs, these characters were sampled only after loudness stabilized in the “plateau” stretch of the song, where notes reached equivalent maximum frequencies. The total number of notes and the duration of longsongs and shortsongs were not evaluated due to much individual variation, possibly related to the motivational state of the different individuals when tape-recorded. Measurements were made using RAVEN PRO 1.3 (Cornell Laboratory of Ornithology, Ithaca, New York).

A Discriminant Function Analysis (DFA) was also performed based on the vocal characters measured for all recordings examined, which were classified *a priori* in natural groups (clades) according to the molecular phylogeny. Because only a smaller portion of specimens included in the vocal analyses were also sequenced for their DNA, and hence used in the molecular analyses, most of the recordings were assigned to groups based on their congruent distributions within the ranges of the identified molecular clades (see results below). Analyses were performed in SYSTAT, version 12, for Windows (Systat Software, San Jose, California). In all tests, statistical significance was accepted at $P \leq 0.05$.

We followed the method of Isler et al. (1998) to test for statistically significant diagnostic vocal features among clades recovered in the molecular analyses. For each vocal character evaluated, the mean (X) and standard deviation (SD) was calculated. We verified normality of distribution of vocal characters through the Shapiro-Wilk test, and considered two continuous and normally distributed characters diagnostic between two clades only if their ranges did not overlap and if the means and SD of the clade with the smaller set of measurements (a) and the clade with the larger set of measurements (b)

met the requirement: Xa + ta SDa ≤ Xb – tb SDb, where ti is the t score at the 97.5 percentile of the t distribution for n – 1 df (Isler et al. 1998).

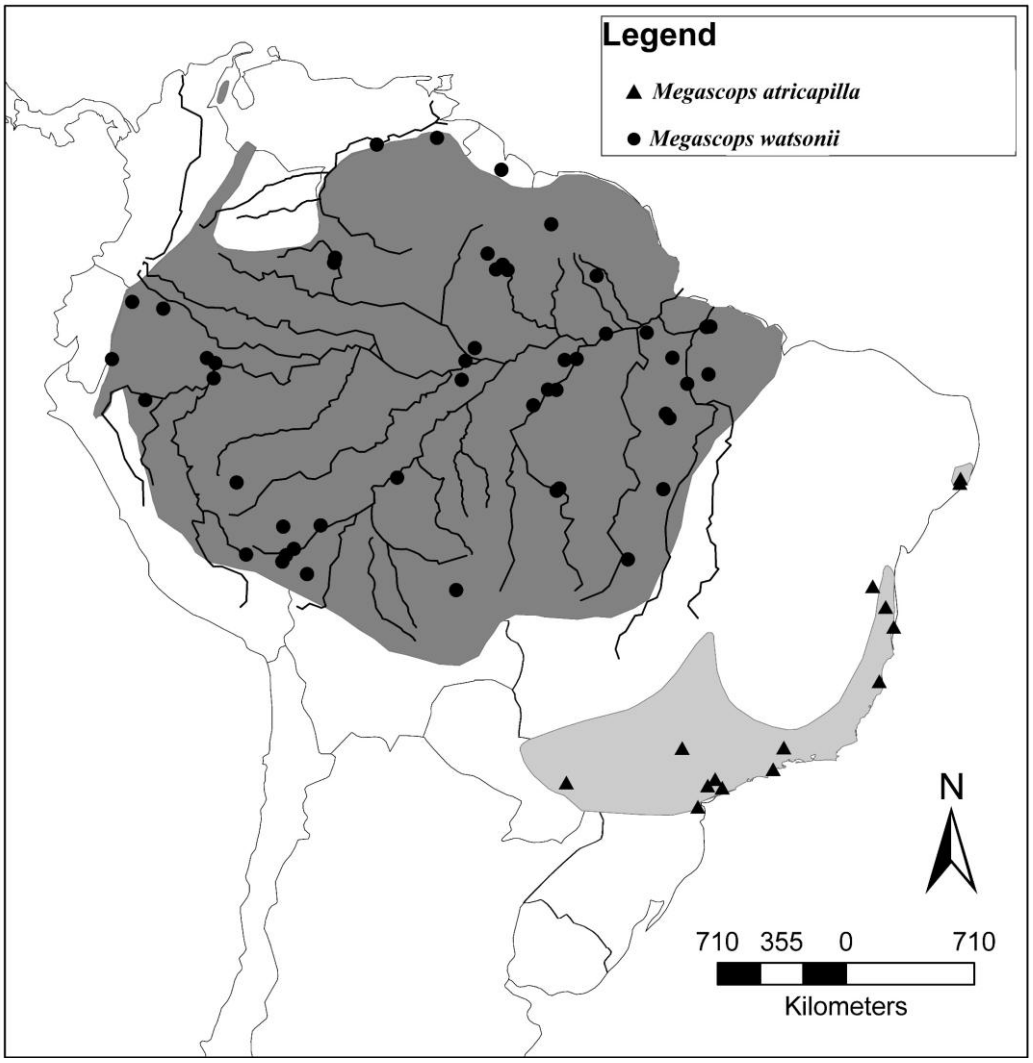

Figure 3. Geographic distribution of vocal samples (longsongs and shortsongs) of *Megascops watsonii* and *M. atricapilla* analyzed in this study. Shaded areas correspond to the allopatric distributions of *M. watsonii* and *M. atricapilla* as in InfoNatura (2007).

3. Results

3.1. Molecular phylogenetics

We obtained a total of 3969 bp of cytb (1035 bp), COI (379 bp), ND2 (1040 bp), BF5 (560 bp), MUSK (605 bp) and CHD (349 bp) sequences for 49 individuals of *Megascops watsonii* and *M. atricapilla* and four outgroup taxa (Appendix 1). Maximum Likelihood, Bayesian Inferences, and Species Tree analyses based on concatenated

mitochondrial and nuclear genes databank generated nearly identical phylogenetic trees, placing *M. watsonii* and *M. atricapilla* as paraphyletic species (Figs. 4 and 6). ML and BI analyses recovered six well-supported clades (A-F, Figs. 4) while the ST uncovered the same clades (except clade E, not included in this particular analysis due to insuficient molecular data) with high statistical support as well, except for the reciprocal monophyly between clades D and E (Figure 6). Samples were nominated according to the Area of Endemism (AEO) where it came from. These areas comprises regions in Amazonia separated by the main rivers, plus the Atlantic Forest region in East South America. The AEOs covered by this study are: Guyanas (between Negro and Amazonas rivers), Imeri (between Negro and Solimões), Inambari (between Solimões and Madeira), Madeira (between Madeira and Tapajós), Tapajós (between Tapajós and Xingu), Xingu (between Xingu and Tocantins), Belem (Amazonia east from Tocantins river) and Atlantic Forest (east coastal Brazil southward).

Clade A contains samples distributed on the Guianan shield east of the Negro / Branco rivers eastward towards Guyana and the Brazilian states of Amazonas and Pará north of the Amazon River (Fig. 5). Clade B includes samples distributed west of the Branco / Negro and both sides of the Amazon / Solimões rivers into eastern Peru, northwestern Bolivia, and the Madeira-Tapajós and the southern parts of the Tapajós-Xingu and Xingu-Tocantins interfluves in southeastern Brazilian Amazonia (Figure 5). Clade C groups samples from the lower Tapajós - Xingu and Xingu-Tocantins interfluves (Figure 5), whereas clade D is made of samples distributed east of the Tocantins River in the Belém area of endemism (*sensu* Silva et al., 2005). Clades E and F, assigned to *Megascops atricapilla*, are distributed in the Atlantic Forest and separated by the São Francisco River in northeastern Brazil. All these clades recognized in *M. watsonii / atricapilla* are apparently alopatric and separated by rivers or non-forest habitats, except for the parapatric B and C clades which come into contact in the middle portions of the Tapajós-Xingu and Xingu-Tocantins interfluves (Figure 5).

Nodal support for the relationships and reciprocal monophyly of clades A - F were high according to both ML and BI phylogeny inferences, except for the basal relationship among clades C, D, and E+F (Figure 4), which was essentially unresolved. The ST also recovered statistically significant values for the reciprocal of most clades, except that between clades C, D, and F, whose basal relationships were not well supported, mirroring the results obtained in the ML and BI analyses (Figure 6).Within clade B, there were three statistically well-supported divisions, one comprising a sample from La

Paz, Bolivia (LSUNM 947), which was the basal most in clade B, followed by a sample from Loreto, Peru (LSUNM 2912), which grouped as sister to all remaining samples in this clade.

Pairwise genetic distances between the clades varied from 1.5% (sister clades E and F) to 7.1% (between clades A and F; Table 2). Within clades, the deepest divergence involved the La Paz sample with respect to the remaining individuals of clade B (2.6%), while in the other clades average internal genetic distances clustered around 0.5% or less.

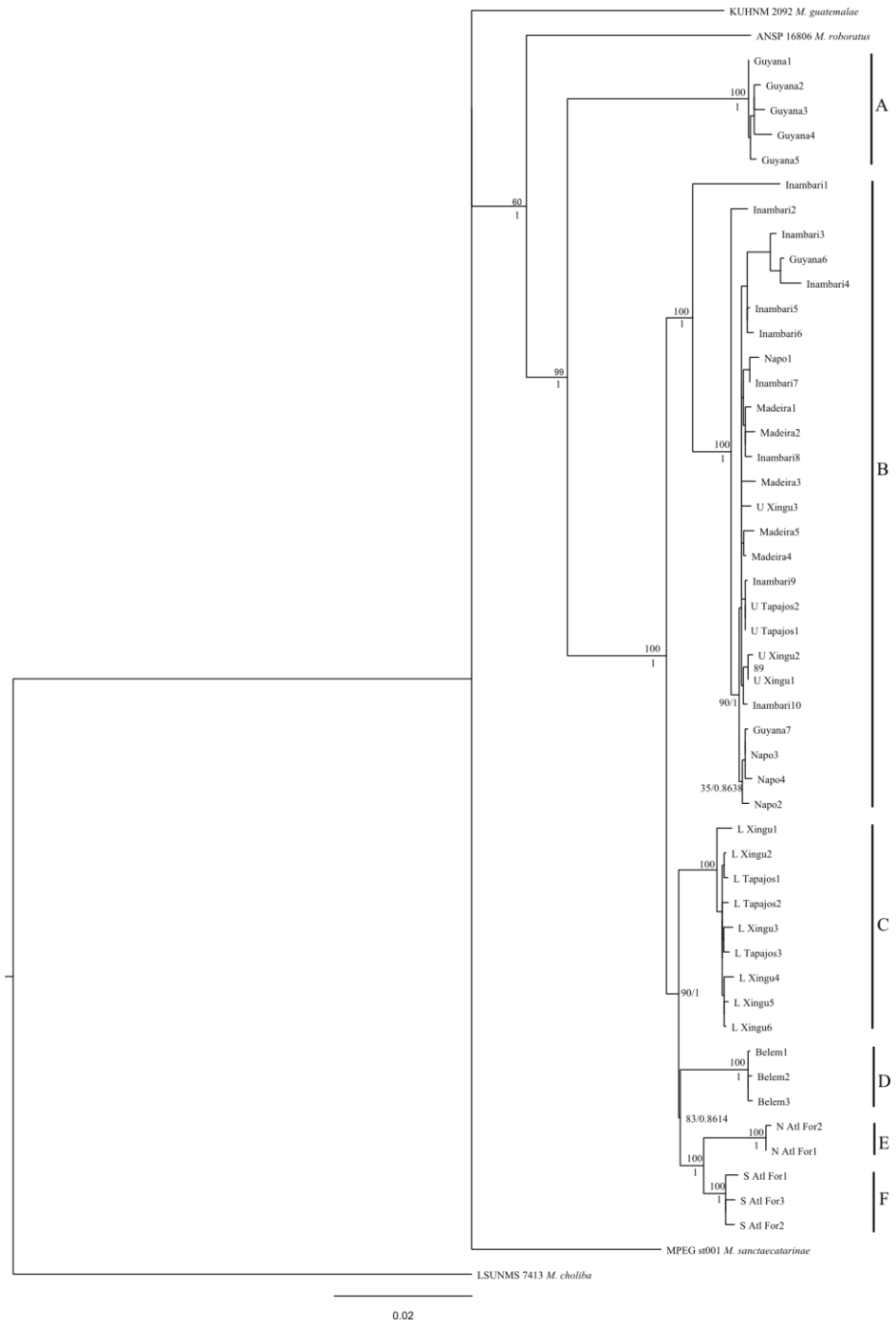

Figure 4. Results of ML and BI phylogenetic analyses based on 3969 bp of the concatenated databank of mitochondrial and nuclear genes sequenced in this study. Numbers associated with nodes represent ML bootstrap (above) and BI posterior probability (below) values. Samples are assigned to areas of endemism (AOE) to which they belong: Guiana, Napo, Imeri, Inambari, Madeira, Tapajós, Xingu, Belém, Northern Atlantic Forest (N Atl For) and Southern Atlantic Forest (S Atl For) (Silva *et al.*, 2005; Carnaval *et al.*, 2009). The Tapajós and Xingu AOs were divided in Upper (U) and Lower (L) portions.

Table 2: Average pairwise uncorrected mitochondrial genetic distances between clades A-F of *M. watsonii / atricapilla* recovered by the molecular phylogenetic analyses.

Clades	**A**	**B**	**C**	**D**	**E**
A	-	-	-	-	-
B	0.067	-	-	-	-
C	0.064	0.032	-	-	-
D	0.067	0.035	0.021	-	-
E	0.066	0.032	0.023	0.023	-
F	0.071	0.035	0.024	0.024	0.015

According to the molecular clock analysis, all splits within *M. watsonii* / *atricapilla* took place during the Plio-Pleistocene (4.5-0.8 mya; Figure 5). The first split separated clade A from the remaining ones between 4.5 and 2.5 million years ago, placing it as basal in *M. watsonii* / *atricapilla*. The second split isolated clade B from the ancestral of clades C-F and took place around 1.3 mya. The splits between eastern Amazonian and Atlantic Forest clades occurred between 0.5 and 1.5 mya (Figure 6).

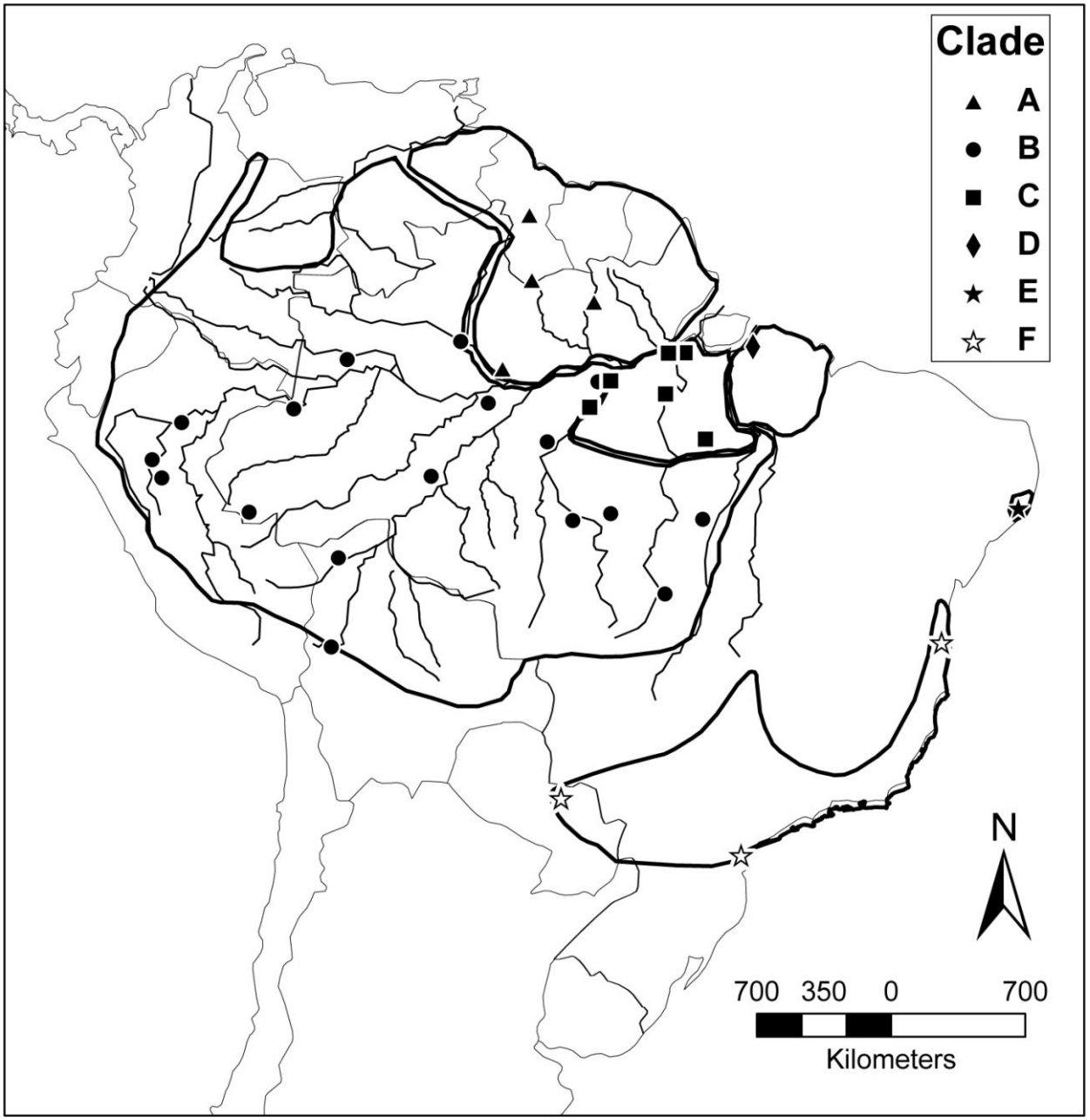

Figure 5. Geographic distribution of clades A-F recovered by ML/BI phylogeny inferences. Clades distributions were sometimes extrapolated based on vocal characters found to be diagnostic among clades.

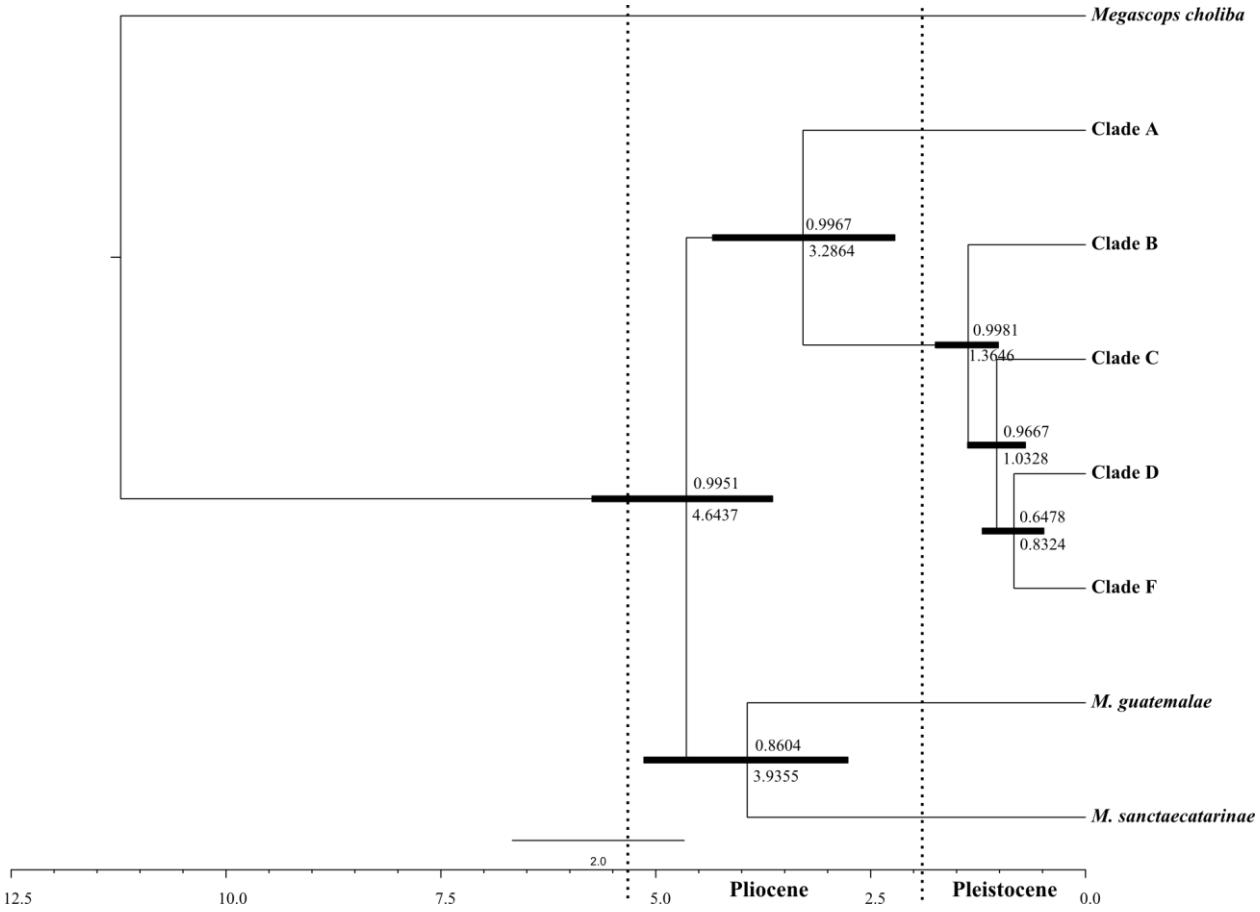

Figure 6. Species tree (ST) chronogram for the diversification of *M. watsonii / atricapilla* in Amazonia and the Atlantic Forest. Black bars represent 95% credibility intervals of ages (in mya) associated with the different nodes. Numbers above and below black bars denote nodal posterior probabilities and node heights (in mya), respectively.

3.2. Morphological Analyses

A DFA analysis among clades A-F recovered by the molecular phylogenies was significant (Wilk's Lambda=0.564; P=0.000). However, the classification matrix could not correctly assign most of the specimens to their respective clades based on the morphometric characters measured (Table 3), and there was broad clade overlap in the morphometric space (Figure 7). One character (bill height) was not used in the analysis because it was highly correlated with other bill measurements (beak length=0.531; beak width=0.431; p=0.354). The first two canonical discriminant variables accounted for 80.5% of total variation among clades. Specimens in clade E were classified with 100% (or 75% in Jacknife matrix) of confidence, but the sample size for this clade was very small (n=4) and consisted of individuals collected at the same site, and there was

overlap with other three groups (Table 2). Specimens in all other clades were classified with much less accuracy (less than 70% of confidence), clade B having the lowest confidence value (26/23%). Total accuracy was low (38/32%).

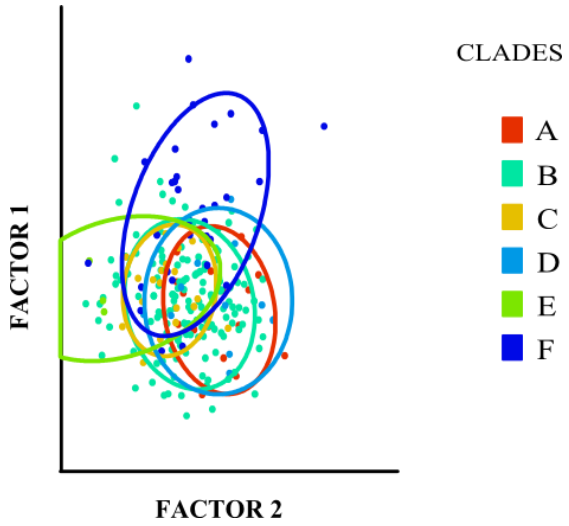

Figure 7. Graphic representation of scores of the first two factors of a Discriminant Function Analysis separating clades A-F of *Megascops watsonii*/*atricapilla* based on measurements of six morphometric characters (wing, tail, tarsus, beak length, beak width, underparts stripes width).

Table 3. Summary of classification accuracy of specimens (study skins) among clades A-F of *Megascops watsonii/ atricapilla* by a Discriminant Function Analysis based on measurements of six morphometric characters (wing, tail, tarsus, beak length, beak width, ventral stripe width). Numbers before and after slashes represent respectively values obtained without and with jackknife procedures.

clades	**A (n=17)**	**B (n=151)**	**C (n=20)**	**D (n=11)**	**E (n=4)**	**F (n=32)**	**%correct**
A	7 / 4	2 / 3	2 / 2	4 / 6	0 / 0	2 / 2	41 / 24
B	36 / 38	39 / 35	28 / 29	25 / 25	11 / 12	12 / 12	26 / 23
C	0 / 0	4 / 5	13 / 11	1 / 2	0 / 0	2 / 2	65 / 55
D	3 / 4	0 / 0	3 / 3	5 / 2	0 / 0	0 / 2	45 / 18
E	0 / 0	0 / 1	0 / 0	0 / 0	4 / 3	0 / 0	100 / 75
F	1 / 2	2 / 2	2 / 2	2 / 3	3 / 3	22 / 20	69 / 63
Total	47 / 48	47 / 46	48 / 47	37 / 38	18 / 18	38 / 38	38 / 32

The striking variation in nearly all plumage characters evaluated makes diagnoses based on plumage among clades A-F impossible. We distinguished red, gray, brown and dark morphs, whose main differences are the predominant colors in under and upperparts

(red, gray, brown and blackish), but many intermediate colored individuals make distinction between morphs a purely subjective task in many cases. Reddish morphs, in particular, tend to have a less contrasting grayish washing in chest than other morphs, and many red individuals have a crown red striped or spotted black, instead of solely black or brownish as in other color phases. Brownish and reddish morphs, and intermediate morphs between them, were more common in most populations (table 4).

Table 4. Proportion (%) of color morphs in the phylogenetic clades.

Color morphs	**A (n=19)**	**B (n =141)**	**C (n=20)**	**D (n=11)**	**E (n=4)**	**F (n=29)**	**Total**
Brown	36.84	48.23	55.00	36.36	25.00	20.69	43.30
Red	10.53	33.33	20.00	9.09	75.00	51.72	32.14
Dark	52.63	-	10.00	36.36	-	3.45	7.59
Gray	-	0.71	-	9.09	-	-	0.89
Cinnamon	-	3.55	-	-	-	-	2.23
Intermediate (Red-brown)	-	8.51	10.00	-	-	10.34	7.59
intermediate (Brown-gray)	-	3.55	-	9.09	-	13.79	4.46
intermediate (Brown-cinnamon)	-	2.13	-	-	-	-	1.34
intermediate (Brown-dark)	-	-	5.00	-	-	-	0.45

Some "tendencies" in plumage variation could be observed in some clades, with some color morphs being more frequent in some populations, but with exceptions in virtually all of them. In clades A and D, most individuals are darker than the darkest morphs of other clades, especially on the back overall color (color 26 – clay color - in Smith, 1975), in addition to having a more contrasting dark chest color. However, at least one clade C individual (MPEG 70647, not sequenced, from Carajás) approaches closely this pattern and could be confounded with clade A or D birds. Clade A dark birds tend to have a more uniform cinnamon colored belly contrasting with the chest, and wider pectoral stripes than those found in dark individuals of clade D. Some clade B morphs possess a more or less distinctive (and variable) cinnamon coloration on the underparts (cinnamon, color 123A; Smith 1975), which is less contrasting in other populations (except some clade A morphs). Apparently, reddish morphs are more frequent in clade B than in other amazonian clades, but similar looking reddish morphs are also common in clade C.

There are other local plumage variations in clade B populations, such as some Peruvian specimens grayer than others from the same population (FMNH 208178, 222284), and a very pale individual from Bolivia, also seemingly smaller than others (LSU 168771). In

the case of *M. atricapilla* (clades E and F), birds of Alagoas and Bahia, belonging respectively to clades E and F, resemble each other much more in plumage than birds from Bahia do with respect to others from the same clade F distributed southwards (São Paulo and Paraná Brazilian States). A simple regression based on weight data from 12 *M. atricapilla* specimens from clades E and F suggests a north-to-south clinal variation in body mass, with southernmost individuals being heavier (R^2=0.684; p=0.001), and a high correlation (0.827) between geographic distance (from the northernmost sampling point) and weight.

3.3. Vocal Analysis

We analyzed 55 longsong and 28 shortsong bouts of *M. watsonii / atricapilla* (Clade A: n=14; clade B: n=27; clade C: n=07; clade D: n=04; clade E: n=04; clade F: n=09). Longsongs in all clades consist on monotonous sequences of equally spaced notes delivered in a variable period of time, gradually rising in volume until reaching a "plateau" where note frequency stabilizes, and becoming a bit slower at the end, usually on the last five or three notes (Fig. 8). However, sometimes longsong increases continually in frequency to the very end of the sequence, especially in clade F birds. Note length, the interval between them, maximum frequency and other vocal traits are usually kept constant along different longsong bouts, sometimes varying very little in pitch. Notes are in general more uniformly shaped at the beginning of the longsong sequence, and can gradually change to slightly downslurred or upslurred notes towards the end of it. Clade B slow-paced populations (pace between 2.5 and 3.9 notes per second), notes are more elongated, uniformly shaped or slightly underslured (falling and then rising in pitch) in the beginning to slightly downslured or upslured to the end. Measurements of the nine longsong characters of clades A-F are found in table 5.

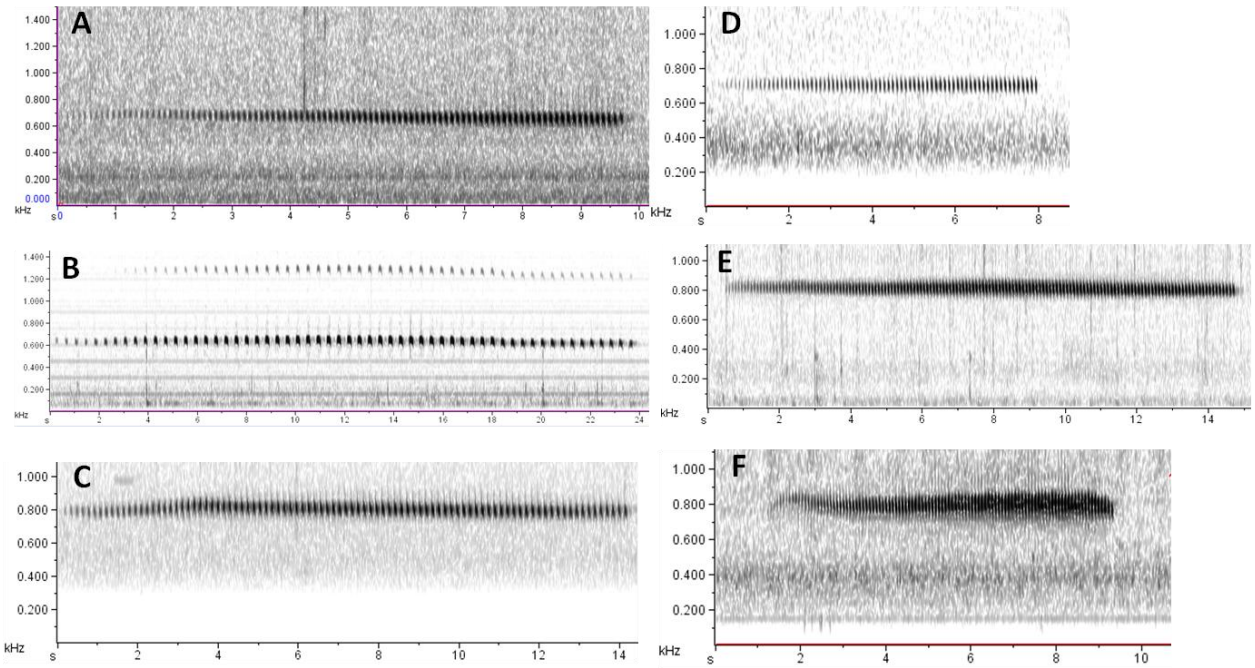

Figure 8. Examples of longsong audio spectrograms from clades A-F of *Megascops watsonii*/*atricapilla*. (A) Clade A: Venezuela, Rio Cuyuni (Paul Schwartz, MLS 59376); (B) Clade B: Peru, Madre de Dios (Ted Parker III, MLS 11496); (C) Clade C: Brazil, Serra dos Carajás (Sidnei Dantas); (D) Clade D: Brazil, IPEAN, Belém (Paul Schwartz, MLS 59395); (E) Clade E: Brazil, Usina Serra Grande, Ibateguara, Alagoas (Curtis Marantz, MLS 127980); (F) Clade F: Brazil, Caetetus Ecological Station, São Paulo (Alexandre Aleixo, MLS 94909). Each sonogram represents its clade as a whole in a general way, except for the example from Clade B, a slow-paced song (less than 4 notes/s) which represents 55% of the samples analysed (15 of 27).

Table 5. mean and SD of 09 quantitative longsong characters measurements for clades A-F *Megascops watsonii / atricapilla*.

Measurements	**A (n=14)**	**B (n=27)**	**C (n=7)**	**D (n=4)**	**E (n=4)**	**F (n=9)**
note length (in seconds)	0.05±0.01	0.12±0.05	0.08±0.01	0.07±0.01	0.06±0.005	0.05±0.01
peak frequency (in kHz)	0.65±0.06	0.65±0.04	0.71±0.05	0.68±0.04	0.78±0.02	0.81±0.05
Pace (n. notes / second)	10.34±1.3	4.66±2.0	7.25±0.89	9.54±0.53	10.62±0.1	13.11±0.9
Maximum power	87.12±15.0	96.25±12.17	92.77±9.2	78.3±8.54	90.2±13.67	103.31±20.8
Change in note length	1.01±0.01	1.01±0.01	1.01±0.01	1.01±0.0	1.01±0.01	1.01±0.0
Change in note interval	1.01±0.01	1.01±0.01	1.01±0.01	1.03±0.02	1.02±0.01	1.01±0.01
Change in peak frequency	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Change in maximum power	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Change in pace	1.01±0.01	1.01±0.02	1.03±0.02	1.01±0.0	1.01±0.01	1.01±0.02

There is great variation in longsong pace within some clades, this being very evident in clade B. In this clade, pace varied from 2.4 notes.sec^{-1} to 8.4 notes.sec^{-1} in a geographic tendency, with slowest-paced songs found from south Ecuador throughout the uppermost portions of the Inambari, Madeira, Tapajós, and Xingu AEOs, while fastest-paced longsongs occur north of Amazon River and in the lowermost portions of Inambari and Madeira AOE. An ANOVA taking into account only longsong pace between clades C and clade B from Tapajós and Xingu endemism centers, where both occur in supposed parapatry (10 samples from 09 localities – Itaituba, Santana do Araguaia, Canarana, Carajás, Trairão, Caxiuanã and Santarem, all in the Brazilian State of Pará), showed a significant difference between them (F=47.989; p=0.000), with clade B birds having a significantly slower longsong in these areas.

A simple regression analysis revealed a north-to-south clinal variation in the pace of longsongs of clades E and F (R^2=0.825; p=0.000), rather than an abrupt change between them. Correlation between longsong pace and geographic distance (from the northernmost sample point) was 0.908.

Clades A and D longsongs were not diagnosable from those in the remaining clades according to the diagnosability test employed. In contrast, longsongs B, C, E and F were statistically diagnosable from each other by at least one trait (Table 6).

A DFA with longsongs of clades A-F showed clade F to differ significantly from all the others, despite some small overlap with the distantly related clade A, with the first two canonical discriminant variables accounting for 93.6% of the total variation (Wilk´s lambda=0.062, P=0.000, Figure 8a). The classification matrix correctly assigned 64-77% of the specimens to their respective clades based on the vocal characters measured (Table 5). Variation was explained most by pace (F=17.078), Max Power (F=3.159) and change in note interval (F=2.434). Otherwise, there is a broad overlap among longsongs of the non-sister and parapatric clades B and C, and the distantly related and alopatric clades A, D, and E (Figure 9A). Hence, three main types of longsong were recovered by the DFA: one unique of clade F, a second shared by clades B and C, and a third found in clades A, D, and E. These three longsong types overlap only marginally with each other (Figure 9A). This is also evident when the analyses are restricted to the closely related clades C-F, with the resulting DFA (first two first canonical discriminant variables accounting for 99.2% of the differences) showing a clear separation among longsongs

of clades C and F from those of clades D and E, which overlap with each other (Wilk´s lambda=0.006, P=0.000, Figure 9B).

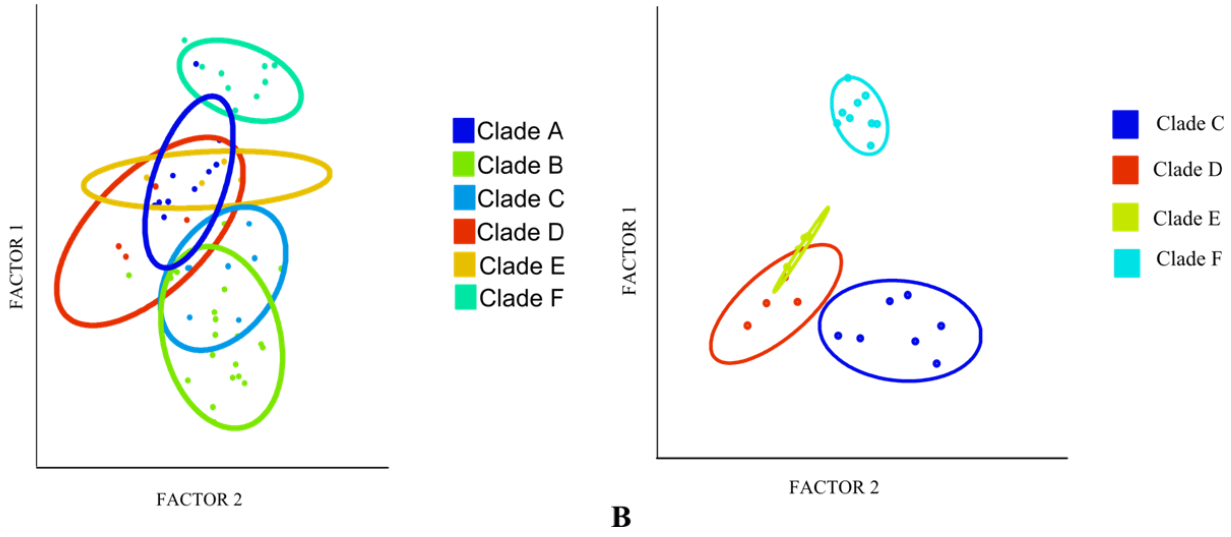

Figure 9. Graphic representation of scores of the first two factors of a Discriminant Function Analysis separating clades A-F of *Megascops watsonii/atricapilla* based on measurements of nine longsong characters (pace, note length, peak frequency, max power, change in note length, change in interval length, change in peak frequency, change in max power and change in pace).

Table 6. Summary of classification accuracy of recordings among clades A-F of *Megascops watsonii/ atricapilla* by a Discriminant Function Analysis based on measurements of nine longsong characters (pace, note length, peak frequency, max power, change in note length, change in interval length, change in peak frequency, change in max power and change in pace). Numbers before and after slashes represent respectively values obtained without and with jackknife procedures.

Clades	**A (n=11)**	**B (n=21)**	**C (n=7)**	**D (n=4)**	**E (n=4)**	**F(n=9)**	**% correct**
A	9/7	0/0	0/0	0/2	1/0	1/2	82/64
B	0/1	14/14	4/3	3/3	0/0	0/0	67/67
C	0/1	1/2	6/3	0/1	0/0	0/0	86/43
D	0/0	0/0	0/1	2/1	2/2	0/0	50/25
E	0/0	0/0	0/0	1/1	3/3	0/0	75/75
F	0/1	0/0	0/0	0/0	0/0	9/8	100/89
Total	9/10	15/16	10/7	6/8	6/5	10/10	77/64

For shortsongs, samples sizes were too small for clades D and E, so they were not included in the analyses. In most cases, shortsongs are monotonous sequences of short notes, upslurred (inverted U or V shaped) towards the end, (Figure 10). In clades A and F, shortsongs were composed of very short notes, downslurred (U or V shaped) or, more commonly upslurred, with a sharp inflection in the middle, giving the note a somewhat A letter shape (Figures 10A and 10D). The inflection is more evident in notes of the second part of the shortsong. Clade B´s shortsong is composed by much longer and less inflected notes, which could be upslurred, downslurred or more uniformly shaped (Figure 10B). In fast-paced populations of clade B and C, notes are shorter, although not so much as in clades A and F´s, and more inflected in the second part of the song (Figure 10C). Measurements of note length, peak frequency and pace values from shortsongs of clades A-F are found in table 7.

Table 7. Values of nine shortsong character measurements for clades A, B, C, and F of *Megascops watsonii / atricapilla* analyzed in this study.

Measures	*A (n=7)*	*B (n=14)*	*C (n=4)*	*F (n=3)*
note legth	0.07±0.01	0.17±0.05	0.10±0.01	0.06±0.01
peak frequency	0.61±0.05	0.64±0.17	0.67±0.03	0.74±0.15
Pace	7.97±0.43	3.21±1.21	4.70±0.30	8.87±1.11
Maximum power	84.44±21.07	84.38±16.4	101.45±43.33	86.06±7.09
Change in note length	1.01±0.01	1.0±0.03	1.02±0.03	1.03±0.03
Change in note interval	1.03±0.01	1.06±0.03	1.1±0.03	1.06±0.02
Change in peak frequency	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Change in maximum power	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Change in pace	0.88±0.03	0.74±0.1	0.82±0.16	0.82±0.05

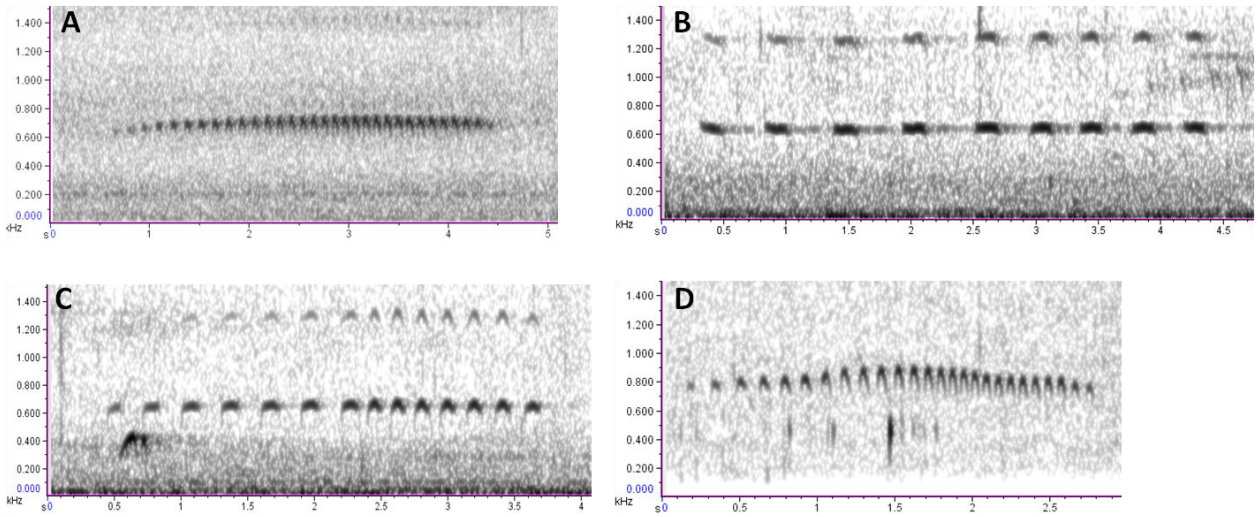

Figure 10. Audiospectrograms of shortsongs of clades A, B, C, and F. (A) Clade A: Venezuela, Rio Grande (Paul Schwartz, MSL 59359); (B) Clade B: Brazil, Mato Grosso (Curtis Marantz, MLS 126830); (C) Clade C: Brazil, Serra dos Carajás (Curtis Marantz, MLS 126657); and (D) Clade F: Brazil, São Paulo (Dan Lane, XC 75524).

Six clade pairs were significantly diagnosable from each other by at least one vocal (longsong and shortsong) trait according to the diagnosability rest employed (Table 8). Four clade pairs (A-B, B-E, C-F and E-F) differed by one character. Two (A-C and B-F) differed by two characters, and one (C-E) differed by three characters.

Table 8. Results of pairwise diagnosability among clades A-F of *Megascops watsonii / atricapilla* based on the longsong and shortsong characters measured.

clades	**A**	**B**	**C**	**D**	**E**	**F**
A	-	-	-	-	-	-
B	(1) pace shortsong	-	-	-	-	-
C	(2) pace shortsong and note interval shortsong	-	-	-	-	-
D		-		-	-	-
E	-	(1) pace longsong;	-	-	-	-
F	-	(2)	(3)	-	(1)	-

clades	A	B	C	D	E	F
		pace longsong and peak frequency longsongs	pace longsong; note interval longsong; note interval shortsongs		pace longsong;	

A DFA conducted with shortsongs of the remaining clades, indicated a separation of clades A and F from clade C (Wilk´s-lambda = 0.029, P=0.000), with the first two canonical discriminant variables accounting for 98.8% of the differences. The DFA correctly classified 89% of the samples (Table 9). Within these two groups there is some overlap between the allopatric clades A and F and the parapatric clades C and B (Fig. 11).

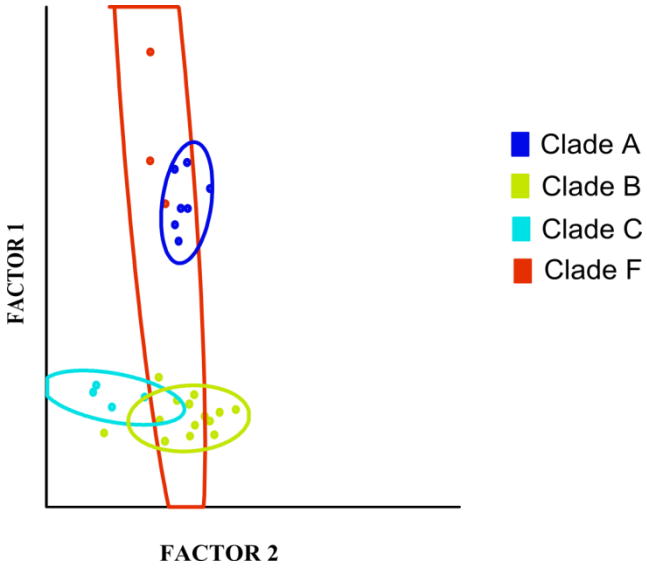

Figure 11. Graphic representation of scores of the first two factors of a Discriminant Function Analysis separating clades A, B, C, and F of *Megascops watsonii*/*atricapilla* based on measurements of nine shortsong characters (pace, note length, peak frequency, max power, change in note length, change in interval length, change in peak frequency, change in max power and change in pace).

Table 9. Summary of classification accuracy of recordings among clades A-F of *Megascops watsonii/ atricapilla* by a Discriminant Function Analysis based on measurements of nine shortsong characters (pace, note length, peak frequency, max power, change in note length, change in interval length, change in peak frequency,

change in max power and change in pace). Numbers before and after slashes represent respectively values obtained without and with jackknife procedures.

	A (n=3)	*B (n=4)*	*C (n=14)*	*F (n=7)*	*% correct*
A	7/6	0/0	0/0	0/1	100/86
B	0/0	12/11	2/3	0/0	86/79
C	0/0	0/3	4/1	0/0	100/25
F	1/2	0/0	0/0	2/1	67/33
Total	8/8	12/14	6/4	2/2	89/68

4. Discussion

4.1. Species limits and the evolution of morphological and vocal characters in *Megascops watsonii / atricapilla*

Results from the molecular analyses revealed a phylogenetic history divergent from the taxonomic treatment currently assigned to *Megascops watsonii/atricapilla* complex (Marks et al., 1999; Remsen et al., 2013; König and Weick, 2008; CBRO, 2011). The traditional taxonomy of this group, based solely on poorly understood variations in plumage and vocal attributes, is in need of a revision, once *Megascops watsonii* and *M. atricapilla*, as currently delimited, were found to be paraphyletic species. Most of the six statistically well-supported clades recovered by the molecular analyses are also reciprocally distinguishable by vocal characters, indicating an advanced degree of independent evolutionary history and speciation among them, and more intricate species limits than currently recognized.

The DFA and the diagnosablity test employed indicated significant diagnoses in at least one vocal character among all clades, except clade D (for which only four longsongs were analyzed). In most cases, for both longsongs and shortsongs, only one vocal trait was significantly diagnosable among clades (comparisons involving clade A versus B, B versus E, C versus F, and E versus F), while in two instances two characters diagnosed clades (clade A versus C and clade B versus F), and in only one instance two clades were diagnosed by three traits (clades C versus E). Despite these differences, not a single clade could be uniquely diagnosed from all the remaining ones by the vocal characters measured, which suggests a considerable level of homoplasy, or maybe retention of ancestral polimorphism in longsong and shortsong structures in *Megascops watsonii* / *atricapilla*. This is further illustrated by the level of variation in vocal

characters within the large clade B, whose reciprocal monophyly with respect to the remaining clades is well supported statistically according to all phylogenetic trees obtained. Alternatively, these results could reflect an inadequate choice of measurable voice characters.

Isler et al. (1998) showed that sympatric species pairs of antbirds (Thamnophilidae) usually differ diagnostically by at least three vocal features, which prompted these authors to propose a minimum number of three diagnostic vocal characters to rank taxa as biological species rather than subspecies. However, Chaves et al. (2010) concluded that only two vocal traits were sufficient to consider two taxa of *Myrmeciza* antbirds as full species, yet they were largely sympatric, but replacing each other elevationally. Carneiro et al. (2012) went even further and found only one statistically diagnosable vocal trait distinguishing reciprocally monophyletic populations of *Hylopezus* antpittas. Reciprocal monophyly and concordant vocal diagnosis are indicative of speciation under the Phylogenetic Species Concept (PSC) or under the General Lineage Species Concept (GLSC; de Queiroz, 1998; Aleixo, 2007), providing the basis for considering those *M. watsonii / atricapilla* clades diverging from each other by at least one significantly diagnosable vocal trait as separate species (i.e., clades A, B, C, E, and F). However, in the case of clade D (not vocally significantly diagnosable from any other clade in *M. watsonii/atricapilla*), non-vocal diagnosability is at odds with statistically significant reciprocal monophyly with respect to all remaining clades (Figure 4). It is difficult to ascertain whether this lack of vocal diagnosability is the result of a very small sample size (n = 4) because the same number of longsongs was available for clade E birds, but which were significantly vocally diagnosable from clades B, C, and F.

Under the Biological Species Concept (BSC), no evidence of gene flow could be detected among the six clades recovered by the molecular phylogenies (i.e., no paraphyletic clades were found; Figs. 4 and 6). The estimated species tree indicated consistent and complete patterns of coalescence for both nuclear and mitochondrial genes associated with clades A, B, and a major clade grouping clades C, D, and F. Within the C+D+E clade, basal relationships mirrored those estimated for the concatenated multi-locus Bayesian tree, but without statistical support, suggesting a lack of complete coalescence for the nuclear genes due either to gene flow or lack of lineage sorting. The fact that clades C+D+E+F are either parapatric (C and D; separated by the Tocantins River) or allopatric (clades C+D with respect to clades F+E, separated by the *Caatinga* and *Cerrado* biomes, and clades E and F separated by the São

Francisco River) favor ancestral polymorphism among recently diverged lineages rather than recent or on-going gene-flow. Therefore, clades A-F could also be regarded as biological species given the overall lack of evidence supporting instances of gene-flow among them.

Nevertheless, samples were missing from most of the area where clades B and C probably meet in the middle part of the Tapajós and Xingu interfluves, and from the upper Branco River and in southern Venezuela, where clades A and B also probably meet. Hence, it is possible that our sampling missed some introgressed individuals. Interestingly, two longsong types have been reported in zones of potential contact between clades B and C (V. Piacentini, pers. comm.; S. Dantas, pers. obs.), and further collecting and recording is needed from these localities. But even if present, introgression must be not sufficient to promote merging of the genotypes between these clades, given the high statistical support for their reciprocal monophyly according to both the concatenated multi-locus Bayesian tree and the multi-locus coalescent species tree. Furthermore, clades B and C are separated by a relatively high average uncorrected mitochondrial distance of 3.2%, which indicates separate evolutionary trajectories for a significant amount of time (see below). In North America, populations of *Strix occidentalis*, a non-migrating species, have limited introgression outside their contact zones (Barrowclough et al., 2005). Rates of secondary sympatry in Furnariidae (ovenbirds) were positively correlated with phylogenetic and ecological distance between species pairs, mainly due to biological and ecological similarity rather than to environmental characteristics (Pigot and Tobias, 2013). Presumably also non-migratory, non-sister and parapatric clades B and C can limit introgression by competitive exclusion. These same clades differ in their longsongs, with clade C being faster-paced than the parapatric populations of clade B. Potentially sympatric *Megascops* species may have different voices, because this trait is very important in species recognition among owls (König and Weick, 2008; Marks et al., 1999). In the Bolivian Andes, *M. marshalli* longsong is different from that of the sympatric *M. ingens*, but similar to the allopatric *M. petersoni´*s (Herzog et al., 2009). Interestingly, longsongs of allopatric populations of clades B and C (from opposite sides of the Tapajós River) sound more similar to one another than where they are potentially sympatric such as in Carajás and Santana do Araguaia (300 km distant). Herzog et al. (2009) reported a geographic cline in the longsong of *M. ingens*, although this may be related to genetic variation in this species (Dantas *et al.*, in prep.), and we also found an apparent clinal variation in the

longsong of *M. atricapilla*. These facts indicate the level of plasticity of vocal characters in *Megascops*, which may or may not be linked to speciation in this group. Fuchs et al. (2008), in a study on scops-wols (*Otus*) from Indian Ocean islands found that "…vocal and morphological differences are indeed associated with distinct evolutionary lineages, but suggest that they are not related in any simple or obvious way to the evolutionary distance between these lineages, and therefore must be used with caution in identifying affinities between taxa (or lineages)".

In the case of *M. watsonii/atricapilla*, longsong traits were useful in distinguishing four of the six clades recovered by the molecular phylogeny (i.e., clades B, C, E, and F), with shortsong traits further diagnosing clade A (which was not diagnosed by any longsong feature) from other two clades. So, a combination of longsongs and shortsongs features is useful to separate most of the clades recovered in *M. watsonii/atricapilla*. The only clade that could not be diagnosed by any vocal character from other clades was clade D, endemic to the Belém AOE. When pace only is considered – the most important trait in distinguishing species pairs according to the diagnosability test employed – average values obtained for clade D are intermediate between those of clades C and F, which could potentially explain this lack of significant diagnosis, added to the fact that only four longsong samples were available for clade D.

Morphological analyses did not detect any fully diagnosable group within *M. watsonii/atricapilla*. There is much overlap among clades in the morphometric space, and also great variation in plumage patterns, although some color morphs tend to be apparently more common in some populations than in others. In particular, dark morphs of clades A and D have a more contrasting chest color, and are in general darker than other populations. In general, plumage and morphological variation in owls are thought to be linked in part to environmental variables rather than to phylogeny alone (Fuchs *et al.*, 2007; Roulin *et al*., 2011). In at least one clade (F - *M. atricapilla*) we found clinal variation in body mass, which increases from north to south, mirroring Bergmann´s rule. North to south clines in size and plumage are also known for other screech-owls, such as *M. asio* and *M. kennicottii* (Proudfoot *et al.*, 2007) and appear not to be linked to any particular phylogenetic structuring in these species as well as in *M. atricapilla*.

4.2. Taxonomic recommendations

Most of the clades recovered by the molecular phylogeny and distinguished by longsong and shortsong characters in *M. watsonii / atricapilla* already have names,

except two (clades C and E). Thus, we suggest splitting and rearranging the species *Megascops watsonii* and *M. atricapilla* in the following taxa:

01) *Megascops watsonii* (Cassin, 1848) (type locality - Orinoco River, Venezuela) corresponding to Clade A, is redefined as the population of the complex Megascops watsonii/ usta/ atricapilla restricted to the Guianan Shield, east from Branco river.
02) *Megascops usta* (type locality - Tefé, Brazil) corresponding to clade B, is distributed over a wide area, west from Branco / Negro rivers throughout Imeri, Napo, Inambari, Madeira and upper stretches of the Tapajos and Xingu AEOs.
03) sp. nov. 01, corresponding to clade C, is distributed along the lower parts of the Tapajos and Xingu AEOs.
04) *Megascops ater* (Hekstra, 1982) (type locality Belém, Pará, Brazil), corresponding to clade D, is restricted to the Belém AEO.
05) *M. atricapilla* (Temminck, 1822) (type locality Brazil), corresponding to clade F, is distributed in the Atlantic Forest from southern Bahia in Brazil to northeastern Argentina and eastern Paraguay.
06) sp. nov. 02, corresponding to clade E, is restricted to isolated forest patches in the Atlantic Forest North of São Francisco River, in east Brazil.

Recognition of this division in the Amazonian and Atlantic Forest *Megascops watsonii / atricapilla* will have effects on conservation issues. The sp. Nov. 02 north of São Francisco (clade E) is known from only four localities in the State of Alagoas (Roda and Pereira, 2006). The forest in this area is extremely fragmented, with the largest forest with a little more than 3,000 ha in area. Clade E population is surely very small and may not survive for a long time unless drastic conservation efforts to be enforced as soon as possible. Clade D from the Belém AOE (*M. ater*) is also under threat, since this is the most deforested sector of the entire Amazon (Silva et al. 2005), with still ongoing deforestation.

4.3. Timing of diversification and biogeography

Speciation within the *Megascops watsonii / atricapilla* complex took place throughout the Plio-Pleistocene (4.5-0.5 mya), a time when other speciation events took place in several Amazonian vertebrate lineages (Aleixo and Rosseti, 2007; Weir and Price, 2011 Alfaro *et al*., 2012). Spatio-temporal patterns of avian diversification in Amazonia vary greatly among groups (Aleixo, 2002, Miller et al., 2008, Ribas et al., 2012, d´Horta et al., 2013), due to multiple geological and climatic events, and biological and ecological

differences among taxa (Patel et al., 2011). Many hypotheses have been proposed to explain these patterns, such as the refuge (Haffer, 1974) and the rivers as barriers (Gascon et al., 2000) hypotheses. Ribas et al. (2012) proposed a paleobiogeographic model for biotic diversification within Amazonia, based on the avian genus *Psophia*. They suggested that vicariant events associated with major Amazonian rivers were the main reason for diversification in this group, mainly during the Pleistocene. Under this model, eastern Amazonia was covered mainly by *terra-firme* upland forest during the Pliocene, while the western part consisted mostly of an extensive fluvial and lacustrine system. Between 2.7 and 2.0 million years ago (mya), this western fluvio-lacustrine system became more entrenched and began to flow eastward, connecting with the lower Amazon River and hence creating the modern trans-continental Amazonian drainage. Later on, it was estimated that the courses of the following main rivers became established or started to work as barriers for *Psophia* lineages:, Madeira (2.0-1.0 mya), Tapajós (1.3-0.8 mya), Negro (1.0-0.7 mya), and Tocantins and Xingu (0.8-0.3 mya).

The most important rivers for the differentiation of Amazonian *Megascops watsonii / atricapilla* were the Amazon, Negro/Branco, Tapajós and Tocantins. Differently from Ribas et al. (2012), populations from north of the upper Amazon / Solimões (Napo and Imeri AOEs) are more closely related to lineages south of this river, rather than those to the east on the Guianan shield (clade A). In fact, populations of clade B birds (*M. usta*) separated by the upper Amazon River are not genetically distinct, despite vocal differences between them. Also, the Branco River, a tributary of the Negro river, separated clades A (*M. watsonii*) and B (*M. usta*). Recently, the Branco River has been shown to be an important barrier in separating populations of many bird taxa (Naka, 2011; Naka et al., 2012; Fernandes et al., 2013).

Rivers have an important role in separating some clades of *Megascops watsonii / atricapilla*, but not in others. Clade B, for example, crosses the middle and upper Tapajós River and comes in parapatry with clade C in the Tapajós - Xingu and Xingu - Tocantins interfluves (Tapajós and Xingu AOEs). Similar patterns of phylogeographic breaks away from the course of main Amazonian rivers have been documented for other lineages along the Tapajós River for instance, such as *Glyphorynchus spirurus* and *Hylophylax naevius* (Fernandes, 2012; Fernandes et al., 2013), among other avian taxa. A possible explanation for parapatry not related to main Amazonian tributaries posits secondary contact via dispersal of at least one of the clades coming together in modern times (Fernandes et al., 2012). So, dispersal of the mainly western Amazonian clade B

(*M. usta*) into eastern Amazonia is a possibility, supported to some extent by the higher level of phylogeographic structure detected among western populations of this species, where an individual from the Andean foothills (Cordillera Oriental Mountains) near La Paz, Bolivia (LSUMNS 947), is separated by an average uncorrected mitochondrial distance of 2.6% from the remaining populations of the same clade, a distance which equals or surpasses that between some clades of the *M. watsonii / atricapilla* complex (clades C-D-E-F). However, a denser sampling of clade B, particularly along the foothills of the Andes, is necessary to test this dispersal scenario in a robust way. Clades C and D split around 1.3 mya (Fig. 6), and are separated by Tocantins River. This more derivate condition (more recent split time) coincides with that of lineages of *Psophia* (Ribas et al., 2012) and *Dendrocolaptes certhia* lineages (Batista *et al.*, in press) by this river, so indicating that Tocantins had a later influence in some lineages of Amazonian birds, compared to other rivers in the region.

The split between Amazonian (AM) and Atlantic Forest (AF) lineages was estimated to have occurred between 0.7 and 1.3 mya. Two or three historical connections between AM and AF have been suggested: an older one (Miocene) linking the Andes to southern AF, and one or two more recent ones (Pliocene to Pleistocene) linking southeastern AM to the northern sector of the AF (Batalha-Filho et al., 2013). AF clades of the *M. watsonii / atricapilla* complex (clades E and F) are closer to clades C and D from southeastern Amazonia (Tapajós, Xingu and Belem AOEs), so a younger (Pleistocene) pathway theory is congruent with our findings. Probably, the Atlantic Forest clades E and F must have descended from an ancestor colonizing the AF from its northern extreme. Again, a denser sampling of clades E and F is necessary to test this hypothesis. Similar ages for AM and AF splits were found in other lineages such as *Ramphastos* and *Pionus* (Patané et al., 2009; Ribas et al., 2007), while in others such as *Brotogeris, Xiphorhynchus, Dendrocincla, Pteroglossus* and *Pionopsitta* divergences between AF and AM lineages took place earlier, i.e., between 3 and 10 mya (Patel et al., 2011; Ebberhard and Bermingham, 2008; Cabanne et al., 2008; Ribas et al., 2009; Weir and Price, 2011). It was not clear according to our results whether the AF lineages (clades E and F) are closer to clade C or D. As suggested by other studies, development of the Tocantins River (which separated clades C and D) and the separation of AM and AF clades may have occurred at approximately the same time (Ribas et al., 2012; Batalha-Filho, 2012). A recent separation among these clades, roughly at the same time, could obscure more basal relationships between them, with more sequences needed to clarify

their relationships. There is also the possibility of a secondary contact between clades C and D after Atlantic forest/Amazonia forms separation, which also could obscure relationship between them.

A later split originated clades E and F, separated by the São Francisco River. This large eastern Brazilian river separates many distinct avian taxa in the AF, isolating to the north the so-called Pernambuco AOE. *Dendrocincla turdina* and *Dendrocincla fuliginosa taunay*, shown to be high-supported sister taxa (Weir and Price, 2011) are also separated by São Francisco River, and this split is estimated as having occurred between 0.5 and 2.0 mya. Other sister taxa separated by this river include *Xiphorhynchus fuscus / X. atlanticus* and *Tangara seledon / T. fastuosa*, and these splits may have taken place around the same time (Cabanne et al., 2008; Weir and Price, 2011). Separation between our clades E and F, although not calculated, seems to have roughly occurred during this time frame. These results reinforce the suggestions of Weir and Price (2011) of a common biogeographic event that might have affected simultaneously populations of these taxa across the São Francisco River. Carnaval et al. (2009) suggested that separate refugia occurred to the north and south of São Francisco River during Pleistocene Glacial maxima, and could have promoted speciation.

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Appendix 1. Tissue samples.

Legend for the source institutions: ANSP = Academy of Natural Sciences of Drexel University; KUNHM = Kansas University Natural History Museum; LSUMNS = Louisiana State University Museum of Natural History; MPEG = Museu Paraense Emilio Goeldi; MZUSP = Museu de Zoologia da Universidade de São Paulo; NMNH = Smithsonian Institution National Museum of Natural History.

source	skin number	species	Clade	code	Locality
LSUMNS	20185	*Megascops watsonii*	A	Guyana1	Brazil; Manaus, Amazonas.
ANSP	21937	*M. watsonii*	A	Guyana2	Guyana; Potaro-Siparuni; Iwokrama Reserve
ANSP	188291	*M. watsonii*	A	Guyana3	Guyana; Potaro-Siparuni; Iwokrama Reserve
NMNH	11476	*M. watsonii*	A	Guyana4	Guyana; west bank upper esequibo river
MPEG	6635	*M. watsonii*	A	Guyana5	Brazil; Óbidos, Pará.
MZUSP	JF297	*M. watsonii*	B	Guyana6	Brazil; Jufari, Amazonas.
MZUSP	JF754	*M. watsonii*	B	Guyana7	Brazil; Jufari, Amazonas.
MPEG	AMA200	*M. watsonii*	B	Napo1	Brazil; Tabatinga, Amazonas.
MPEG	62428	*M. watsonii*	B	Napo2	Brazil; Japurá, Amazonas.
MPEG	AMA55	*M. watsonii*	B	Napo3	Brazil; Tabatinga, Amazonas.
MPEG	AMA56	*M. watsonii*	B	Napo4	Brazil; Tabatinga, Amazonas.
LSUMNS	947	*M. watsonii*	B	Inambari1	Bolivia; La Paz
LSUMNS	2912	*M. watsonii*	B	Inambari2	Peru; Loreto Department.
LSUMNS	2829	*M. watsonii*	B	Inambari3	Peru; Loreto Department; 1km N Rio Napo
LSUMNS	46287	*M. watsonii*	B	Inambari4	Peru; San Martin.
LSUMNS	9665	*M. watsonii*	B	Inambari5	Bolivia; Pando.
KUNHM	944	*M. watsonii*	B	Inambari6	Peru
MPEG	71378	*M. watsonii*	B	Inambari7	Brazil; Jordão, Acre.
MPEG	70987	*M. watsonii*	B	Inambari8	Brazil; Humaitá, Amazonas.
MPEG	70997	*M. watsonii*	B	Inambari9	Brazil; Humaitá, Amazonas.
MPEG	62429	*M. watsonii*	B	Inambari10	Brazil; Careiro, Amazonas.
MPEG	70663	*M. watsonii*	B	Madeira1	Brazil; Porto Velho, Rondônia.
MPEG	70660	*M. watsonii*	B	Madeira2	Brazil; Porto Velho, Rondônia.
MPEG	ARAII03	*M. watsonii*	B	Madeira3	Brazil; Santarém; RESEX Tapajós-Arapiuns.
MPEG	ITA001	*M. watsonii*	B	Madeira4	Brazil; Jacareacanga, Pará.
MPEG	ARAII01	*M. watsonii*	B	Madeira5	Brazil; Santarém; Pará.
MPEG	70206	*M. watsonii*	B	H Xingu1	Brazil; Querência, Mato Grosso.
MPEG	70205	*M. watsonii*	B	H Xingu2	Brazil; Querência, Mato Grosso.
MZUSP	88058	*M. watsonii*	B	H Xingu3	Brazil; Santana do Araguaia, Pará.
MPEG	57930	*M. watsonii*	B	H Tapajos1	Brazil; Novo Progresso, Pará.
MPEG	67246	*M. watsonii*	B	H Tapajos2	Brazil; Paranaíta, Mato Grosso.
MPEG	71331	*M. watsonii*	C	L Xingu 1	Brazil; Melgaço, Pará.
MPEG	71332	*M. watsonii*	C	L Xingu 2	Brazil; Melgaço, Pará.

source	skin number	species	Clade	code	Locality
MPEG	70678	*M. watsonii*	C	L Tapajos 1	Brazil; Belterra, Pará.
MPEG	70845	*M. watsonii*	C	L Tapajos 2	Brazil; Itaituba, Pará.
MZUSP	83559	*M. watsonii*	C	L Xingu 3	Brazil; Porto de Moz, Pará.
MPEG	70846	*M. watsonii*	C	L Tapajos 3	Brazil; Itaituba, Pará.
MPEG	70632	*M. watsonii*	C	L Xingu 4	Brazil; Parauapebas, Pará.
NMNH	7020	*M. watsonii*	C	L Xingu 5	Brazil; Altamira, Pará.
MPEG	70627	*M. watsonii*	C	L Xingu 6	Brazil; Parauapebas, Pará.
MPEG	70268	*M. watsonii*	D	Belem 1	Brazil; Benevides, Pará.
MPEG	70433	*M. watsonii*	D	Belem 2	Brazil; Benevides, Pará.
MPEG	70434	*M. watsonii*	D	Belem 3	Brazil; Benevides, Pará.
MPEG	70437	*M. atricapilla*	E	N Atl Forest 1	Brazil; Ibateguara, Alagoas.
MPEG	70438	*M. atricapilla*	E	N Atl Forest 2	Brazil; Ibateguara, Alagoas.
KUNHM	157	*M. atricapilla*	F	S atl For 1	Paraguay; Concepción.
MPEG	64808	*M. atricapilla*	F	S atl For 2	Brazil; Quatro Barras, Paraná.
MZUSP	BA226	*M. atricapilla*	F	S atl For 3	Brazil; Camacan, Bahia.
KUNHM	2092	*M. guatemalae*	Outgroup		Mexico; Campeche; Silvituc.
ANSP	16806	*M. roboratus*	Outgroup		Ecuador; Loja Province.
MPEG	st001	*M. sanctaecatarinae*	Outgroup		Brazil; Chapecó, Santa Catarina.
LSUNMS	7413	*M. choliba*	Outgroup		Venezuela; Amazonas territory.

Appendix 2. Skin samples.

Legend for the source institutions: ANSP = Academy of Natural Sciences of Drexel University; KUNHM = Kansas University Natural History Museum; LSUMNS = Louisiana State University Museum of Natural History; MHNCI = Museu de historia Natural do Capão da Imbuia; MNRJ = Museu Nacional do Rio de Janeiro; MPEG = Museu Paraense Emilio Goeldi; MZUSP = Museu de Zoologia da Universidade de São Paulo; NMNH = Smithsonian Institution National Museum of Natural History.

source	skin number	species	clade	endemism center	locality
AMNH	476787	*Megascops watsonii*	A	Guyana	Venezuela; Caura.
AMNH	277583	*M. watsonii*	A	Guyana	Brazil; Parintins, Amazonas.
NMNH	622354	*M. watsonii*	A	Guyana	Guyana; Parabara Savvanah.
NMNH	625110	*M. watsonii*	A	Guyana	Guyana; Sipu river.
NMNH	622139	*M. watsonii*	A	Guyana	Guyana; Parabara Savvanah.
NMNH	625366	*M. watsonii*	A	Guyana	Guyana; west bank upper Essequibo river.
ANSP	188289	*M. watsonii*	A	Guyana	Guyana; Iwokrama Reserve; Turtle Mountain.
ANSP	188291	*M. watsonii*	A	Guyana	Guyana; Iwokrama Reserve; Turtle Mountain.
ANSP	188292	*M. watsonii*	A	Guyana	Guyana; Iwokrama Reserve; Turtle Mountain.

source	skin number	species	clade	endemism center	locality
ANSP	188290	*M. watsonii*	A	Guyana	Guyana; Iwokrama Reserve; Turtle Mountain.
ANSP	2444	*M. watsonii*	A	Guyana	Orinoco
MPEG	101573	*M. watsonii*	A	Guyana	Brazil; Óbidos, Pará.
FMNH	260181	*M. watsonii*	A	Guyana	Surinam; Zuid River.
MZUSP	JF 754	*M. watsonii*	A	Guyana	Brazil; Boa Vista, RR.
MZUSP	JF 297	*M. watsonii*	A	Guyana	Brazil; Boa Vista, RR.
MNRJ	45807	*M. watsonii*	A	Guyana	Brazil; Mazagão, AP
MPEG	66635	*M. watsonii*	A	Guyana	Brazil; Óbidos, PA; ESEC Grão-Pará.
MPEG	52948	*M. watsonii*	A	Guyana	Brazil; Manaus, Amazonas.
MPEG	66608	*M. watsonii*	A	Guyana	Brazil; Óbidos, Pará.
MPEG	66424	*M. watsonii*	A	Guyana	Brazil, Almeirim, Pará.
AMNH	270463	*M. watsonii*	B	Guyana	Venezuela; Rio Cassiquiare.
AMNH	270460	*M. watsonii*	B	Guyana	Venezuela; Duida Mountains.
AMNH	431818	*M. watsonii*	B	Guyana	Venezuela; Rio Cassiquiare.
AMNH	476786	*M. watsonii*	B	Guyana	Venezuela; Soapure, Caura.
AMNH	270462	*M. watsonii*	B	Guyana	Venezuela; Duida Mountains.
AMNH	115739	*M. watsonii*	B	Imeri	Colombia; La Morelia, Caqueta.
AMNH	115738	*M. watsonii*	B	Imeri	Colombia; La Morelia, Caqueta.
NMNH	325915	*M. watsonii*	B	Imeri	Brazil; Serra Imeri.
NMNH	325916	*M. watsonii*	B	Imeri	Brazil; Serra Imeri.
NMNH	328958	*M. watsonii*	B	Imeri	Venezuela; Cerro Yapacana, upper Orinoco.
AMNH	239445	*M. watsonii*	B	Inambari	Sta. Rosa, alto Ucayali.
AMNH	237708	*M. watsonii*	B	Inambari	Peru; Parayacu, R. Ucayali.
AMNH	820837	*M. watsonii*	B	Inambari	Peru; Rio Ene (at mouth of R. Quipachiari).
AMNH	820810	*M. watsonii*	B	Inambari	Peru; Rio Ene (at mouth of R. Chiquireni).
AMNH	820912	*M. watsonii*	B	Inambari	Peru; Depto. Huanuco, rio Llulla Pichis.
AMNH	781789	*M. watsonii*	B	Inambari	Peru; Cuzco, Apumarimac River.
AMNH	820913	*M. watsonii*	B	Inambari	Peru; Depto. Huanuco, rio Llulla Pichis.
AMNH	406864	*M. watsonii*	B	Inambari	Peru; Rio Pisqui.
AMNH	818044	*M. watsonii*	B	Inambari	Bolivia; Sta. Rosa.
LSUNMS	156207	*M. watsonii*	B	Inambari	Peru; Dept. Ucayali.
LSUNMS	114632	*M. watsonii*	B	Inambari	Peru; Dept.Loreto; S. Rio Amazonas.
LSUNMS	114633	*M. watsonii*	B	Inambari	Peru; Dept.Loreto; S. Rio Amazonas.
LSUNMS	63961	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Río Curanja, Balta.
LSUNMS	51612	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Río Curanja, Balta.
LSUNMS	63959	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Río Curanja, Balta.
LSUNMS	63960	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Río Curanja, Balta.
LSUNMS	34023	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Río Curanja, Balta.
LSUNMS	37024	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Balta.
LSUNMS	62155	*M. watsonii*	B	Inambari	Peru; Dept. Loreto; Balta.
LSUNMS	132015	*M. watsonii*	B	Inambari	Bolivia; Pando; Prov. Nicolás Suarez.
FMNH	293363	*M. watsonii*	B	inambari	Peru; Depto. Pasco; Asolis.
FMNH	397727	*M. watsonii*	B	Inambari	Peru; Dept. Madre de Dios.

source	skin number	species	clade	endemism center	locality
FMNH	295081	*M. watsonii*	B	Inambari	Peru; Dept. Junin; Conchapen Mt.
FMNH	320454	*M. watsonii*	B	Inambari	Peru; Depto. Madre de Dios; Hda. Amazonia.
FMNH	297888	*M. watsonii*	B	Inambari	Peru; Depto. Pasco, Prov. Oxapampa.
FMNH	299045	*M. watsonii*	B	Inambari	Peru; Dept. Junin; Peyeñtesoñe.
FMNH	297889	*M. watsonii*	B	Inambari	Peru; Dept. Pasco, Prov. Oxapampa.
FMNH	208177	*M. watsonii*	B	Inambari	Peru; Cuzco; Marcapata.
FMNH	208178	*M. watsonii*	B	Inambari	Peru; Cuzco; Marcapata.
FMNH	247144	*M. watsonii*	B	Inambari	Peru; Loreto, Iquitos, Sta. Rita.
FMNH	222284	*M. watsonii*	B	Inambari	Peru; Cuzco; Marcapata.
MZUSP	3592	*M. watsonii*	B	Inambari	Brazil; Rio Juruá, Amazonas.
MPEG	71376	*M. watsonii*	B	Inambari	Brazil; Jordão, Acre.
MPEG	70987	*M. watsonii*	B	Inambari	Brazil; Humaitá, Amazonas.
MPEG	71375	*M. watsonii*	B	Inambari	Brazil; Jordão, Acre.
MPEG	71378	*M. watsonii*	B	Inambari	Brazil; Jordão, Acre.
MPEG	71377	*M. watsonii*	B	Inambari	Brazil; Jordão, Acre.
MPEG	71374	*M. watsonii*	B	Inambari	Brazil; Jordão, Acre.
MPEG	63692	*M. watsonii*	B	Inambari	Brazil; Porto Acre, Acre.
MPEG	16790	*M. watsonii*	B	Inambari	Brazil; Estirao do Equador, Amazonas.
MPEG	60463	*M. watsonii*	B	Inambari	Brazil; Floresta Estadual do Aretimary, Acre.
MPEG	59772	*M. watsonii*	B	Inambari	Brazil; Assis Brasil, Acre.
MPEG	61521	*M. watsonii*	B	Inambari	Brazil; Plácido de Castro, Acre.
MPEG	61994	*M. watsonii*	B	Inambari	Brazil; Porto Walter, Acre.
MPEG	63691	*M. watsonii*	B	Inambari	Brazil; Feijó, Acre.
MPEG	15998	*M. watsonii*	B	Inambari	Brazil; Rio Juruá, Acre.
MPEG	62429	*M. watsonii*	B	Inambari	Brazil; Careiro, Amazonas.
MPEG	72973	*M. watsonii*	B	Inambari	Brazil; Atalaia do Norte, Amazonas.
AMNH	281397	*M. watsonii*	B	Madeira	Brazil; Rosarinho, Rio Madeira.
AMNH	281392	*M. watsonii*	B	Madeira	Rosarinho, Lago Sampaio, rio Madeira, Brasil
AMNH	819830	*M. watsonii*	B	Madeira	Peru; Rio Aputimac, Luisiana.
AMNH	34597	*M. watsonii*	B	Madeira	Brazil; Chapada, Mato Grosso.
LSUNMS	101691	*M. watsonii*	B	Madeira	Bolivia: La Paz; Rio Beni.
LSUNMS	37315	*M. watsonii*	B	Madeira	Bolivia; Cochabamba; Prov. Chapare.
LSUNMS	123553	*M. watsonii*	B	Madeira	Bolivia; Depto. Santa Cruz.
LSUNMS	168771	*M. watsonii*	B	Madeira	Bolivia; Dept. Santa Cruz.
FMNH	101084	*M. watsonii*	B	Madeira	Brasil; Rio Arapiuns; Arúan, Pará.
MZUSP	66536	*M. watsonii*	B	Madeira	Brazil; Urucurituba, rio Tapajós, Pará.
MZUSP	84594	*M. watsonii*	B	Madeira	Brazil; Comunidade São Benedito, Amazonas.
MZUSP	62160	*M. watsonii*	B	Madeira	Brazil; Rio Aripuanã, Amazonas.
MZUSP	20354	*M. watsonii*	B	Madeira	Brazil; Lago do Batista, Amazonas.
MZUSP	65918	*M. watsonii*	B	Madeira	Brazil; Pedra Branca, Rondônia.
MZUSP	81659	*M. watsonii*	B	Madeira	Brazil; Sapizal, Mato Grosso.
MZUSP	78048	*M. watsonii*	B	Madeira	Brazil; Vila Bela da Santíssima Trindade, Mato Grosso.
MPEG	J1507	*M. watsonii*	B	Madeira	Brazil; Mutum, Rondônia.

source	skin number	species	clade	endemism center	locality
MPEG	J1633	*M. watsonii*	B	Madeira	Brazil; Ex.pediçao Jirau
MNRJ	4779	*M. watsonii*	B	Madeira	Brazil; Pinhel, Rio Tapajós, Amazonas.
MNRJ	4785	*M. watsonii*	B	Madeira	Brazil; Rio Jamari, Rondônia.
MPEG	70997	*M. watsonii*	B	Madeira	Brazil; Machadinho d'oeste, Rondônia.
MPEG	70660	*M. watsonii*	B	Madeira	Brazil; Porto Velho, Rondônia.
MPEG	70670	*M. watsonii*	B	Madeira	Brazil; Porto Velho, Rondônia.
MPEG	70663	*M. watsonii*	B	Madeira	Brazil; Porto Velho, Rondônia.
MPEG	36538	*M. watsonii*	B	Madeira	Brazil; Rio Paraíso, Rondônia.
MPEG	39408	*M. watsonii*	B	Madeira	Brazil; Cachoeira Nazaré, Rondônia.
MPEG	74006	*M. watsonii*	B	Madeira	Brazil; Jacareacanga, Pará.
MPEG	74096	*M. watsonii*	B	Madeira	Brazil; Santarém, Pará.
MPEG	74094	*M. watsonii*	B	Madeira	Brazil; Santarém, Pará.
MPEG	74097	*M. watsonii*	B	Madeira	Brazil; Santarém, Pará.
AMNH	255106	*M. watsonii*	B	Napo	Ecuador; Noca.
AMNH	255102	*M. watsonii*	B	Napo	Ecuador; Noca.
AMNH	708675	*M. watsonii*	B	Napo	Ecuador; Montalvo Oriente.
AMNH	172969	*M. watsonii*	B	Napo	Ecuador; near River Napo.
AMNH	172971	*M. watsonii*	B	Napo	Ecuador; near River Napo.
AMNH	238841	*M. watsonii*	B	Napo	Peru; Alto rio Ucayali.
AMNH	255105	*M. watsonii*	B	Napo	Ecuador; Noca.
AMNH	708677	*M. watsonii*	B	Napo	Ecuador; Montalvo Oriente.
AMNH	255193	*M. watsonii*	B	Napo	Ecuador; Noca.
AMNH	708676	*M. watsonii*	B	Napo	Ecuador; Montalvo Oriente.
AMNH	708678	*M. watsonii*	B	Napo	Ecuador; Rio Rutuno, Oriente.
AMNH	255104	*M. watsonii*	B	Napo	Ecuador; Noca.
AMNH	708674	*M. watsonii*	B	Napo	Ecuador; Rio Bobonaza, Oriente.
AMNH	185226	*M. watsonii*	B	Napo	Ecuador; Rio Suno Abajo.
AMNH	255107	*M. watsonii*	B	Napo	Ecuador; Noca.
AMNH	406865	*M. watsonii*	B	Napo	Peru; Rio Mazán.
ANSP	184585	*M. watsonii*	B	Napo	Ecuador; prov. Napo.
ANSP	186787	*M. watsonii*	B	Napo	Ecuador; prov. Sucumbios.
ANSP	52222	*M. watsonii*	B	Napo	Colombia; Morelia Caquetá.
ANSP	52221	*M. watsonii*	B	Napo	Colombia; Morelia Caquetá.
LSUNMS	75122	*M. watsonii*	B	Napo	Peru; Dept. Amazonas.
LSUNMS	114634	*M. watsonii*	B	Napo	Peru; Dept. Loreto; Lower Rio Napo region.
LSUNMS	91698	*M. watsonii*	B	Napo	Peru; Dept. Amazonas.
LSUNMS	87281	*M. watsonii*	B	Napo	Peru; Dept. Amazonas; vinicity of Huampani.
LSUNMS	84357	*M. watsonii*	B	Napo	Peru; Dept. Amazonas; vinicity of Huampani.
LSUNMS	70499	*M. watsonii*	B	Napo	Ecuador; Prov. Oriente, Limoncocha.
LSUNMS	23042	*M. watsonii*	B	Napo	Peru; Dept. Loreto; ca. 86 km SE Juanjui.
LSUNMS	23529	*M. watsonii*	B	Napo	Peru; Dept. Loreto.
LSUNMS	119341	*M. watsonii*	B	Napo	Peru; Dept. Loreto; Quebrada Óran.
LSUNMS	109334	*M. watsonii*	B	Napo	Peru; Dept. Loreto; 1 km N Rio Napo.

source	skin number	species	clade	endemism center	locality
LSUNMS	109335	*M. watsonii*	B	Napo	Peru; Dept. loreto; 1.5 km S Libertad.
LSUNMS	50293	*M. watsonii*	B	Napo	Ecuador; Prov. Napo; Limoncocha.
FMNH	247145	*M. watsonii*	B	Napo	Peru; Loreto, Iquitos.
FMNH	248551	*M. watsonii*	B	Napo	Colombia; Meta, La Macarena.
FMNH	248550	*M. watsonii*	B	Napo	Colombia; Meta, La Macarena.
FMNH	102527	*M. watsonii*	B	Napo	Ecuador; Rio Copataza-Oriente.
FMNH	102979	*M. watsonii*	B	Napo	Colombia; Morelia Caquetá.
FMNH	248552	*M. watsonii*	B	Napo	Colombia; Meta, La Macarena.
FMNH	277679	*M. watsonii*	B	Napo	Peru; Dept. Amazonas: Puerto Galilea.
MPEG	42462	*M. watsonii*	B	Napo	Brazil; Maraã, Amazonas.
MPEG	62428	*M. watsonii*	B	Napo	Brazil; Maraã, Amazonas.
MPEG	72556	*M. watsonii*	B	Napo	Brazil; Tabatinga, Amazonas.
MPEG	72557	*M. watsonii*	B	Napo	Brazil; Tabatinga, Amazonas.
MPEG	72619	*M. watsonii*	B	Napo	Brazil; Tabatinga, Amazonas.
MZUSP	38322	*M. watsonii*	B	Tapajós	Brazil; Serra do Cachimbo, Pará.
MZUSP	88778	*M. watsonii*	B	Tapajós	Brazil; Paranaíta, Mato Grosso.
MPEG	69086	*M. watsonii*	B	Tapajós	Brazil; Paranaíta, Mato Grosso.
MPEG	69086	*M. watsonii*	B	Tapajós	Brazil; Paranaíta, Mato Grosso.
MPEG	67245	*M. watsonii*	B	Tapajós	Brazil; Paranaíta, Mato Grosso.
MPEG	67247	*M. watsonii*	B	Tapajós	Brazil; Paranaíta, Mato Grosso.
MPEG	15284	*M. watsonii*	B	Tapajós	Brazil; Serra do Cachimbo, Pará.
MPEG	67246	*M. watsonii*	B	Tapajós	Brazil; Paranaíta, Mato Grosso.
MPEG	33870	*M. watsonii*	B	Tapajós	Brazil; Matupá, Mato Grosso.
MPEG	57930	*M. watsonii*	B	Tapajós	Brazil; Serra do Cachimbo, Pará.
MZUSP	88058	*M. watsonii*	B	Xingu	Brazil; Santana do Araguaia, Pará.
MZUSP	32311	*M. watsonii*	B	Xingu	Brazil; Xavantina, Mato Grosso.
MZUSP	32310	*M. watsonii*	B	Xingu	Brazil; Xavantina, Mato Grosso.
MNRJ	45806	*M. watsonii*	B	Xingu	Diananíu (?) - Alto Xingu
MNRJ	45805	*M. watsonii*	B	Xingu	Brazil; Jacaré, Alto Xingu, Mato Grosso.
MNRJ	30566	*M. watsonii*	B	Xingu	Brazil; Garapu, Alto Xingu, Mato Grosso.
MNRJ	30565	*M. watsonii*	B	Xingu	Brazil; Garapu, Alto Xingu, Mato Grosso.
MNRJ	30560	*M. watsonii*	B	Xingu	Brazil; Jacaré, Alto Xingu, Mato Grosso.
MPEG	70205	*M. watsonii*	B	Xingu	Brazil; Querência, Mato Grosso.
MPEG	70206	*M. watsonii*	B	Xingu	Brazil; Querência, Mato Grosso.
MPEG	48526	*M. watsonii*	B	Xingu	Brazil; Santana do Araguaia, Pará.
MPEG	34663	*M. watsonii*	B	Xingu	Brazil; Conceição do Araguaia, Pará.
MPEG	34664	*M. watsonii*	B	Xingu	Brazil; Conceição do Araguaia, Pará.
MZUSP	93276	*M. watsonii*	C	Tapajós	Brazil; Porto de Moz, Pará.
MZUSP	83558	*M. watsonii*	C	Tapajós	Brazil; Porto de Moz, Pará.
MZUSP	81758	*M. watsonii*	C	Tapajós	Brazil; Jacareacanga, Pará.
MZUSP	81757	*M. watsonii*	C	Tapajós	Brazil; Jacareacanga, Pará.
MZUSP	83559	*M. watsonii*	C	Tapajós	Brazil; Porto de Moz, Pará.
MPEG	53840	*M. watsonii*	C	Tapajós	Brazil; Belterra, Pará.

source	skin number	species	clade	endemism center	locality
MPEG	40578	*M. watsonii*	C	Tapajós	Brazil; Belterra, Pará.
MPEG	72216	*M. watsonii*	C	Tapajós	Brazil; Moraes Almeida, Pará.
MPEG	70846	*M. watsonii*	C	Tapajós	Brazil; Itaituba, dist. Miriticuba, Pará.
MPEG	70845	*M. watsonii*	C	Tapajós	Brazil; Itaituba, dist. Miriticuba, Pará.
MPEG	70674	*M. watsonii*	C	Tapajós	Brazil; Belterra, Pará.
MPEG	70684	*M. watsonii*	C	Tapajós	Brazil; Belterra, Pará.
MPEG	70678	*M. watsonii*	C	Tapajós	Brazil; Belterra, Pará.
NMNH	562192	*M. watsonii*	C	Xingu	Brazil; Altamira, Pará.
NMNH	541330	*M. watsonii*	C	Xingu	Brazil; Altamira, Pará.
NMNH	562193	*M. watsonii*	C	Xingu	Brazil; Altamira, Pará.
MZUSP	64307	*M. watsonii*	C	Xingu	Brazil; Altamira, Pará.
MPEG	70632	*M. watsonii*	C	Xingu	Brazil; Parauapebas, Pará.
MPEG	71332	*M. watsonii*	C	Xingu	Brazil; Flona Caxiuanã, Pará.
MPEG	70647	*M. watsonii*	C	Xingu	Brazil; Parauapebas, Pará.
MPEG	70627	*M. watsonii*	C	Xingu	Brazil; Parauapebas, Pará.
MPEG	71331	*M. watsonii*	C	Xingu	Brazil; Flona Caxiuanã, Pará.
MPEG	35994	*M. watsonii*	C	Xingu	Brazil; Jacundá, Pará.
MPEG	36079	*M. watsonii*	C	Xingu	Brazil; Jacundá, Pará.
MPEG	55335	*M. watsonii*	C	Xingu	Brazil; Altamira, Pará.
AMNH	430283	*M. watsonii*	D	Belém	Brazil; Baião, Pará.
NMNH	513892	*M. watsonii*	D	Belém	Brazil; Belém, Pará.
MZUSP	77127	*M. watsonii*	D	Belém	Brazil; Tailândia, Pará.
MZUSP	77128	*M. watsonii*	D	Belém	Brazil; Tailândia, Pará.
MZUSP	1983	*M. watsonii*	D	Belém	Brazil; Belém, Pará.
MNRJ	4782	*M. watsonii*	D	Belém	Brazil; Cametá, Pará.
MPEG	70433	*M. watsonii*	D	Belém	Brazil; Benevides, Pará.
MPEG	70435	*M. watsonii*	D	Belém	Brazil; Benevides, Pará.
MPEG	70434	*M. watsonii*	D	Belém	Brazil; Benevides, Pará.
MPEG	70268	*M. watsonii*	D	Belém	Brazil; Benevides, Pará.
MPEG	70267	*M. watsonii*	D	Belém	Brazil; Benevides, Pará.
MPEG	19786	*M. watsonii*	D	Belém	Brazil; Belém, Pará.
MZUSP	79948	*M. atricapilla*	E	Pernambuco	Brazil; Ibateguara, Alagoas.
MZUSP	79947	*M. atricapilla*	E	Pernambuco	Brazil; Ibateguara, Alagoas.
MPEG	70437	*M. atricapilla*	E	Pernambuco	Brazil; Ibateguara, Alagoas.
MPEG	70438	*M. atricapilla*	E	Pernambuco	Brazil; Ibateguara, Alagoas.
NMNH	608026	*M. atricapilla*	F	Atl F S	Brazil; Faz. Barreiro Rico, São Paulo.
NMNH	576981	*M. atricapilla*	F	Atl F S	Paraguay; Amamby Dept. Cerro Gora Nat. Park.
ANSP	2451	*M. atricapilla*	F	Atl F S	?
ANSP	2455	*M. atricapilla*	F	Atl F S	Brazil
FMNH	356565	*M. atricapilla*	F	Atl F S	Brazil; Icapara, São Paulo.
MHNCI	4697	*M. atricapilla*	F	Atl F S	Brazil; Curitiba, Paraná.
MHNCI	4846	*M. atricapilla*	F	Atl F S	Brazil; Guabirotuba, Paraná.
MHNCI	345	*M. atricapilla*	F	Atl F S	Brazil; Vale do Ivaí, Paraná.

source	skin number	species	clade	endemism center	locality
MHNCI	3232	*M. atricapilla*	F	Atl F S	Brazil; Foz do Iguaçu, Paraná.
MZUSP	2426	*M. atricapilla*	F	Atl F S	Brazil; Iguape, São Paulo.
MZUSP	68392	*M. atricapilla*	F	Atl F S	Brazil; Icapara, São Paulo.
MZUSP	61986	*M. atricapilla*	F	Atl F S	Brazil; Estação Biológica de Boracéia, São Paulo.
MZUSP	61741	*M. atricapilla*	F	Atl F S	Brazil; Icapara, São Paulo.
MZUSP	30993	*M. atricapilla*	F	Atl F S	Brazil; Lageado (Iporanga), São Paulo.
MZUSP	47577	*M. atricapilla*	F	Atl F S	Brazil; Onça Parda, São Paulo.
MZUSP	49437	*M. atricapilla*	F	Atl F S	Brazil; Rocha, São Paulo.
MZUSP	49442	*M. atricapilla*	F	Atl F S	Brazil; Ribeirão Fundo, São Paulo.
MZUSP	49439	*M. atricapilla*	F	Atl F S	Brazil; Rocha, São Paulo.
MZUSP	60605	*M. atricapilla*	F	Atl F S	Brazil; Nazaré Paulista, São Paulo.
MZUSP	49440	*M. atricapilla*	F	Atl F S	Brazil; Rocha, São Paulo.
MZUSP	49436	*M. atricapilla*	F	Atl F S	Brazil; Rocha, São Paulo.
MZUSP	61740	*M. atricapilla*	F	Atl F S	Brazil; Icapara, São Paulo.
MZUSP	5173	*M. atricapilla*	F	Atl F S	Brazil; Itapira (?),São Paulo.
MZUSP	66545	*M. atricapilla*	F	Atl F S	Brazil; Icapara, São Paulo.
MZUSP	49438	*M. atricapilla*	F	Atl F S	Brazil; Rocha, São Paulo.
MZUSP	90983	*M. atricapilla*	F	Atl F S	Brazil; Camacan, Bahia.
MZUSP	13967	*M. atricapilla*	F	Atl F S	Brazil; Rio Gongogi, BA.
MNRJ	31121	*M. atricapilla*	F	Atl F S	Brazil; Faz. Barreiro Rico, São Paulo.
MNRJ	43651	*M. atricapilla*	F	Atl F S	Brazil; Ilha Grande, Rio de Janeiro.
MNRJ	4362	*M. atricapilla*	F	Atl F S	Brazil; Ilha Grande, Rio de Janeiro.
MNRJ	44276	*M. atricapilla*	F	Atl F S	Brazil; Ilha Grande, Rio de Janeiro.
MPEG	71820	*M. atricapilla*	F	Atl F S	Brazil; Camacan, Bahia.
MPEG	71819	*M. atricapilla*	F	Atl F S	Brazil; Camacan, Bahia.
MPEG	64808	*M. atricapilla*	F	Atl F S	Brazil; Quatro Barras, Paraná.

Appendix 3. Vocalization samples.

Source legend: AA = Alexandre Aleixo; CB = Christian Borges Andretti; GT = Gregory Thom; ML = Macaulay Library; SD = Sidnei Dantas; WA = Wikiaves (www.wikiaves.com.br); XC = Xeno-Canto (www.xeno-canto.org).

source	catalog name	type	clade	locality
XC	XC120065	longsong	A	Brazil; Manaus, Amazonas.
ML	54362	longsong	A	Guyana; N of Parabara savannah
ML	134576	longsong	A	Suriname, Sipaliwini, Bakhuis Gebergte
ML	59376	longsong	A	Venezuela, Rio Cuyuni + 10 km
ML	59394_edited	longsong	A	Brazil; Manaus, Amazonas.
ML	131016	longsong	A	Guyana, Upper Takutu-Upper Essequibo, Sipu River
AA	Rebio Maicuru 5nov2008	longsong	A	Brazil; Rebio Maicuru, Almeirim, Pará.
ML	134352	longsong	A	Guyana, Upper Takutu-Upper Essequibo
ML	59358	longsong	A	Venezuela, 0.5 km E of river; Rio Grande; El Palmar
WA	WA583160	longsong	A	Brazil; Prainha, Pará.
ML	73042	longsong	A	Guyana, Upper Takutu-Upper Essequibo
SD	*Megascops usta* Arapiuns	longsong	B	Brazil; Resex Tapajos-Arapiuns, Santarem, Pará.
SD	*Megascops usta* Jacareacanga	longsong	B	Brazil; PARNA Amazônia, Itaituba, Pará.
ML	59397	longsong	B	Venezuela; Pica San Carlos-Solano.
ML	75206	longsong	B	Peru; Madre de Dios; Tambopata Jungle Lodge.
ML	49255	longsong	B	Ecuador, Morona-Santiago.
ML	35599	longsong	B	Brazil; PARNA Amazônia, Itaituba, Pará.
XC	XC20907	longsong	B	Brazil; Fazenda Fartura, Santana do Araguaia, Pará.
ML	59396	longsong	B	Venezuela, Amazonas, left bank Rio Negro.
ML	89115	longsong	B	Brazil; Alta Floresta, Mato Grosso.
ML	29235	longsong	B	Peru; Loreto.
ML	74930	longsong	B	Peru; Madre de Dios; Collpa de Guacamayos.
SD	*Megascops usta* Porto Velho	longsong	B	Brazil; Porto Velho, Rondônia.
ML	53376	longsong	B	Ecuador, Napo, La Selva-lodge.
XC	XC35367	longsong	B	Peru, Loreto, ACTS
SD	M usta 6	longsong	B	Brazil; Jordão, Acre.
AA	*Megascops* Careiro	longsong	B	Brazil; Tupana Lodge, Careiro, Amazonas
SD	*Megascops usta* Acre	longsong	B	Brazil. Jordão, Acre
ML	78330	longsong	B	Ecuador, Napo, Zancudo Cocha
SD	1007 *Megascops usta* Tanguro	longsong	B	Brazil; Fazenda Tanguro, Canarana, Mato Grosso.
ML	112831	longsong	B	Brazil; Terra Verde Lodge, Amazonas.
ML	126692 parte ML	longsong	C	Brazil; Flona Carajas, Salobo road, Parauapebas, Pará.
SD	*Megascops usta* Salobo_A2	longsong	C	Brazil; Projeto Salobo, Carajas, Parauapebas, Pará.
CB	*Megascops watsonii* Trairao	longsong	C	Brazil; Trairao, Pará.
SD	*Megascops usta* trilha bacaba 01	longsong	C	Brazil; Rebio Tapirape-Aquiri, Carajas, Parauapebas, Pará.

source	catalog name	type	clade	locality
AA	Indivíduo 4 coletado	longsong	C	Brazil; Flona Caxiuana, Pará.
AA	indivíduo 5	longsong	C	Brazil; Flona Caxiuana, Pará.
XC	XC94645	longsong	C	Brazil; Flona Tapajos, Santarem, Pará.
ML	59395_edited	longsong	D	Brazil; Belem, Pará.
SD	*Megascops watsonii* Base 04.mp3	longsong	D	Brazil; Tucuruí, east bank of Tocantins river, Pará.
GT	*Megascops usta* individuo 1.	longsong	D	Brazil; Benevides, Pará.
SD	*Megascops usta* Mata Pirelli	longsong	D	Brazil; Benevides, Pará.
ML	127980	longsong	E	Brazil; Usina Serra Grande, Engenho Coimbra, Alagoas.
ML	128031 edited	longsong	E	Brazil; Murici, Alagoas.
ML	127911 edited	longsong	E	Brazil; Usina Serra Grande, Engenho Coimbra, Alagoas.
ML	127829	longsong	E	Brazil; Usina Serra Grande, Engenho Coimbra, Alagoas.
XC	XC7159	longsong	F	Brazil; PN Itatiaia, Rio de Janeiro.
ML	101565	longsong	F	Paraguay, Caninde, Reserva Natural del Bosque Mbaracayu
ML	94909	longsong	F	Brazil; Estação Ecológica De Caetetus, São Paulo.
XC	XC85265	longsong	F	Brazil, Boa Nova, Bahia.
XC	XC99919	longsong	F	Brazil; Quatro Barras, Paraná.
WA	WA126698	longsong	F	Brazil; Veracel, Porto Seguro, Bahia.
XC	XC46306	longsong	F	Brazil; RPPN Serra Bonita, Camacan, Bahia.
XC	XC25671	longsong	F	Brazil; Miracatú, São Paulo.
XC	XC91649	longsong	F	Brazil; Ubatuba, São Paulo.
ML	59359	shortsong	A	Venezuela, 0.5 km E of river; Rio Grande; El Palmar
ML	59369	shortsong	A	Venezuela, Rio Grande; 1 km N of km 9.5; El Palmar
ML	59376	shortsong	A	Venezuela, Rio Cuyuni + 10 km
ML	59378	shortsong	A	Venezuela, Rio Caura; Cano La Urbana; main camp
ML	73045	shortsong	A	Guyana, Upper Takutu-Upper Essequibo, Maipaima Camp
ML	77917	shortsong	A	Guyana, Upper Demerara-Berbice, Shanklands
ML	87589	shortsong	A	Guyana, Upper Demerara-Berbice.
ML	29294	shortsong	B	Peru, Loreto, 1.0 km S of Libertad; south bank Rio Napo
ML	30868	shortsong	B	Peru, loreto, N. bank Rio Napo; Sucusari Camp
ML	29469	shortsong	B	Peru, Madre de Dios, Tambopata Reserve
ML	33783	shortsong	B	Peru, Loreto, N of Sucusari Camp, North Bank Rio Napo
ML	34372	shortsong	B	Peru, Loreto, W of Sucusari Camp; North Bank Of Rio Napo
ML	51923	shortsong	B	Bolivia, Santa Cruz; Noel Kempff Mercado National Park.
ML	84479	shortsong	B	Ecuador, Napo, 34.0 km N of Coca
ML	89060	shortsong	B	Brazil, MT, Reserva Ecologica Cristalino, Trilha das Rochas
ML	11052	shortsong	B	Bolivia, La Paz, Serrania Tequeje near Rio Undumo
ML	126830	shortsong	B	Brazil, MT, Cristalino Jungle Lodge, Trilha das Rochas, Saleiro
ML	132280	shortsong	B	Peru,madre de Dios, 6.0 km W of Iberia at Oceania
ML	135024	shortsong	B	Bolivia, Pando
ML	135176	shortsong	B	Peru, madre de Dios, 13.0 km NW of Atalaya.
XC	XC121151	shortsong	B	Brazil; Itaituba, PA.
ML	126657	shortsong	C	Brazil, Pará, Floresta Nacional de Carajas; Salobo Road
SD	*Megascops watsonni* precious woods	shortsong	C	Brazil; Portel, Pará.

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SD	*Megascops usta* trilha bacaba	shortsong	C	Brazil; Rebio Tapirape-Aquiri, Carajas, Parauapebas, Pará.
SD	*Megascops usta* Caxiuana Team plot 2	shortsong	C	Brazil; Flona Caxiuana, PA.
XC	XC84114	shortsong	D	Brazil; Paragominas, Pará.
XC	XC75524	shortsong	F	Brazil; Parque Estadual Intervales, São Paulo.
XC	XC102229	shortsong	F	Brazil; São Miguel Arcanjo, São Paulo.
ML	113381	shortsong	F	Brazil; Reserva Florestal de Linhares, Espírito Santo.