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Evolução vocal em suboscines: complexidade na variação vocal e discriminação acústica do complexo *Synallaxis ruficapilla*

Sidnei Sampaio dos Santos

Salvador, BA
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Sidnei Sampaio dos Santos

Orientador: Dr. Henrique Batalha-Filho,

Coorientador: Dr. Marcos Maldonado-Coelho

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Prof. Dr: Guilherme Henrique Silva de Freitas	Prof. Dr.: Luiz Pedreira Gonzaga
Prof. Dr.: Marcelo Felgueiras Napoli	Prof. Dr.: Mario Cohn-Haft
Prof. Dr.: Henrique Batalha-Filho Orientador	Prof. Dr. Marcos Maldonado-Coelho Coorientador

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

Arthur Schopenhauer (1778-1860)

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Índice

Resumo (geral)

Sinais acusticos tem muitas funções e intermediam várias interações em animais. Por exemplo, podem ser importantes na escolha de parceiros e na defesa do território. Os processos de deriva (i.e., estocástico) e seleção (i.e., determinístico) podem moldar a variação e divergência em sinais acústicos. Por sua vez, o grau de divergência acústica muitas vezes pode determinar os padrões de discriminação comportamental. A discriminação acústica de sinais divergentes pode estar por trás da origem de barreiras ao fluxo gênico e, portanto, desempenhar um papel crucial durante o processo de especiação. No entanto, nosso entendimento de como a deriva e a seleção conduzem à divergência acústica e influenciam a discriminação comportamental ainda é pouco conhecida, principalmente em aves com vocalizações inatas. Os suboscines representam uma grande radiação de passeriformes tropicais cujas vocalizações são consideradas inatas. Por isso, a influência da evolução cultural na variação e divergência do canto neste grupo é menos provável do que em aves que aprendem seus cantos (por exemplo, passeriformes oscines). Por isso, suboscines constituem um excelente sistema para testar os efeitos de processos estocásticos e deterministas na evolução acústica e na discriminação comportamental sem o efeito do aprendizado cultural. No Capítulo 1, testei se os processos estocásticos de deriva genética e determinísticos de seleção desempenharam um papel na divergência acústica em suboscines do complexo *Synallaxis ruficapilla*. Este complexo é composto por duas espécies endêmicas da Mata Atlântica sul-americana. Para tal, analisei cantos gravados em uma ampla área dentro da distribuição geográfica dessas espécies. No Capítulo 2, usei experimentos de playback recíproco de canto para testar se a similaridade acústica ou a história filogenética influenciam a discriminação comportamental neste complexo. Os experimentos de playback foram realizados em três populações representando três das principais linhagens evolutivas (i.e., clados) encontradas neste complexo. Meus resultados indicam que a variação vocal no complexo *ruficapilla* não reflete as relações filogenéticas. Em vez disso, a deriva genética, a seleção sexual e o ambiente surgem como candidatos para explicar uma variação vocal que é intrigante. Além disso, meus resultados mostram que a discriminação de canto é simétrica e todas as linhagens do complexo *ruficapilla* exibem respostas comportamentais mais fortes a cantos locais (própria população) em relação a cantos estrangeiros (outras populações) divergentes. No entanto, a resposta aos cantos estrangeiros distintos não foi estatisticamente significativa. Nesse contexto, descobri que nem a similaridade acústica nem a relação evolutiva influenciaram significativamente a resposta comportamental. No entanto, essa tendência de discriminação heterogênea não significativa para cantos estrangeiros distintos pode fornecer

pistas sobre o papel da divergência acústica na resposta comportamental em linhagens evolutivas que divergiram recentemente. Finalmente, meus resultados sugerem que diferentes processos evolutivos podem moldar elementos distintos do canto de maneiras específicas. Isso pode potencialmente desempenhar um papel importante no processo de discriminação acústica em aves suboscines.

Abstract

Acoustic signals have many functions and mediate many interactions in animals. For example, they can be important in mate choice and territory defense. The processes of drift (i.e., stochastic) and selection (i.e., deterministic) can shape the variation and divergence in acoustic signals. In turn, the degree of acoustic divergence often determines the patterns of behavioral discrimination. Effective auditory discrimination of divergent signals might underly the origin of barriers to gene flow and therefore, play a crucial role during the speciation process. Nonetheless, our understanding of how drift and selection drive acoustic divergence and influence behavioral discrimination is still poorly known, mostly in birds with innate vocalizations. The suboscine birds is a large radiation of tropical passerine birds of which vocalizations are thought to be innate. Thus, the influence of cultural evolution on song variation and divergence in this group is less likely than in birds that learn their songs (e.g., oscine passerines). As a consequence, suboscine birds constitute an excellent system to test the effects of stochastic and determinist processes in acoustic evolution and behavioral discrimination without the confounding effect of cultural learning. In Chapter 1, I tested whether the stochastic and deterministic processes of genetic drift and selection have played a role in shaping acoustic divergence in suboscine spinetails of the *Synallaxis ruficapilla* complex. This species complex consists of two species thar are endemic to the South American Atlantic Forest. To this end, I analyzed songs recorded over a broad region within the geographical range of these species. In Chapter 2, I used reciprocal song playback experiments to test if either acoustic similarity or phylogenetic history influence behavioral discrimination in this species complex. Here, the playback experiments were performed in three populations representing three main evolutionary lineages (i.e., clades) found in this species complex. My results indicate that vocal variation in the *ruficapilla* complex does not mirror phylogenetic relationships. Instead, genetic drift, sexual selection, and the environment emerge as candidates to explain a puzzling vocal variation. Additionally, my results show that song discrimination is symmetric, and all lineages of the *ruficapilla* complex exhibit stronger behavioral responses to local songs relative to divergent foreign songs. Yet, the response to distinct foreign songs were not statistically meaningful. In this context, I found that neither acoustic similarity nor evolutionary relatedness significantly influenced the behavioral response. However, a non-significant heterogenous discrimination trend towards distinct foreign songs can provide insights into the role of acoustic divergence on behavioral response in recently diverged evolutionary lineages. Finally, my results suggest that different evolutionary processes can

shape distinct elements of the song in specific ways. This can potentially play an important role in the process of acoustic discrimination in suboscine birds.

Introdução Geral

Sinais e comunicação

Sinas de comunicação são utilizados por animais em uma grande diversidade de situações e de variadas formas. O display de cores, formas, feromônios, expressões faciais, sinais elétricos e vocalizações, entre outros, são apenas alguns exemplos da diversidade de sinais utilizados na comunicação animal (Parr & Waller, 2006; Jackson & Ratnieks, 2006; Oller & Griebel, 2008; Girard et al., 2011; Laidre & Johnstone, 2013; Ligon et al., 2018). Um conceito mais amplo de sinal na comunicação, independente da forma ou situação que o sinal é utilizado, considera que é uma ação ou estrutura que modifica o comportamento de outro animal (Bradbury & Vehrencamp, 2011). Uma definição mais específica trata o sinal como qualquer aspecto (comportamento, morfologia, fisiologia) que altera o comportamento de outro animal em favor do emissor, que evolui com essa função (mudar o comportamento) e que tem seus efeitos através da evolução de respostas correlatas do receptor (Maynard & Harper, 2003; Searcy & Nowicki, 2005; Vitousek et al., 2014). Em outras palavras, os sinais são traços que permitem ao animal transmitir (emissor) informações pelo meio ambiente e que ajudam outros animais (receptores) a decidir se e como responder ao sinal (Irschick et al., 2015).

Considerar que sinais estão diretamente ligados ao contexto de comunicação implica supor a existência de sistemas que evoluíram em concerto para emissão, percepção e decodificação (Irschick et al., 2015). É nesse contexto que despontam muitas questões ligadas ao papel dos sinais, sua diversificação e a evolução de comunicação, que há muito interessam as mais diversas questões de cientistas em todo o mundo, como por exemplo: como são utilizados e quais contextos? Como são produzidos? Quais estruturas evoluem para maximizar ou restringir sua transmissão, percepção e decodificação? Que tipo de informação pode ser transmitida? De que forma se diversificam? Obviamente que não há uma resposta única para cada questão, embora alguns padrões possam ser observados (Bradbury & Vehrencamp, 2011; Irschick et al., 2015). É um campo promissor de investigação, com muitos desafios, que embora há muito estudado cresceu nas últimas décadas, principalmente porque há muitos paralelos com a linguagem humana e a cada dia há mais dados disponíveis (em bases online) para ampliar nosso escopo de investigação (Eliason, 2018; Searcy & Nowicki, 2019).

Comunicação nas aves

Em se tratando de sinais de comunicação nas aves, sem dúvida os acústicos são de longa data os mais estudados, ainda que existam outras formas de comunicação, como visual e tátil (Gill, 2007; Lovette & Fitzpatrick, 2016). De modo geral as aves produzem sons em um órgão específico e único no reino animal, que é a siringe (Catchpole & Slater, 2008). Mas também fazem uso de estruturas externas para a produção de sons, como asas, tamborilar do bico (batendo sobre troncos) e pés (batendo contra o solo ou outras estruturas) (Byers & Kroodsma, 2016). Os sinais acústicos produzidos pela siringe são, em geral, categorizados em dois grupos: sons e chamados (Kroodsma et al., 1982; Catchpole & Slater, 2008). Os sons, ou cantos, são sinais acústicos que carregam maior complexidade e são de longo alcance, ou seja, percorrem o ambiente com maior distância, e estão associados a interações na escolha de parceiros e defesa territorial (Kroodsma et al, 1982; Catchpole & Slater, 2008). Já os chamados são sinais de menor complexidade, de curto alcance e empregados para intermediar interações sociais não ligadas a escolha de parceiros (Marler, 2004). Obviamente que há exceções e outras categorizações (Odom et al., 2021), mas de modo geral esse é o padrão de categorização mais aplicado na vasta maioria dos trabalhos com o grupo. Ambos os sinais são extremamente importantes para as aves, já que mediam as principais interações intra e interespecíficas, como escolha de parceiros, defesa de recursos e sociabilidade (Slabbekoorn & Smith, 2002; Podos et al., 2004).

Outra distinção importante em relação aos sinais acústicos nas aves é ligada a diversificação que envolve aprendizado. Aqui podemos separar as aves em dois grandes grupos: primeiro, aves que aprendem os sons, ou seja, requerem tutores para desenvolver seu repertório vocal; e segundo, o grupo que tem som estereotipado, atribuído ser de base genética, ou seja, não depende de tutores para desenvolver seu repertório vocal (Slabbekoorn & Smith, 2002). A capacidade de aprendizado está bem documentada em três grupos, que são os Psittacidae (periquitos, papagaios), Trochilidae (beija-flores) e Oscines, um grupo de Passeriformes (Searcy & Nowicki, 2019) e recentemente outros grupos estão indicando alguma capacidade de aprendizado e ampliando a discussão sobre o alcance desse processo para o grupo (Ten Cate, 2021). Essa distinção é importante porque há processos específicos ligados a diversificação de sinais acústicos entre os dois grupos (som estereotipado ou dependente do aprendizado).

Diversificação de sinais acústicos

A diversificação dos sinais acústicos em aves é resultado da ação de diferentes forças e processos de seleção ou restrição (Podos et al., 2004; Wilkins et al., 2013). Podemos identificar cenários onde a evolução dos sinais acústicos parece ser mediada via processos estocásticos (deriva) ou ligados a seleção (por exemplo, ecológica), ou ainda pela combinação de ambos (Podos & Moseley, 2009; Wilkins et al., 2013). Cabe ressaltar que por trás desse cenário sempre deve ser considerado também os fatores que atuam com potencial de limitar (restrição) a diversificação (Wiley & Richards, 1982; Ryan & Brenowitz, 1985; Wilkins et al., 2013).

Como qualquer outro fenótipo, os sinais acústicos exibem variação intra e interespecífica em diferentes níveis, como por exemplo no espaço, ou seja, variação geográfica (Wilczynski & Ryan, 1993; Podos & Warren, 2007). A variação geográfica oferece excelentes oportunidades para entender a ação de variadas forças atuando na diversificação dos sinais acústicos e testar hipóteses ligadas a evolução (Wilczynski & Ryan, 1993). Nessa direção, apresento cenários ligados a diversificação dos sinais acústicos, exibindo como processos estocásticos ou de seleção e/ou restrição atuam na sua diversificação e os resultados em escala geográfica. O objetivo maior é evidenciar os padrões de variação geográfica nos sinais acústicos das aves e possíveis explicações para alguns cenários.

Entre os processos estocásticos podemos citar dois cenários; o primeiro é a deriva cultural. Está associado, principalmente, ao grupo de aves que exibem aprendizado. Nesse caso o resultado da diversificação depende da extensão de acurácia do aprendizado (Krebs & Kroodsma, 1980). Quanto menor a acurácia, maior serão as mudanças do sinal ao longo das gerações. Isso ocorre durante o estágio de desenvolvimento do repertório com "erros" de aprendizado, ou seja, com exclusão, inserção ou combinação de sons distintos de sua referência de tutores (Podos & Moseley, 2009). O segundo cenário associado a processos estocásticos é o de deriva genética, ou seja, mudanças nos locos gênicos que ancoram a expressão do fenótipo. Aqui poderíamos esperar que mudanças aleatórias nas estruturas da anatomia, fisiologia ou sistemas neurais, ligadas a produção e aprendizado do som, estariam sob maior efeito de deriva gênica, principalmente em populações pequenas (Podos & Moseley, 2009; Wilkins et al., 2013). Outra possível influência de efeitos da deriva gênica seria pequenas mudanças aleatórias que se acumulam com a distância entre as populações, um efeito associado ao isolamento por distância. Em outras palavras, a diversificação dos sons seria resultado do grau de isolamento entre as populações, onde populações mais próximas exibiriam maior similaridade quando

comparadas com populações mais distantes (Wright, 1943; Wei et al., 2014). Uma clássica maneira de testar o efeito da deriva gênica é correlacionar em conjunto a divergência genética neutra com a vocal (Irwin et al., 2008). Quando há correlação significativa entre ambas assumimos que, pelo menos em parte, a diversificação acústica pode ter ocorrido por deriva (Irwin et al., 2008; Wilkins et al., 2013).

Em relação a processos de seleção podemos destacar pelo menos dois cenários. O primeiro cenário envolve a seleção ecológica, que pode atuar, pelo menos, de duas formas: a primeira ligada as diferenças nas propriedades de transmissão do sinal em relação ao habitat (Morton, 1975). Nesse caso deve existir ação direta da seleção dependente do habitat sobre propriedades do sinal em direção a optimização de transmissão (Lemon et al., 1981; Brumm & Naguib, 2009). Dessa forma, tem sido tratada como Hipótese de Adaptação Acústica (Ey & Fischer, 2009). A segunda forma nesse cenário considera que a seleção atua em estruturas ligadas, ou que influenciam, a produção dos sons (por exemplo, tamanho do bico e corpo) e como consequência agem de forma indireta na diversificação (Derryberry et al., 2018). Por exemplo, bicos estão sob forte seleção em relação a disponibilidade de alimento e manipulação: seleção no tamanho do bico pode levar a mudanças de som, já que o trato vocal depende da coordenação de movimentação do bicho durante a emissão do sinal acústico (Podos, 2001).

O segundo cenário é ligado a seleção social (que aqui inclui também a seleção sexual), onde os sinais acústicos são considerados para mediar interações sociais, como competição ou guiar a escolha de parceiros (Searcy & Andersson, 1986; Wilkins et al., 2013). No contexto de competição dois modelos de deslocamento de caráter chamam a atenção, principalmente em populações simpátricas: primeiro, convergência de sinais para mediar competição (Tobias & Seddon, 2009); segundo, diferenciação para evitar hibridização (Kirschel et al., 2009). Em se tratando dos modelos no contexto de seleção sexual, temos por exemplo, o sinal acústico indicando qualidade do parceiro (Searcy & Andersson, 1986). Complexidade do canto tem sido atribuído como um indicador de qualidade, ou seja, quanto mais complexo o canto maior seria o vigor (fitness) e, portanto, maiores seriam as chances do parceiro ser escolhido (Searcy & Andersson, 1986; Searcy & Brenowitz, 1988).

Não podemos deixar de falar de outros fatores que atuam durante a diversificação dos sinais acústicos, especialmente atuando de forma limitante (*restrição*): composição da comunidade, ou seja, a presença de outras espécies que produzem sinal acústico pode conduzir a seleção divergente para evitar sobreposição do sinal (Krause, 1993). Essa restrição está

diretamente ligada a hipótese de nicho acústico, com a predição que os sons seriam um produto da seleção para um espaço acústico com faixas temporais e espectrais especificas para diminuir a sobreposição e interferência entre espécies (Mullet et al., 2017). Outro fator que limita a diversificação é a história filogenética. Nesse caso estruturas derivadas ou ancestrais (morfologia do bico, tamanho do corpo) que evoluíram em outro contexto ecológico e social limitam a variação acústica porque há influencia ancestral, ou melhor, sinal filogenético no sinal acústico (Ryan & Brenowitz. 1985). Temos ainda a morfologia e a neurofisiologia, tanto do emissor como receptor, que também impõem algumas restrições na diversificação sonora (DeVoogd, 2004, Derryberry et al., 2012). Restrições impostas por morfologia estão associadas ao tamanho do corpo ou do aparato vocal (principalmente tamanho e forma do bico) (Ballentine, 2006; Derryberry et al., 2018). Em relação ao tamanho do corpo há inúmeras evidências de que há uma relação negativa entre tamanho do corpo e frequência do sinal acústico, ou seja, quanto maior o corpo menor a frequência emitida (Ryan & Brenowitz, 1985; Price et al., 2006). Isso decorre do fato do tamanho das estruturas vocais de produção do som. Corpos maiores implicam em estruturas de maior tamanho (mais especificamente a siringe) e, portanto, maior dificuldade de movimentação (siringe vibra com menor intensidade), o que resulta em emissão de sons mais graves. Já em relação ao tamanho do bico, se mantem uma relação similar, ou seja, bicos longos tem um aparelho vocal maior que funciona como um filtro para sons mais graves (Nowicki, 1987; Huber & Podos. 2006).

Essa miríade de fatores tem sido evocada para explicar os distintos padrões de variação geográfica observado nos sinais acústicos (Slabbekoorn & Smith, 2002; Podos et al., 2004; Podos & Warren, 2007; Price, 2008; Wilkins et al., 2013). Aqui é preciso mencionar um importante pano de fundo ao longo do curso de qualquer diversificação: o padrão de distribuição das populações, destacadas aqui: (i) populações isoladas (alopátricas); (ii) populações ao longo de um contínuo de distribuição, sem barreiras geográficas aparentes de isolamento; (iii) populações espacialmente sobrepostas (simpatria). O primeiro contexto aplica-se a variação acústica tanto intra como interespecífica, o segundo intraespecífico e o terceiro interespecífico (Wilczynski & Ryan, 1993). Esses contextos funcionam como um importante pano de fundo da variação sonora que pode ser tratado como similar ao que ocorre na variação genética (Koetz et al., 2007).

Em populações isoladas algumas características do som poderiam surgir como resultado da deriva (cultural ou genética) ou seleção (ecológica, sexual), ou ambas. Por exemplo, a variação vocal em populações isoladas de *Batara cinerea* (Aves: Thamnophilidae – grupo que

não possui aprendizado do canto) possivelmente ocorre sob o efeito de seleção ecológica e restrição morfológica (Sementili-Cardoso & Donatelli, 2019). A espécie ocorre em áreas florestais em populações disjuntas (Mata Atlântica e Andes). Diferenças temporais e espectrais entre os grupos indicaram que a população dos Andes produziu vocalizações mais longas, com taxa de repetição de *trill* maiores, sílabas mais curtas e com frequência mais alta. As características ambientais entre as áreas (Andes de Mata Atlântica) são diferentes, o que segundo os autores poderia suportar a hipótese de influência ambiental atuando na transmissão sonora, ou seja, nos parâmetros espectrais (seleção ecológica). O principal suporte para essa hipótese é a correlação entre variáveis climáticas e estrutura vocal. Uma hipótese complementar a frequência mais alta na população dos Andes estaria relacionada à morfologia. Em Thamnophilidae, o tamanho do corpo exibe correlação negativa com a frequência vocal, ou seja, quanto maior o corpo, mais alta a frequência (Seddon, 2005). Embora não testado, as populações dos Andes são menores em tamanho e peso (Sementili-Cardoso & Donatelli, 2019). Outro exemplo de variação vocal em populações isoladas, só que agora com efeito de isolamento seguido por deriva cultural, genética e restrição morfológica ocorre em *Campylorhynchus rufinucha* (Aves: Troglodytidae – grupo com aprendizagem vocal) (Sosa-López et al., 2013). A espécie ocorre em florestas deciduais do México ao nordeste da Costa Rica. Dados de genética e morfologia indicam que há pelo menos três grupos isolados com uma estreita faixa de contato entre duas populações (Sosa-López et al., 2013). Há relação entre divergência vocal e divergência genética, mas não entre estas e variáveis climáticas, sugerindo que a variação vocal está acoplada ou segue o mesmo padrão da variação genética. A hipótese levantada sugere ausência de forças ambientais atuando na diversificação sonora e influência de eventos vicariantes, seguido por acumulações aleatórias na estrutura dos sons através de erros no processo de aprendizado (deriva cultural – Lynch, 1996). Os autores não descartam ainda que tenham ocorrido mudanças aleatórias ou seleção natural em mecanismos ligados a produção do som (deriva gênica ou seleção ecológica indireta) (Slabbekoorn & Smith, 2002; Podos & Warren, 2007).

No segundo contexto, em populações contínuas, o isolamento por distância poderia resultar na acumulação de pequenas diferenças ao longo da distribuição. Quanto mais distante, maiores seriam as diferenças entre as populações. Um exemplo de diferença vocal relacionada a distância geográfica foi documentado em *Drymophila ferruginea* (Aves: Thamnophilidae), espécie que ocorre na Floresta Atlântica. Parâmetros de tempo e frequência exibiram maior dissimilaridade com o aumento do espaçamento geográfico, ou seja, quanto mais distantes ao

longo da distribuição maior a diferença vocal entre as populações (Cardoso & Donatelli, 2016). Em *Phylloscopus trochiloides* (Aves: Phylloscopidae) a divergência vocal (sons e chamados) foi correlacionada com a distância geográfica e divergência genética, sem relação com seleção ecológica ou morfológica (Irwin et al., 2008). Outra possibilidade nesse contexto de distribuição contínua está relacionada a característica especificas do habitat ao longo da ocorrência. Nesse caso a divergência vocal pode estar relacionada a seleção ecológica imposta por particularidades do ambiente. Por exemplo, ao longo de um gradiente altitudinal (1300-2500m) *Chiroxiphia boliviana* (Aves, Pipridae) exibiu variação vocal relacionada a altitude e aspectos de transmissão (reverberação e atenuação) (Villegas et al., 2018). Esses resultados são concordantes com a hipótese de adaptação acústica. E ainda temos outra possibilidade nesse contexto que é a variação clinal. Ainda que populações isoladas por distância sejam mais distintas entre si, ao longo da distribuição não exibem contrastes ou categorias, mas sim um contínuo de variação. Esse padrão foi documentado no complexo *Thamnophilus caerulescens* que ocorre do norte da Bolívia ao nordeste da Argentina (Isler et al., 2005). Apesar de algumas variáveis quantitativas vocais distinguir populações nos extremos da distribuição, valores intermediários em populações adjacentes não permitiram distinguir grupos distintos (Isler et al., 2005).

No último contexto de distribuição, simpatria, talvez ocorra cenários mais complexos. Poderíamos esperar convergência de sinais (se por exemplo, os sinais estão sob mesmo efeito de seleção ecológica) ou não são discriminados porque em parte são incorporados pelo aprendizado (Qvarnström et al., 2006). Ou seja, em alguns casos não poderíamos separar as espécies pelos sinais acústicos quando em simpatria (Kenyon et al., 2011). Por exemplo, em populações simpátricas de *Oporornis tolmiei* e *O. hiladelphia* (Parulidae) não foi encontrado discriminação nos parâmetros acústicos, embora populações alopátricas tenham exibido diferenças significativas (Kenyon et al., 2011). Outro resultado interessante foi a ausência de correlação entre variação vocal e genética, principalmente em relação aos híbridos. Ou seja, provavelmente ocorra aprendizado vocal tanto de coespecíficos como heteroespecíficos na zona de simpatria. Um cenário oposto ao anterior é esperado se em simpatria ocorre forte seleção contra hibridização. Neste caso poderíamos observar divergência vocal distinta entre as espécies, o que pode estar associado a mecanismos de *deslocamento de caráter* ou reforço, ou seja, divergência em simpatria (Kirschel et al., 2009). Por exemplo, em *Pogoniulus bilineatus* e *P. subsulphureus* (Lybiidae), tanto parâmetros espectrais como comportamentais (resposta ao *playback*) exibiram maior diferença em simpatria do que em alopatria (Kirschel et al., 2009).

É preciso chamar atenção ainda para um padrão de variação que talvez seja a variação geográfica mais estudado em aves, os dialetos (Baker & Michael, 1985). Os dialetos indicam a variação de aprendizado dentro da mesma espécie exibido de forma agrupada em regiões distintas (Byers & Kroodsma, 2016). De forma específica, dialetos ocorrem quando populações ou grupos de indivíduos compartilham repertório com variantes espécie-específicos (diferença discreta em sinais) com limites geográficos bem definidos (Baker & Michael, 1985; Podos & Warren, 2007). Há modelos clássicos de estudo de dialetos, como *Zonotrichia leucophrys pugetensis*, com décadas de observação, exibindo uma variação complexa em aspectos estruturais e qualitativos do sinal acústico (Nelson et al., 2004, Nelson & Soha, 2004; Soha et al., 2004).

Em resumo, sinais acústicos são importantes por mediar as principais interações nas aves, como escolha de parceiros, defesa de recursos e sociabilidade. Sujeitos a diferentes forças de seleção, como seleção natural (ecológica e social), deriva (genética e cultural) e muitas restrições (história filogenética, composição da comunidade, morfologia, neurofisiologia) que quando considerados em contextos de distribuição espacial distintos (distribuição contínua, alopatria, simpatria) exibem variadas respostas de diversificação. Ao examinar este cenário fica evidente que não há uma única resposta para o concerto entre forças de seleção e contexto geográfico. Mas sem dúvidas, os sinais acústicos desempenham papel fundamental para processos de diversificação relacionados a comunicação, comportamento e especiação.

Escopo da tese

No contexto geral essa tese investiga como alguns dos processos e mecanismos apresentados acima poderiam explicar a diversificação de sinais sonoros em suboscines. Como modelo dentro do grupo utilizamos duas espécies endêmicas à Floresta Atlântica pertencentes ao complexo *Synallaxis ruficapilla* (descrição do modelo abaixo). A tese foi dividida em dois capítulos para testar especificamente: (I) o quanto forças estocásticas e/ou determinísticas moldam a variação vocal no complexo *Synallaxis ruficapilla*; (II) qual a influência da história evolutiva e da similaridade acústica na discriminação do comportamento nesse complexo em resposta a playback. Os capítulos são apresentados no formato submetido ou requerido para publicação. O Capítulo I, intitulado "Solving an evolutionary puzzle: disentangling the drivers of geographic variation in songs of Atlantic Forest spinetails" está em revisão na *Animal Behaviour*. Já o segundo Capítulo, intitulado "The role of evolutionary history and acoustic

similarity on song discrimination in the *Synallaxis ruficapilla* complex: fitting the other piece of the puzzle" será submetido ao periódico *Behavioral Ecology*.

Modelo de estudo - O "complexo *ruficapilla*," como tratado aqui, compreende duas espécies, o pichororé *Synallaxis ruficapilla* Viellot, 1819 e o joão-baiano *Synallaxis cinerea* Wied 1831, que são endêmicas da Mata Atlântica (Figura 1). *Synallaxis ruficapilla* se distribui da Argentina (Missiones) e leste do Paraguai até o sudeste do Brasil (Minas Gerais e Espírito Santo), desde o nível do mar até 1400 m (Remsen Jr, 2020), enquanto *S. cinerea* ocorre no Brasil ao norte de Minas Gerais e Bahia, sendo a Chapada Diamantina o extremo norte da distribuição, entre 200-1000 m (Remsen Jr, 2020). Estas espécies utilizam o sub bosque denso de florestas úmidas a estacionais (Ridgely & Tudor, 1994; Pacheco & Gonzaga, 1995).

Dois debates interessantes nas últimas décadas foram motivados por essas espécies. O primeiro envolveu a interpretação do código internacional de nomenclatura zoológica (CINZ) e um outro sobre a validade de *S. cinerea* (Whitney & Pacheco, 2001; Stopiglia & Raposo, 2006; Aleixo, 2008; Stopiglia et al., 2013; Batalha-Filho et al., 2013; Bauernfeind et al., 2014). O centro da discussão sobre a nomenclatura foi se *S. whitneyi* Pacheco & Gonzaga, 1995, era um sinônimo júnior de *S. cinerea* Wied 1813 (Aleixo, 2008; Stopiglia & Raposo, 2006, 2008; Whitney & Pacheco, 2001). A luz da interpretação de Bauernfeind et al. (2014), que sugeriu a validade de *S. cinerea*, a sinonímia foi reconhecida pelo CBRO (Comitê Brasileiro de Registros Ornitológicos) e pelo SAAC (South American Classification Committee) (Aleixo, 2008; SACC, 2015; Pacheco et al., 2021). A partir de então as principais referências e comitês ornitológicos reconheceram *S. cinerea* como nome válido.

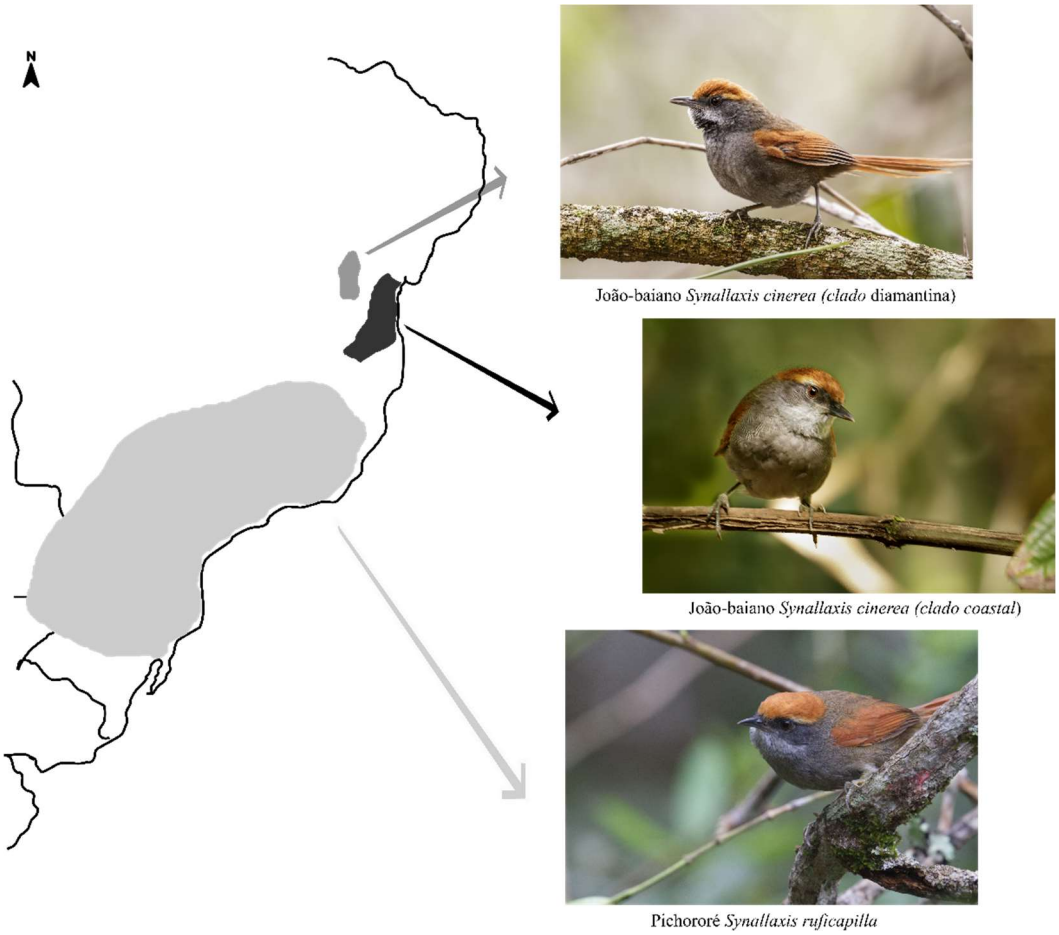

Figure 1. Distribuição das populações do complexo *Synallaxis ruficapilla* investigadas. Clados seguem Batalha-Filho et al. (2019). Fotos (Sidnei Sampaio).

Já a discussão sobre a validade da espécie foi iniciada por Stopiglia et al. (2013). Dessa vez os autores propuseram a invalidade de *S. cinerea* (ainda tratado por eles como *S. whitneyi*), com base no padrão de variação morfológica e vocal, que na opinião dos autores seria apenas uma variação geográfica de *S. ruficapilla* (Stopiglia et al., 2013). No entanto, estudos filogenéticos e filogeográficos, utilizando genes mitocondriais e nucleares, sugerem que *S. cinerea* e *S. ruficapilla* constituem linhagens evolutivas distintas (Batalha-Filho et al., 2013; 2019), fato este que reacende o debate sobre o status específico destas populações geneticamente e vocalmente (veja Capítulo 1) divergentes. Por fim, uma zona híbrida entre estas espécies foi descrita para o interflúvio entre os rios Doce e Jequitinhonha, provavelmente fruto de contato secundário decorrente das mudanças climáticas do Quaternário (Batalha-Filho et al., 2019).

Para além desse contexto, o complexo parece um bom modelo para testar hipóteses ligadas a processos responsáveis pela variação vocal e discriminação acústica por alguns

motivos: (i) são suboscines, de canto inato, ou seja, a ação de influência da deriva cultural não é esperada. Portanto, permite testar hipóteses de variação vocal sem efeitos de deriva cultural e trazer *insights* para outros grupos de canto inato que são pouco estudados; (ii) pertencem a família Furnariidae, que é considerada conservativa no padrão de coloração e espera-se que cantos tenham grande importância, principalmente para espécies florestais que vivem no sub-bosque com pouca visibilidade de luz. Assim, os sons aparentam ser fundamentais no reconhecimento individual e especifico; (iii) ocorrem ao longo de um gradiente climático e de habitat (florestas úmidas a estacionais) ao longo da Mata Atlântica que permite testar hipóteses ligadas a variação vocal em relação ao ambiente; (iv) exibem estrutura filogeográfica que permite testar influência da divergência genética e relações filogenéticas na variação vocal e discriminação; (v) a divergência entre *S. ruficapilla* e *S. cinera* é recente, cerca de 0.902 mya (Batalha-Filho et al., 2019), o que pode trazer insights sobre a variação vocal e discriminação acústica em populações com divergência recente e possivelmente em estados inicias do processo de especiação; (vi) por fim, testar diferentes hipóteses ligadas a variação vocal nesse complexo pode ajudar a elucidar sua história evolutiva.

Aqui nós levamos em conta a estrutura filogeográfica proposta por Batalha-Filho et al. (2019) e consideramos três clados nas nossas análises: clado *cinerea* (*coastal clade* de *S. cinerea*) clado diamantina (*Chapada Diamantina clade* de *S. cinerea*) e clado *ruficapilla* (que representa o *southern clade* de *S. ruficapilla*) (Figura 1). Excluímos das análises o clado *northern* de *S. ruficapilla*, que está restrito ao interflúvio do Rio Doce e Jequitinhonha e parte da população do clado *cinerea,* no extremo sul da distribuição da espécie. Excluímos das análises sons dessas regiões porque: (i) ocorre hibridação entre as duas espécies e não conseguimos determinar qual a identidade genética da amostra vocal e (ii) evitamos a influência homogeneizadora de hibridação na variação vocal. No caso de *S. ruficapilla*, assumimos que as populações externas a esse interflúvio não são hibridas e a amostra vocal é representativa do seu respectivo clado. Em relação aos estudos de discriminação acústica, optamos por executar os experimentos de playback fora da zona hibrida, com o mesmo objetivo de evitar a influência homogeneizadora da introgressão genética, que estava fora do escopo de investigação.

Capítulo 1

Solving an evolutionary puzzle: disentangling the drivers of geographic variation in songs of Atlantic Forest spinetails

Sidnei Sampaio[1], Marcos Maldonado-Coelho[2], Rômulo Ribon[3], Bret M. Whitney[4], & Henrique Batalha-Filho[1,5]

[1] Programa de Pós-Graduação em Biodiversidade e Evolução, Instituto de Biologia, Universidade Federal da Bahia, Salvador, Brazil

[2] Smörbollsgatan 2, 24756 Dalby, Sweden

[3] Universidade Federal de Viçosa

[4] Louisiana State University, Department of Biological Sciences, Museum of Natural Science, Baton Rouge, Louisiana, USA

[5] National Institute of Science and Technology in Interdisciplinary and Transdisciplinary Studies in Ecology and Evolution (INCT IN-TREE), Instituto de Biologia, Universiade Federal da Bahia, Brazil

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Abstract

Acoustic signals provide essential cues in mate choice and territorial defense. These signals might evolve under the influence of stochastic and deterministic processes, which may result in barriers to gene flow and speciation. Yet, the role of the evolutionary forces of drift and selection on acoustic variation still remains poorly understood in birds with innate songs, such as in suboscine passerine birds. Here, we tested whether stochastic and deterministic forces shaped acoustic variation in an avian complex endemic to the South American Atlantic Forest, the *Synallaxis ruficapilla* complex. To this end, we integrated vocal variation, environmental variation and phylogeographic structure along the geographical range of the complex. Our analyses indicate that i) vocal variation in the *ruficapilla* complex does not mirror phylogenetic relationships and ii) the two syllables of the song exhibit distinct patterns of variation. Genetic drift, sexual selection, and spatial heterogeneity in forest cover emerge as candidates to explain the puzzling vocal variation in the *ruficapilla* complex. In addition, the distinct elements of the song seem to have evolved under the influence of different selective regimes.

Keywords: bioacoustics, Neotropics; song convergence; suboscines

It is widely known that acoustic signals mediate interactions in many animal groups and investigating their pattern of variation provides valuable insights into evolutionary biology (Bradbury & Vehrencamp, 2011). These signals can be used for, among other functions, territory defense and mate choice and are expected to be shaped by ecological and social selective forces, drift (genetic and cultural) and constraints (Slabbekoorn & Smith, 2002; Mason et al., 2017; Podos & Warren, 2007; Wilkins et al., 2013, Wilkins et al., 2018). Therefore, describing the geographic variation of acoustic signals and disentangling its ecological and evolutionary drivers is a matter of utmost importance to understand the dynamics of reproductive isolation and speciation (Wilczynski & Ryan, 1993; Ptacek, 2000; Edwards et al., 2005; Podos & Warren, 2007; Wilkins et al., 2013, 2018).

The environment is one of the main factors influencing acoustic signal variation and design. It can drive acoustic diversification by favoring acoustic properties that optimize propagation efficiency (Ryan & Kime, 2002; Tubaro & Lijtmaer, 2006; Mullet et al., 2017). Specifically, acoustic signals can be degraded by physical barriers (trees, leaves, rocks) and attenuated by obstacles and atmospheric absorption. Accordingly, denser habitats can select vocalizations with longer temporal aspects, slower-paced and with lower frequency while hot and dry habitats select for lower frequency signals (Wiley & Richards, 1978; Ryan & Kime, 2002; Barker, 2008). Environmental noise can also contribute to vocal diversification (Krause, 1987; Brumm & Slabbekoorn, 2005), being more important when the overlap between noise and signal is high (Krause, 1987; Brumm, 2013). In other words, vocalizations cast outside the noise frequency range of the habitat (i.e., songs from insects, wind, and rain) can be selectively favored as they optimize transmission (Ryan & Brenowitz, 1985; Luther & Gentry, 2013). For example, low-frequency signals can be selected against in habitats with higher intensity of rain or wind (Brumm & Slabbekoorn, 2005; Tubaro & Lijtmaer, 2006; Naguib, 2013). If habitat structure imposes selective constraints on acoustic signals, we

should find an association between spectral and temporal properties of vocalizations with habitat density and climate (temperature and precipitation). Hence, populations inhabiting structurally similar habitats should exhibit similar acoustic signals.

Stochastic processes such as cultural and genetic drift can also underly vocal diversification (Sosa-López et al., 2013; Wilkins et al., 2018). Cultural drift results from the random process of incorporating new elements in a vocally learned repertoire due to copying errors and innovation (Williams, 2004; Podos & Warren, 2007). On the other hand, evolutionary changes resulting from genetic drift relate to random changes in genetic loci underpinning the morphological apparatus associated with sound production (i.e., bill and body size) or genes linked directly to vocal expression (Podos 2001; Podos & Warren, 2007; Derryberry et al., 2012). Genetic drift can influence the variation of acoustic signals through isolation-by-distance and geographic isolation. In the former, random changes in vocalizations accumulate with increasing spatial distancing as a consequence of geographically restricted gene flow (Slatkin, 1993; Hutchison & Templeton, 1999). On the other hand, random changes in acoustic signals randomly accumulate over time due the cessation of gene flow in geographically isolated populations (Blomberg & Garland, 2002). In addition, if evolutionary history has a strong influence on vocal variation, populations sharing a more recent common ancestor would be expected to exhibit higher acoustic similarity (Losos, 2008; Price & Lanyon, 2002; Mejías et al., 2020). Thus, vocal signals would be more similar among populations that are geographically closer, that have been recently isolated and that are phylogenetically closely related (Koetz et al., 2007).

Although closely related species might share similar vocalizations, acoustic divergence under sexual selection can be unpredictable and prone to evolve fast (Andersson, 1994; Price, 2008). As a consequence, labile acoustic traits are often decoupled from

phylogeny (Blomberg et al., 2003; Benz and Robbins, 2011). This pattern is more common in birds that learn their songs (e.g., oscine passerines; Irwin et al., 2001; Price et al., 2007) but also has been found in birds with innate songs (Joseph et al., 2004; Benz and Robins, 2011).

Vocal complexity could also influence vocal geographic variation and divergence. For instance, most bird species exhibit structural differences among elements or notes in a given vocalization (i.e. temporal, spectral, or shape). Distinct notes or syllables in a song are more likely to have evolved as a consequence of unrelated processes. That is, when distinct elements in a vocalization exhibit different patterns of geographic variation, this heterogeneity can be a consequence of disparate evolutionary processes (Benedict & Najar, 2019; Lee et al., 2019; Backhouse et al., 2021).

Despite advances in our knowledge of patterns and processes underlying geographic variation in the vocalizations of birds, most studies were conducted in the temperate region with oscine passerines (Tobias et al., 2012; Crouch & Mason-Gamer, 2019; Najar & Benedict, 2019). In contrast, there are major gaps in our knowledge concerning song variation in birds with innate songs from the Neotropical region (e.g. suboscine passerines). In fact, the role of stochastic and deterministic processes in driving vocal geographic divergence in this group remains poorly known (Sementili-Cardoso & Donatelli, 2019; Capelli et al., 2020; Acero-Murcia et al., 2021). In addition, there are striking differences in environmental and biological factors between wet tropical and temperate regions, such as seasonality, reproductive behavior, and community composition (Stutchbury & Morton, 2001; Reboreda et al., 2019), which could lead to distinct diversification scenarios in acoustic traits.

In this study, we used multivariate analyses and generalized additive models to assess the pattern of geographic variation and divergence in songs of two sister species of suboscine birds endemic to the South American Atlantic Forest: the *Synallaxis ruficapilla* and *S. cinerea*

spinetail complex – hereafter *ruficapilla* complex. This complex exhibits strong phylogeographic structure, with two clades found in each species (Figure 1B; Batalha Filho et al., 2019). Although vocal geographic variation in the *ruficapilla* complex has been studied (Stopiglia et al., 2013), the influence of phylogenetic history and the environment on vocal divergence was not formally tested. Our study investigated the pattern of vocal variation within and across the geographic range of divergent populations in the *ruficapilla* complex. We first assessed the vocal distinctiveness of each clade and then tested which evolutionary processes (deterministic or stochastic) better explain vocal variation throughout the landscape. To this end, we used a vocal dataset encompassing most of the range of the *ruficapilla* complex (Figure 1A).

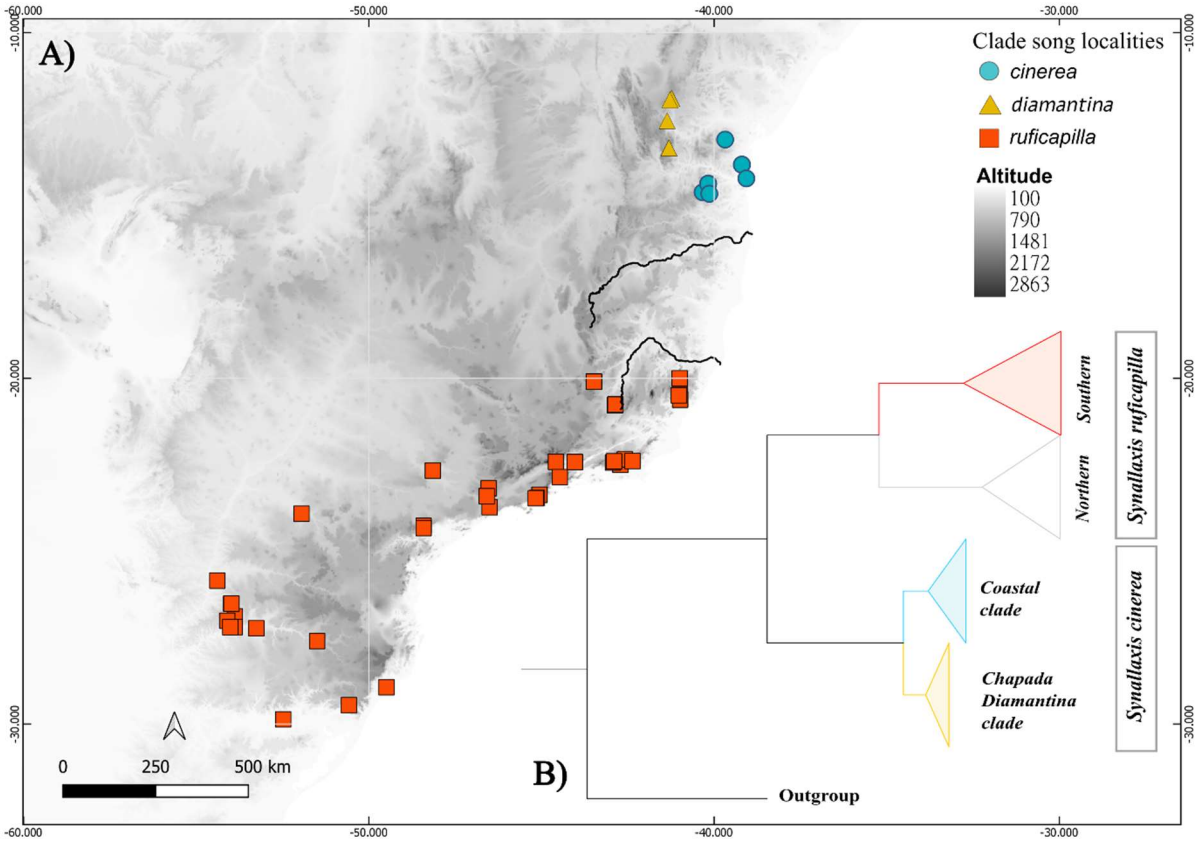

Figure 1. A) Map showing localities with vocal records of the *S. ruficapilla* complex. B) Phylogenetic relationships reported in Batalha-Filho et al. (2019). Black lines correspond to Doce and Jequitinhonha rivers, to the south and north, respectively

We aimed to answer the following questions: (i) Do clades in the *ruficapilla* complex exhibit diagnosable vocal signature? (ii) Is there a correspondence between vocal variation and phylogenetic history? If not, what factors could explain such discrepancy: (iii) geographic distance or (iv) habitat structure? In (i), we expected an association between vocal and genetic divergence (i.e. clades). In (ii), we predicted that vocal variation would track phylogeny; then evolutionary relationships and vocal similarity should be congruent, independent of geographic distancing between populations. In (iii), if isolation-by-distance explained vocal variation, we expected that latitude (a proxy of geographical space) would explain most of the vocal variation in the system. Alternatively, the environment could underly vocal variation, regardless of phylogenetic history or geographical space. However, these hypotheses are not mutually exclusive. For instance, populations can exhibit higher vocal similarity because they share both a common ancestor and a similar habitat.

METHODS

Study system – The *ruficapilla* complex*,* comprises two species endemic to the coastal Atlantic Forest: *Synallaxis ruficapilla* Viellot, 1819 (Rufous-capped spinetail) and *Synallaxis cinerea* Wied 1831 (Bahia spinetail). *Synallaxis ruficapilla* occurs from Argentina (Missiones) and east Paraguay north through southeastern Brazil to points in the states of Minas Gerais and Espírito Santo, from near sea level to 1400m. *Synallaxis cinerea* occurs in the northeasternmost region of the state of Minas Gerais into the state of Bahia, from 200 to 1200 m, and the northernmost known population is found in the Chapada Diamantina mountains (Batalha-Filho et al. 2019, Remsen Jr, 2020). Both species are fairly common in the understory edges of humid, semideciduous and dry forests (Ridgely & Tudor, 1994; Pacheco & Gonzaga, 1995), especially those with at least some bamboos in the understory.

The northern clade of *S. ruficapilla* is syntopic and hybridizes with the southernmost population of the coastal clade of *S. cinerea*. The Diamantina clade in *S. cinerea* inhabits the semideciduous forests on the eastern flank of the Chapada Diamantina mountains; it is geographically isolated from the coastal clade of *S. cinerea* to the west and from *S. ruficapilla* to the north (Batalha-Filho et al., 2019, Figure 1).

Pacheco and Gonzaga (1995) described *Synallaxis whitneyi* (which name was later corrected to *S. cinerea* by Whitney and Pacheco, 2001) as a species distinct from closely related *S. ruficapilla* on the basis of clear diagnostic differentiation in morphology and vocalizations. A subsequent taxonomic review of the *S. ruficapilla* complex based on morphology and vocal variation led Stopiglia et al. (2013) to consider *S. cinerea* as only a geographic variant of southern *S. ruficapilla*. Nonetheless, phylogenetic studies using mitochondrial and nuclear genes identified divergent clades that are geographically congruent with the proposed ranges of *S. cinerea* and *S. ruficapilla*, with a hybrid zone where their ranges meet (Batalha-Filho et al., 2013; 2019).

The *ruficapilla* complex constitutes a good model to test hypotheses related to the process of vocal diversification because: (i) songs are supposedly innate (Kroodsma 1984; Kroodsma & Konishi 1991; Touchton et al. 2014). Hence, it allows us to test hypotheses without the influence of cultural evolution and to gain insights into the factors underlying vocal evolution in avian groups with innate songs; (ii) they belong to the Furnariidae family, a radiation with dull plumage coloration (Marcondes & Brumfield 2019). Accordingly, vocalizations can be essential in mediating intra- and inter-specific recognition in this group, mainly in species inhabiting the dimlylit forest understory; (iii) the *ruficapilla* complex occur across a gradient of climate and habitats in the Atlantic Forest that allows testing the influence of the environment on vocal variation; (iv) the complex is phylogeographically structured,

which allows testing the role of evolutionary history on vocal variation; and (v) the divergence between *S. ruficapilla* and *S. cinerea* is relatively recent (estimated at roughly 902 kya; Batalha-Filho et al., 2019), which provides an opportunity to disentangle the processes shaping vocal variation in recently diverged lineages.

Here we used the phylogeographic structure proposed by Batalha-Filho et al. (2019) and considered three clades in our analysis: the *cinerea* clade (coastal clade of *S. cinerea*, type from Boa Nova, Bahia,), the diamantina clade (Chapada Diamantina clade of *S. cinerea*, unnamed population) and the *ruficapilla* clade (the southern clade of *S. ruficapilla*, type from Rio de Janeiro) (Figure 1B). We excluded both the northern clade of *S. ruficapilla* and the southernmost population of the *cinerea* coastal clade from our analysis because a hybrid zone between these two species was found in this region (Batalha-Filho et al., 2019), and we were unable to ascribe songs to the correct genetic/species identity there. Moreover, this minimizes any possible homogenizing influence of hybridization on vocal variation, which was beyond the scope of our study. For the *ruficapilla* clade, we assume that populations outside the hybrid zone region (i.e south of the Doce River, Figure 1) are under small or negligible influence of vocal introgression, and that vocal samples included here are representative of their respective clades.

Acoustic analyses – We used songs recorded by us and from online databases: Xeno Canto (XC, xeno-canto.org), Macaulay Library (ML, macaulaylibrary.org - Cornell Lab of Ornithology, Ithaca, NY, US), Wikiaves (WA, wikiaves.com.br), Fonoteca Neotropical Jacques Vieillard (FNJV) and Arquivo Sonoro Elias Coelho (ASEC) (Supplementary material – Table 1). We selected recordings with the highest signal-noise ratio. Each recording was normalized to 0dB and standardized at a sampling rate of 44 kHz and 16-bit amplitude resolution using Audacity 2.1.3 (https://www.audacityteam.org/). Spectrograms were

quantified in Raven Pro v.1.6 (Bioacoustic Research Program, Cornell Lab of Ornithology, Ithaca, NY) with the following presets: Window type Hann, size 5.8 ms, 3dB filter bandwidth 248 Hz; Time grid Overlap 50 %, Hope size 128 samples; Frequency grid with DFT size of 256. We randomly selected five to 10 songs in each recording and estimated the mean of each measured variable. Each song element was classified into syllables accounting for a similar shape and repetition sequence (Odom et al., 2021). Songs in the *ruficapilla* complex were considered to comprise two distinct syllables (Figure 2). The song in the *ruficapilla* complex has two distinct syllables (Figure 2). The 18 measured variables are listed in Table 1.

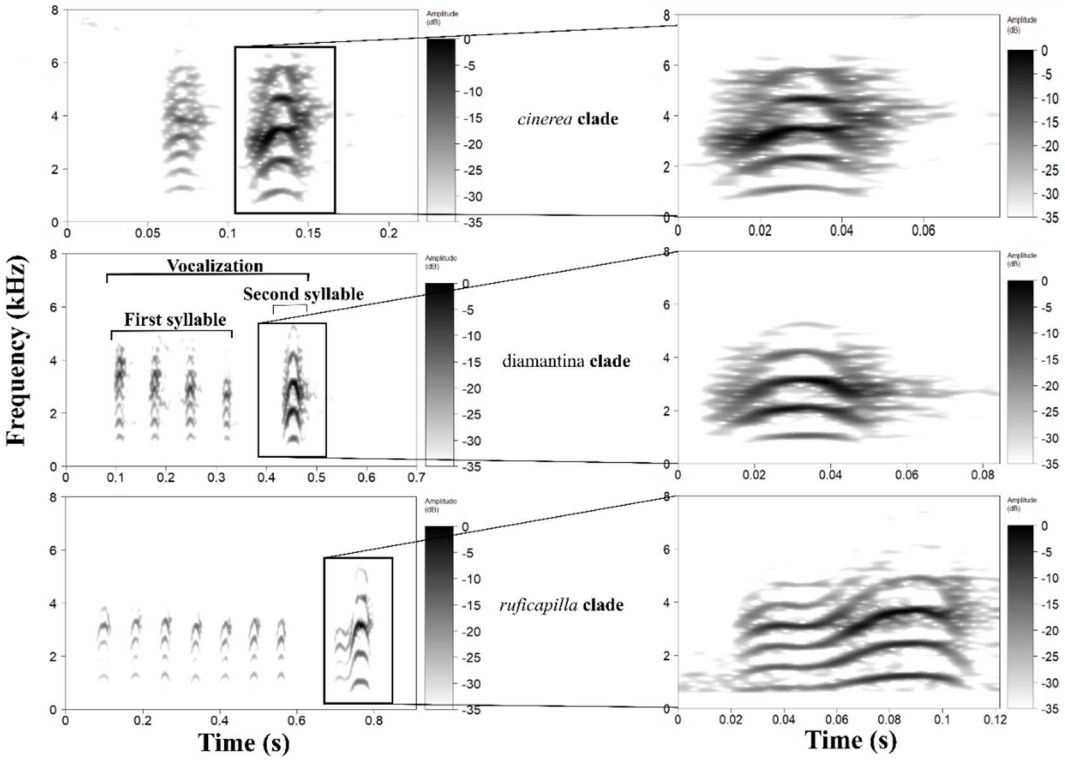

Figure 2. Sonogram of a typical song of each clade of the *S. ruficapilla* complex, highlighting variation in the second note.

Our vocal sampling covered a broad area within the geographic distribution of each clade (Figure 1A). We analyzed 902 songs in total (98 individuals; individual mean 9.2, SD = 1.3), of which 521 are from S. *ruficapilla* (56 individuals; mean 9.3, SD = 1.2), 224 are from *S. cinerea* (26 individuals; mean = 8.9, SD = 1.17) and 158 are from the diamantina clade of *S. cinerea* (17 individuals; mean 9.2, SD = 1.1) (Table 1).

Ethical note - We used songs recorded in natural conditions and from online databases. No licences were required to conduct this research. However, all song recordings used in this study were made noninvasively from a distance.

Clade	Descriptive statistics	Song note number	Song length	Song pace	Internote length	First syllable note number	First syllable length	First syllable pace	Ssecond syllable note number	Second syllable duration	Center Time*	Center frequency*	Max frequency	Freq 5%*	Freq 25%*	Freq 75%*	Freq 95%*	BW50 %*	BW90 %*
	min	1.00	0.03	15.55	0.01	0.00	0.03	0.00	1.00	-0.01	0.56	2670.12	2859.61	1033.59	2067.19	3152.46	3824.30	275.63	1670.98
	max	2.60	0.14	33.34	0.08	1.60	0.04	133.13	2.00	0.06	2.28	3933.40	4163.09	2928.52	3565.90	4421.48	4938.28	1705.43	3652.03
cinerea	mean	1.96	0.10	21.81	0.05	0.92	0.03	56.75	1.05	0.02	1.47	3432.27	3518.90	1941.97	2993.95	3769.30	4420.74	775.34	2478.77
	sd	0.27	0.02	4.83	0.02	0.31	0.00	29.78	0.21	0.01	0.39	291.48	319.21	475.79	370.89	320.36	408.07	335.25	518.52
	cv	13.85	24.73	0.22	42.75	33.92	7.92	0.52	20.39	71.19	26.83	8.49	9.07	24.50	12.39	8.50	9.23	43.24	20.92
	min	2.11	0.21	10.16	0.10	1.11	0.03	16.64	1.00	0.02	1.68	2153.32	2205.00	913.01	1894.92	2308.36	3049.10	344.53	1399.66
	max	9.67	0.76	13.55	0.21	8.67	0.06	55.71	1.14	0.51	4.69	3273.05	3402.25	2105.47	3051.56	3617.58	5374.69	1501.17	4461.68
diamantina	mean	4.02	0.33	12.20	0.14	3.01	0.04	23.78	1.01	0.15	3.17	2806.73	2874.87	1451.82	2329.05	3110.97	3783.34	781.92	2331.52
	sd	1.78	0.13	0.96	0.03	1.78	0.01	9.23	0.03	0.12	0.81	292.99	303.56	332.08	316.69	304.01	515.18	376.51	734.71
	cv	44.33	41.19	0.07	22.03	58.97	15.62	0.38	3.44	78.27	25.61	10.44	10.56	22.87	13.60	9.77	13.62	48.15	31.51
	min	3.11	0.24	10.53	0.05	2.11	0.05	13.76	0.90	0.09	1.72	2721.80	2583.98	981.91	1860.47	2997.42	3445.31	206.72	878.55
	max	11.30	0.83	21.55	0.15	10.30	0.12	26.39	2.20	0.65	8.49	4323.87	4375.55	2980.20	3807.07	4461.68	5271.33	1528.86	3255.82
ruficapilla	mean	5.57	0.43	13.01	0.10	4.55	0.08	19.31	1.02	0.25	4.86	3421.40	3528.37	1930.31	2997.84	3709.03	4259.58	711.19	2329.26
	sd	1.96	0.14	1.60	0.02	1.98	0.02	2.88	0.16	0.12	1.69	321.14	334.26	452.83	387.98	302.41	402.47	282.90	528.40
	cv	35.11	32.31	0.12	18.15	43.38	20.06	0.15	15.80	50.70	34.76	9.39	9.47	23.46	12.94	8.15	9.45	39.78	22.69

Table 1. Song variables of the *S. ruficapilla* complex. SD = standard deviation; CV = coefficient of variation. Song pace = ratio of the total number of elements and the duration time of the entire song; Internote length = time interval between syllables (ms); First syllable duration = time interval from the beginning of the first element to the end of the last element (ms); First syllable pace = ratio of the number of elements and the duration time; Second syllable duration = time interval from the beginning of the second element to end of the last element (ms). * Measurements considered "robust signal measurements" following Charif et al., 2010. Details of these measurements and Maximum frequency are provided in Charif et al., 2010.

Environmental variables – We used temperature (BIO1) and precipitation (BIO12) from Bioclim (Fick & Hijmans, 2017) as abiotic variables. We used NDVI (Normalized Difference Vegetation Index) to quantify habitat heterogeneity. The NDVI is a satellite-based vegetation index that correlates with aboveground net primary productivity and has been used to indicate vegetation density and cover (Pettorelli, 2013). These variables have been used to assess the influence of the environment on vocal variation (Barker, 2008; Medina & Francis, 2012). We used the R package Dismo (version 1.1-4, Hijmans et al., 2011) to extract these variables based on our records at a resolution of 2.5 min (~5 Km) (Figure A1). Bioclimatic data were downloaded from the WorldClim version 2.1 (https://www.worldclim.org), for the period 1970-2000. For the NDVI, we used the mean from 2000 to 2010 accessed from ORNL DAAC in the Google Earth Engine platform.

Statistical analyses

Vocal variation and signature – We performed a principal component analysis (PCA*)* to reduce collinearity and redundancy of the vocal variables. Before the PCA analysis, the acoustic variables were normalized to zero mean and unit variance (z-scores). We conducted the analysis using a correlation matrix of the 18 vocal variables and retained four axes with eigenvalues > 1. These components explained 86.94% of the total variance of the vocal data (Table A1). The PCA allowed us to explore the multivariate vocal variation in the *ruficapilla* complex and to assess the correspondence between vocal variation and phylogeny. We tested whether clades differed in a multivariate acoustic space (PC scores) with the Kruskal-Wallis test, followed by the Dunn *post hoc* test for multiple comparisons among groups. The vocal PC scores were further used to

test the influence of stochastic and deterministic effects on vocal variation (see additive models below).

Congruency between vocal variation and phylogeny – To evaluate the congruence between phylogenetic history and vocal variation, we applied four distinct clustering methods. One was a supervised learning method (SL) and three were unsupervised learning methods (UL): (i) discriminant analysis (SL); (ii) K-means clustering (UL); (iii) partitioning around medoids clusters (PAM, UL); and (iv) hierarchical cluster analysis (HC, UL). In these analyses, we used only vocal variables with correlation (r) < 0.70 (Dormann et al., 2012). Employing and contrasting results of more than one classification technique allowed us to rule out idiosyncrasies of each method. If our results are method-independent, we expected a general congruency among them regardless of their accuracy.

SL method – The discriminant analysis is a dimensionality reduction technique that considers *a priori* groups to maximize the difference between-class relative to within-class variance in order to find maximal separability (Hair et al., 2009). Some vocal variables (mostly temporal ones) failed to fit a normal distribution and hence we applied the nonparametric Flexible Discriminant Analysis (FDA, Hastie et al., 1994) using z-scores. In FDA, we assumed three of the clades recovered in Batalha-Filho et al. (2019): the *cinerea* and diamantina clades (*S. cinerea*) and the southern *ruficapilla* clade (*S. ruficapilla*). To evaluate the chance-adjusted classification accuracy of FDA between the groups, we used Cohen's kappa coefficients (K) via R package vcd (Meyer et al., 2020). We used the *fda* function of the R package mda to perform the FDA analysis (Hastie & Tibshirani, 2020).

UL methods – K-means is an algorithm that partitions a given data set into k groups (k clusters). Here, the classification considers the high intra-class similarity within the same cluster and how they are as dissimilar as possible among clusters (i.e. low inter-class similarity). Each cluster coincides with the mean of points assigned to the centroid (i.e. cluster center; Kassambara, 2017). The PAM is an algorithm that seeks K representative objects (medoids) in a given data set. The medoid represents the most centrally placed object of the cluster, with minimal mean dissimilarity for all cluster elements. PAM deal with outliers better than K-means (Kassambara, 2017). To find the optimal number of clusters using K-means and PAM algorithms, we used the average silhouette width with R packages factoextra v.1.0.7 (Kassambara & Mundt, 2020) and NbClust v.3.0.1 (Charrad et al., 2014). The HC is an approach to clustering unlabeled grouping objects based on their similarity. We used the most common HC method, agglomerative clustering (AC), which cluster objects considering the similarity and does not require cluster predefinition (Kassambara, 2017). The AC starts considering each object as a single group. Then, the algorithm combines pairs of groups until all clusters merge into a big cluster, including all objects (Kassambara, 2017). The result is a dendrogram that shows the similarity among groups. We used the Euclidian distance to measure the similarity among objects and ward.D2 (= ward) as a linkage method. In the unsupervised methods analysis, we used the R packages cluster v.2.1.2 (Maechler et al., 2021) and factoextra (Kassambara & Mundt, 2020) to visualize graphically the results.

Latitude, habitat, genetic divergence and vocal variation – To test the influence of deterministic and stochastic processes on vocal variation, we used generalized additive models (GAM). In GAMs, we considered the following scenarios: (i) four independent models including latitude, genetic divergence/phylogenetic clusters, habitat (NDVI), and climatic variables as predictors, and the vocal PCs (PC1 and PC2) and spectral

variables (maximum frequency and bandwidth 90%) as the response variables; (ii) two additional models including latitude, habitat (NDVI) and phylogenetic clusters as predictors and the temporal variables (song time and song pace) as the response variables. We used the R package mgcv v1.8-48 (Wood, 2011) to fit GAMs. In each model, latitude was modeled as a smooth spline given its non-linear association with the dependent vocal variables, while the other predictors were modeled as parametric/linear variables. The reasoning here is that we have theoretical expectations of a linear relationship between environmental variables (habitat and climate) and song variables. In addition, the *concurvity* function indicated that many predictors exhibit prohibitive concurvity levels (~1; i.e., the smooth generalization of collinearity). This was especially true when most predictors were modeled with latitude as a smooth term. The collinearity between parametric variables in each model was assessed with the R package mgcv.help v.0.1.9 (Clifford, 2019) using the variance inflation factor (VIF). We kept variables with VIF < 3 (Zuur et al., 2010).

RESULTS

Vocal variation and signature – All clades in the *ruficapilla* complex have a unique vocal signature, even though the diamantina and *ruficapilla* clades overlap at some extent in a two-dimensional multivariate space (Figure 3, Table A2). The PC1 distinguishes *cinerea* from the other two groups (*ruficapilla* and diamantina), while the PC2 indicates that *cinerea* and *ruficapilla* are more similar to each other relative to diamantina. The vocal PC1 represents an axis of temporal variation with the variables song length, first syllable length, and number of notes positively correlated; the vocal PC2 is an axis of spectral variation with center frequency, maximum frequency, and frequency at 25% positively correlated; the PC3 also represents spectral variation, with

the bandwidth at 50% and 90% positively correlated and frequency at 5% negatively correlated; the PC4 represents temporal variation, with the song pace positively correlated and internote length negatively associated.

The PC1 (Kruskal-Wallis test, $P < 0.05$; Table S2) and the PC2 (Kruskal-Wallis test, $P < 0.001$; Table A2) scores were significantly distinct among clades (Figure A2A). The *cinerea* clade was the most divergent in PC1 scores, being significantly distinct from the diamantina and *ruficapilla* clades (Dunn *post hoc* test). The diamantina clade had the smallest PC2 score values, and all clades were significantly different from each other along this axis (Dunn *post hoc* test, Table A2; Figure A2B). The PC3 scores did not exhibit significant differences among clades (Tabela A2; Figure A2C). The PC4 scores differ significantly among all clades (Kruskal-Wallis test, $P < 0.001$; Table A2; Figure A2D), with the diamantina clade being the most divergent one. Here, all clades were also significantly different from each other (Dunn *post hoc* test, Table A2).

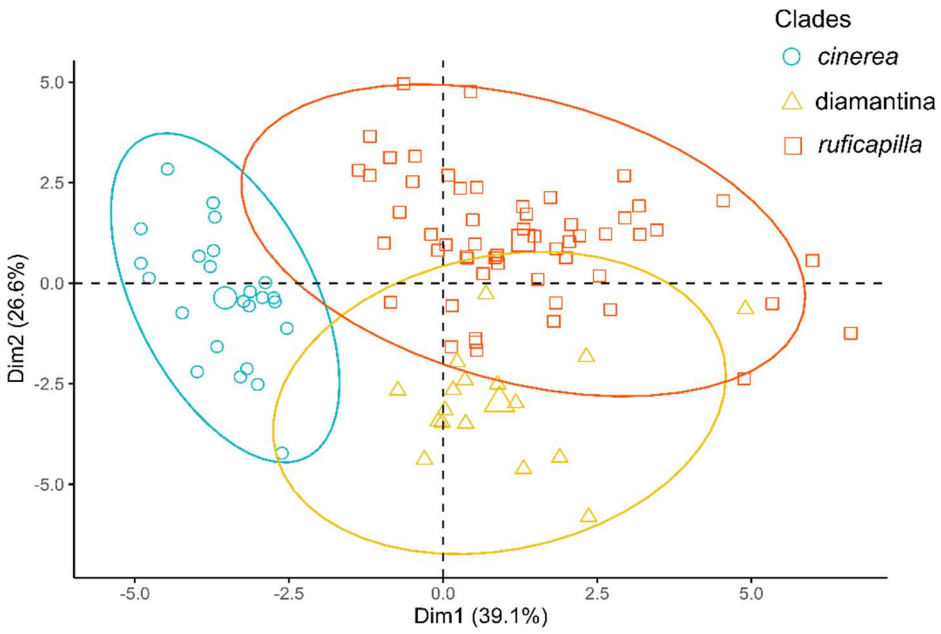

Figure 3. Scatter plot from a principal component analysis (vocal PC1 vs. vocal PC2) of the *S. ruficapilla* complex. Group colors represent the three phylogenetic clades according to figure 1.

Congruency between vocal variation and phylogeny – All classification methods suggest a lack of correspondence between vocal variation and phylogenetic structure in the *ruficapilla* complex. Specifically, the FDA, the K-means, PAM and the HC indicate that songs of the non-sister diamantina and *ruficapilla* clades are more similar to each other relative to the *cinerea* clade (Figure 4). The two FDA discriminant functions explained 100% of the variance (Figure 4A). The overall classification accuracy of the original clades was 94%, with 92 out of 98 songs correctly classified to their original clade, which is significantly higher than expected in a randomly assigned classification (Cohen's $k = 0.89$, $p < 0.05$, 95% CI: 0.81-97; Table A3). Specifically, only three songs from the diamantina clade were incorrectly assigned to the *ruficapilla* clade and *vice versa* (Figure 4A, Table A3).

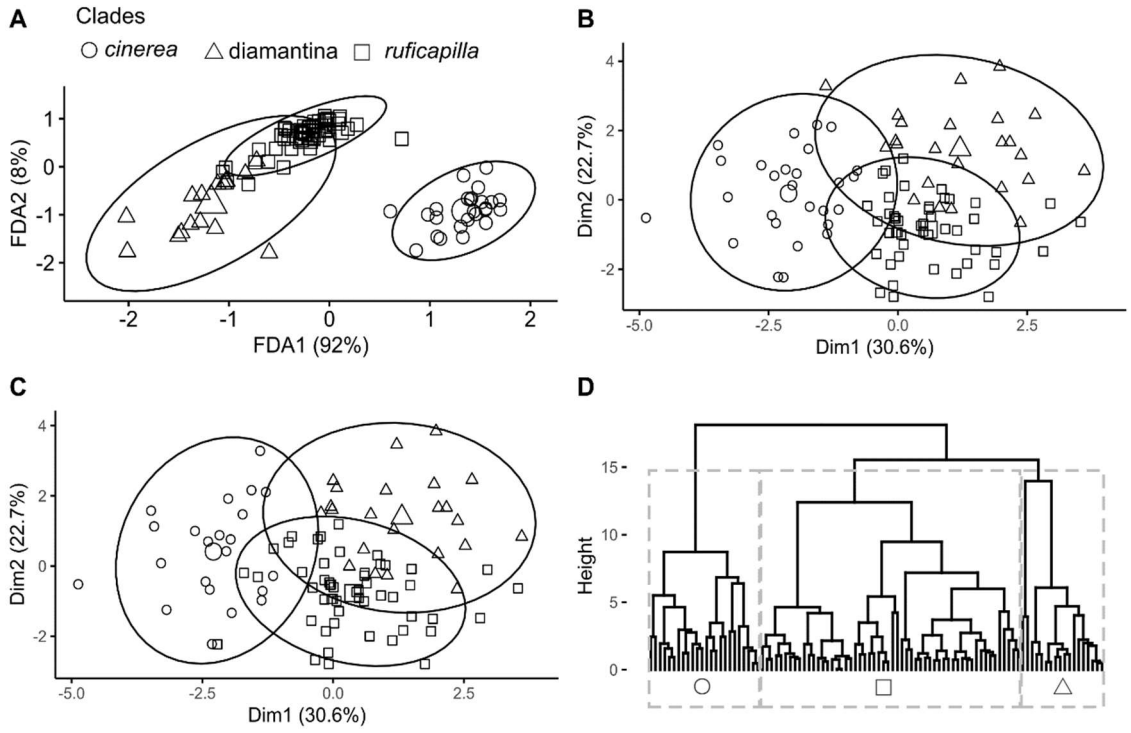

Figure 4. Cluster analyses of song elements of the *S. ruficapilla* complex. A) Distribution of scores of the discriminant analysis (Flexible Discriminant Analysis); B) K-means cluster: C) Partitioning Around Medoids (PAM) cluster; D) dendrogram of the hierarchical cluster (grouping by Ward method). The

ellipses correspond to the 95% confidence interval of the classification. All analyses were performed using the same data set (only variables with r < 0.70 were included).

The K-means recovered three groups associated with their original clades (Figure 4B, Table A3). The first and second axes represent 30.6% and 22.7 % of the variation, respectively, and 86% of songs were correctly assigned to the original clades (Figure 4B, Table A3). The *cinerea* clade had higher classification accuracy (96%), followed by the diamantina (94%) and *ruficapilla* (80%) clades (Table A3). The K-means had the lower classification accuracy among all clustering methods (Table A3). The PAM also recovered three groups associated with the original clades (Figure 4C, Table A3). The first and second axes represent 30.6% and 27.7% of the variation, respectively. Here, the classification correctly assigned 89% of the songs to each original clade (Table A3). The classification accuracy of the *cinerea*, *ruficapilla* and diamantina clades were respectively 9%, 89% and 88% (Table A3). Finally, the HC recovered three groups that concur with clades of the *ruficapilla* complex, with an overall classification accuracy of 88.7% (Figure 4D, Table A3). The classification accuracy of the *cinerea* clade was 96%, followed by the *ruficapilla* (91%) and diamantina (70%) clades (Table A3).

Latitude, habitat, genetic divergence and vocal variation – We found a significant effect of latitude (GAM: $edf = 4.1.$, $F = 10.67$, $P < 0.001$, n = 98) and genetic divergence (clades: *cinerea* - $\beta = 5.79$, $t = 8.88$, $P < 0.001$; diamantina - $\beta = 10.97$, $t = 11.79$, $P < 0.001$; *ruficapilla* - $\beta = 5.75$, $t = 10.53$, $P < 0.001$) on PC1 scores (Figure 5, see Table A4 for other model parameters). Forest cover had a significantly positive effect on PC2 scores (NDVI, $\beta = 0.46$, $t = 2.02$, $P < 0.05$). Genetic divergence also had

a significant effect on PC2 scores (clades: *cinerea* - $\beta = 6.57$, $t = 12.32$, $P < 0.001$; diamantina - $\beta = 3.62$, $t = 6.81$, $P < 0.001$; *ruficapilla* - $\beta = 7.95$, $t = 19.04$, $P < 0.001$; Table A4). Only genetic divergence had a significant effect on raw spectral variables; specifically on maximum frequency (clade: *cinerea* - $\beta = 1.45$, $t = 21.55$, $P < 0.001$; diamantina - $\beta = 1.01$, $t = 12.47$, $P < 0.001$; *ruficapilla* - $\beta = 1.44$, $t = 29.34$, $P < 0.001$) and bandwidth 90% (clade: *cinerea* - $\beta = 4.02$, $t = 7.25$, $P < 0.001$; diamantina - $\beta = 3.84$, $t = 4{,}40$, $P < 0.001$; *ruficapilla* - $\beta = 3.61$, $t = 7.98$, $P < 0.001$; Table A5). Finally, we found a significantly positive effect of forest cover ($\beta = 0.08$, $t = 3.62$, $P < 0.001$), latitude (GAM: $edf = 1.69$, $F = 10.67$, $P < 0.001$) and genetic divergence (clade: *cinerea* - $\beta = 0.60$, $t = 6.49$, $P < .001$; diamantina - $\beta = 1.30$, $t = 10.01$, $P < 0.001$; *ruficapilla* - $\beta = 0.90$, $t = 11.47$, $P < 0.001$) on song length (Table A6). Only genetic divergence had a significant effect on song pace (clade: *cinerea* - $\beta = 1.23$, $t = 18.10$, $P < 0.001$; diamantina - $\beta = 0.35$, $t = 4.27$, $P < 0.001$; *ruficapilla* - $\beta = 0.47$, $t = 9.57$, $P < 0.001$; Table A6).

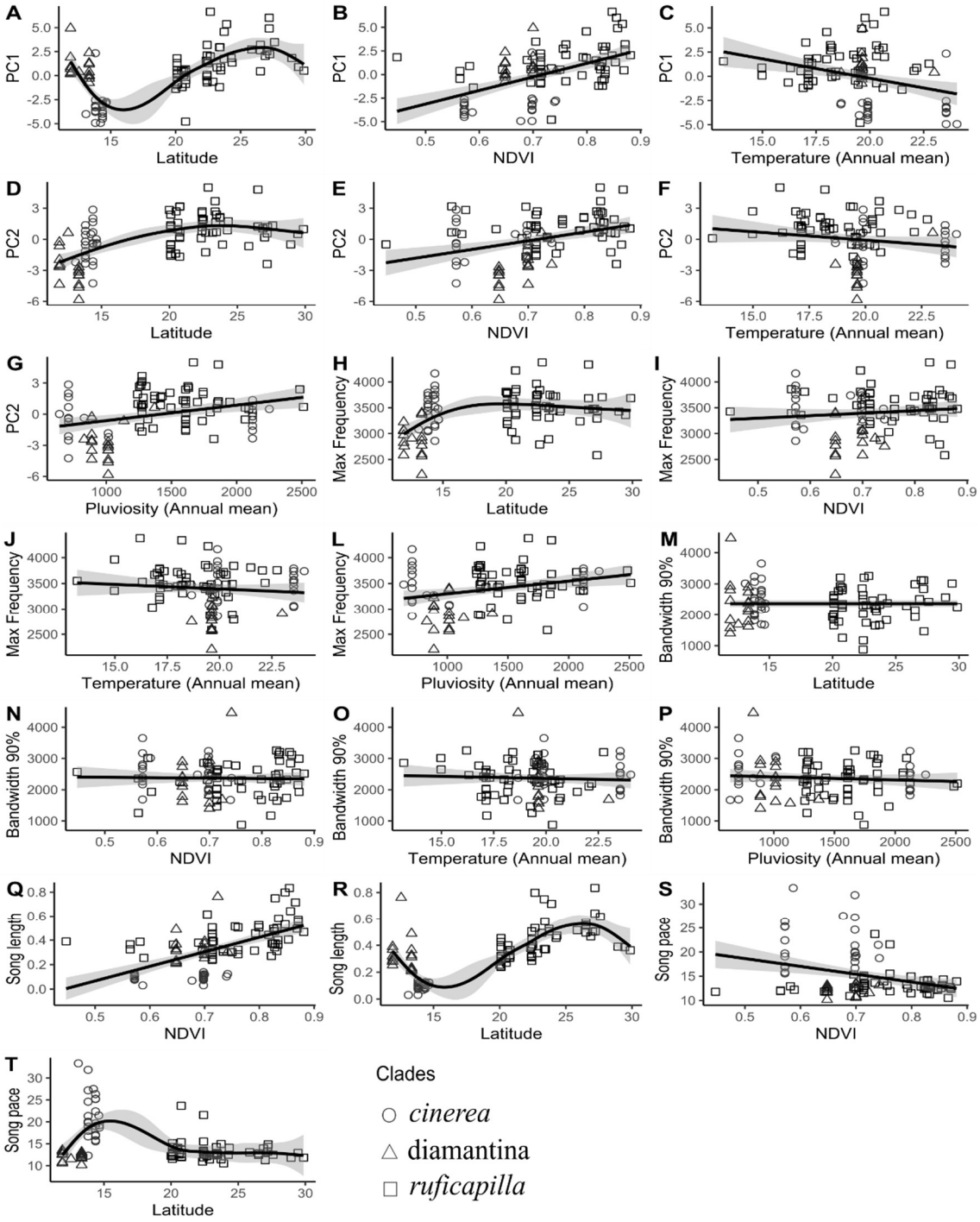

Figure 5. The association between song PCs and raw song elements with predictor variables in the *S. ruficapilla* complex. The plots depict: PCI with latitude, NDVI, and temperature (A, B, and C); PC2 with latitude, NDVI, temperature, and pluviosity (D, E, F and G); Maximum frequency with latitude, NDVI, temperature, and pluviosity (H, I, J and L); Bandwidth 90% with latitude, NDVI, temperature, and pluviosity (M, N, O and P); Song length (duration) with NDVI and latitude (Q and R); Song pace with NDVI and latitude (S and T).

DISCUSSION

We used a large vocal sampling and multivariate analyses to partition the relative contributions of deterministic and stochastic factors influencing song variation in the *ruficapilla* complex. Specifically, we tested whether there was an association between vocal variation with phylogenetic history, the environment and isolation-by-distance. Our results revealed that: (1) each clade has a distinct vocal signature; (2) vocal variation is not congruent with phylogenetic relationships; (3) isolation-by-distance, phylogenetic history, and forest cover seemingly influence vocal variation.

The vocal diversification of the Furnariidae was shaped by phylogenetic history, social selection, morphology, and habitat (Tobias et al., 2014; Derryberry et al., 2012, 2018). Despite the high diversity in this group (ca. 295 species), little research has focused on vocal diversification at a microevolutionary scale, exceptions being studies on *Aphrastura spinicauda* (Ippi et al., 2011), *Synallaxis albencens* (Lindell, 1998), and the *ruficapilla* complex (Stopiglia et al., 2013). Our study further expands the importance of ecological and evolutionary drivers of geographic variation in songs at a microevolutionary scale in this family and more generally, in suboscine birds. In the following, we discuss our results in the light of evolutionary forces and/or constraints.

Vocal variation and signature – Pacheco and Gonzaga (1995) documented clear vocal diagnosability of songs of *S. cinerea* from southern (nominate) *S. ruficapilla*. Our analyses further documented that each clade of the *ruficapilla* complex has a distinct vocal signature (Figures A2 A-D; Table A2). Temporal and spectral aspects of each song syllable differ among clades. The most divergent vocal elements in this complex comprise temporal aspects, with song length the variable that differs the most (PC1, Figure 3). Length of the first syllable and song note number also contribute significantly

to this pattern of vocal divergence (Table A2). While the *cinerea* clade is vocally distinct from the diamantina and *ruficapilla* clades (Figure 2-3), the last two did not differ statistically. That is, the diamantina and *ruficapilla* clades are similar regarding temporal aspects. Another source of vocal variation encompasses the spectral characteristics (PC2). Specifically, center frequency of the second syllable (Table A2) differed significantly among all clades (Table A2). This heterogeneity in divergence patterns may suggest that selective forces and/or constraints have acted in distinct ways on each song component (i.e., syllables).

It is conceivable that the higher the magnitude of the difference between elements (i.e., notes or syllables) in a specific signal (i.e., song), the higher the odds of evolving under different selective regimes. In the *ruficapilla* complex, heterogeneity in patterns of geographic variation of temporal relative to spectral aspects of the song suggests a role of distinct selective forces. Indeed, particular elements of the song have been suggested to be shaped by distinct selective forces or constraints (Benedict & Bowie, 2012; Roach & Phillmore, 2017; Benedict & Najar, 2019; Lee et al., 2019; Backhouse et al., 2021). Nevertheless, it is a challenge to disentangle which diversifying forces acted either in the entire signal/song or in specific song elements (syllables or notes). In addition, it is widely unknown how selection could shape interactions among song elements. Further work investigating the evolution of different song elements in the genera *Synallaxis* in a macroevolutionary perspective is warranted.

Vocal similarity and phylogeny – In one hand, behavior has been considered a labile trait prone to homoplasy (Blomberg et al., 2003). On the other, behavior also can provide historical information about evolutionary relationships (de Queiroz & Wimberger, 1993; Price & Lanyon, 2002). Vocal variation in the *ruficapilla* complex was not congruent

with the evolutionary history of the group (Batalha-Filho et al., 2019). The non-sister clades diamantina and *ruficapilla* were vocally more similar to each other than either is to the *cinerera* clade, as documented by all classification methods (Figure 4). Song note number and associated temporal variables (i.e., song length, internote length, and first syllable length) contributed largely to this pattern. On the other hand, a lower number of notes in *cinerea* relative to the other two clades was seemingly the main source of discrepancy between vocal variation and phylogeny. However, it is noteworthy that the diamantina and *ruficapilla* clades did not show significant differences in two vocal PCs (Table A2). These results are surprising as one would expect acoustic variation mirroring phylogenetic relationships in groups with innate vocalizations, mostly when they have recently diverged. In fact, trait lability is expected to be more frequent in groups that learn their songs (e.g., oscines - Irwin et al., 2001; Price et al., 2007), and our findings provide further evidence that it can be more common than previously thought in vocally non-learning birds (Joseph et al., 2004; Benz and Robins, 2011). This scenario leads us to the second question: could geographic isolation and/or habitat explain vocal variation in the *ruficapilla* complex and be the source of incongruency between phylogeny and vocal variation?

Latitude, habitat, and vocal variation – Vocal variation in agreement with a latitudinal gradient or isolation-by-distance has been reported elsewhere (Isler et al., 2005; Irwin et al., 2008; Xing et al., 2017; Capelli et al., 2020). Our findings suggest that temporal and spectral variation of songs in the *ruficapilla* complex were associated with latitude in the Atlantic Forest. However, the pattern of temporal and spectral variation was distinct between the two species. In *S. ruficapilla*, temporal elements showed a non-linear association with latitude (Figure 5A, Table A4), with longer songs found southwards. Meanwhile, the same pattern was observed in the northernmost population of *S. cinerea*.

That is, each species exhibits clinal variation but in the opposite direction throughout their ranges (Figure 5A). Convergence in temporal aspects of the songs in the extreme of their distribution is unexpected given an isolation-by-distance model. Under this model, genetic drift would cause populations further apart to be acoustically more divergent. Alternatively, random changes could have been important during the process of divergence after the fragmentation of the ancestral range. Accordingly, drift could have had a stronger influence on the vocal differentiation of the *cinerea* clade. Indeed, the *cinerea* clade shows the most distinct temporal pattern of variation among all clades (but see below). Here, we found a nearly linear association between spectral variables and latitude (vocal PC2; Figure A5D). Specifically, PC2 scores increase southwards (Figure A5B), thereby supporting an isolation-by-distance pattern and implying that gene flow is geographically restricted (Koetz et al., 2007). Thus, we cannot rule out the effect of stochastic factors on the divergence of spectral elements, although it is difficult to invoke drift when explaining vocal similarity between non-sister clades.

Similarities in habitat also can underly convergence of the acoustic temporal structure (Morton, 1975; Ey & Fischer; Hard, 2009; Mullet et al., 2017). In the *ruficapilla* complex, the spectral (vocal PC2) and temporal (song length) elements were positively associated with forest cover (i.e., NDVI). Specifically, songs with higher values of center frequency and maximum frequency were typical of sampling localities with denser vegetation. However, the sister clades diamantina and *cinerea* exhibited higher overlap in NDVI values relative to values across the range of the *ruficapilla* clade (Figure 5E, I and N). That is, vegetation density *per se* cannot explain the higher vocal similarity between the non-sister clades diamantina and *ruficapilla*. In addition, temperature was not significantly associated with any spectral vocal characters. Thus,

our results underscore the idea that the environment often cannot satisfactorily predict vocal variation (Hardt & Benedict, 2021).

Other processes – Introgressive hybridization is one potential process that can result in acoustic similarity/homogenization. As acoustic signals are used in mate choice, it is expected that they homogenize in contact zones due to hybridization, especially in recently diverged species (Qvarnström et al., 2006; Willis et al., 2014). Thus, under a hybridization scenario, we would expect higher vocal similarity between the coastal *cinerea* and *ruficapilla* clades, which interbreed along their contact zone (Batalha-Filho et al., 2019). On the other hand, the *ruficapilla* and the diamantina clades are geographically isolated, implying that hybridization is unlikely to explain their higher vocal similarity. In addition, when individuals compete by space or resources, acoustic signals can evolve convergently through social selection (Cody 1969, West-Eberhard, 1983, Rainey and Grether 2007, Tobias & Seddon, 2009; Kirschel et al., 2019). However, sympatry, indeed syntopy, is a necessary condition in considering such social selection scenarios (West-Eberhard, 1983; Tobias & Seddon, 2009). Likewise, we also would expect higher vocal similarity between the sympatric *ruficapilla* and *cinerea* clades (Batalha-Filho et al., 2019) under a social selection scenario. Thus, the higher vocal similarity between allopatric clades (diamantina and *ruficapilla*) rules out an influence of either hybridization or vocal mimicry.

Another possible explanation is ancestral variation in the first syllable of the song. In this context, stabilizing selection in the first syllable in *ruficapilla* and diamantina clades, with divergence in the *cinerea* clade, may underly the higher vocal similarity of the two non-sister clades. Indeed, the first syllable has fewer notes in the *cinerea* clade. Consequently, its song is shorter. Thus, we suggest two non-mutually

exclusive explanations for the diversification of the first syllable in the *cinerea* clade: genetic drift and/or sexual selection. Under these scenarios, the evolution of a smaller number of notes in this clade could be a consequence of (i) random changes in loci underpinning song expression during periods of population size reduction in allopatry (Batalha-Filho et al., 2019) and/or (ii) sexual selection in the *cinerea* clade, whereby female choice favored songs with fewer notes. Yet, song divergence resulting from reinforcement (Coyne & Orr 2004, Hudson & Price 2014) could have driven character displacement in the *ruficapilla* complex. Specifically, a reduction in song note number in the *cinerea* clade after it resumed secondary contact with *ruficapilla* (Batalha-Filho et al. 2019) could have been a consequence of selection against hybridization and homogenization.

Finally, the spectral variation in the second syllable of the song suggests that diversification of song elements is asymmetric. Specifically, the spectral variables of the second syllable in the diamantina clade, showing significantly lower values, are distinct from the *ruficapilla* and *cinerea* clades (Figure A3). This scenario is opposite to the one proposed for the first syllable, which shows a distinct signature in temporal variables (Figure A4). In the multivariate space, *ruficapilla* is more similar to the diamantina clade. Thus, the first and second syllables may have had distinct selection regimes and rates of evolution during the diversification of the *ruficapilla* complex (Figure 2).

In conclusion, our results indicate that distinct evolutionary forces can drive vocal variation in complex ways in suboscine birds. In the *ruficapilla* complex, specific elements of the song (i.e., syllables) have seemingly evolved under the influence of distinct selective pressures. Also, some results were unexpected. Specifically, contrary to what found in a macroevolutionary study of the ovenbird radiation (Derryberry et al.,

2018), vocal variation in the *ruficapilla* complex is decoupled from phylogeny. This underscores the fact that even innate behaviors can be evolutionary labile. Stochastic processes, sexual selection, geographic space, and habitat emerged as candidates to explain a puzzling vocal variation in the complex. Additional integrative studies should further enhance our understanding on the role of evolutionary forces underlying vocal variation in suboscine passerines.

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Appendix

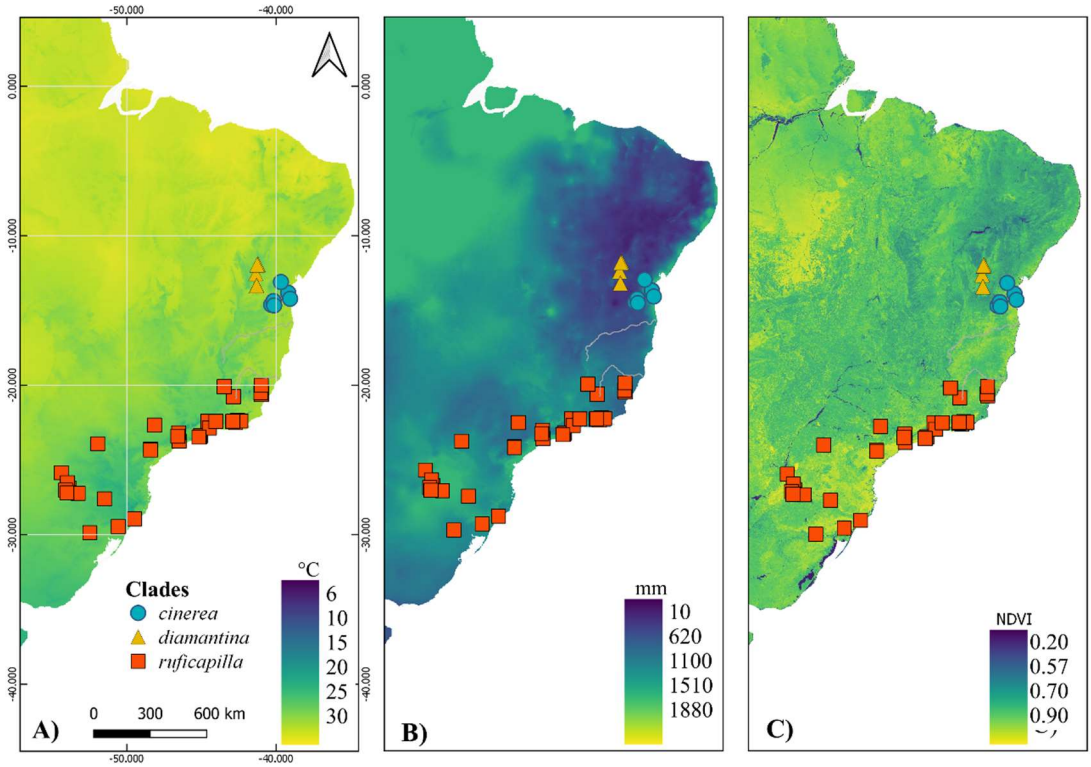

Figure A1. Maps of abiotic and biotic variables covering the vocal sampling localities within the range of each of the three phylogenetic clades of the *S. ruficapilla* complex. Bioclimatic variables from WorldClim (version 20.1) correspond to the average for 1970-2000 (Fick & Hijmans, 2017). A) BIO 1 = Annual mean temperature (°C); B) BIO12 = Annual mean pluviosity (mm); C) NDVI (Normalized Difference Vegetation Index) – mean.

Song element measured	PC1	PC2	PC3	PC4
Song note number	**0.9019**	0.2496	0.0632	0.2788
Song length	**0.9596**	0.2227	0.0296	0.0825
Song pace	**-0.6837**	-0.1014	0.1039	**0.6538**
Internote length	**0.7129**	-0.0925	-0.0619	**-0.5856**
First syllable note number	**0.9008**	0.2528	0.0634	0.2782
First syllable length	**0.9153**	0.2313	0.0387	0.2586
First syllable pace	**-0.6553**	-0.1027	0.0088	0.2191
Second syllable length	**0.7169**	0.4606	0.0928	0.0327
Center time	**0.8377**	0.2884	0.1128	0.0644
Center frequency	-0.2955	**0.9213**	0.0758	-0.0420
Max frequency	-0.2737	**0.8915**	0.1721	-0.0470
Frequency 5%	-0.2607	**0.6568**	**-0.6387**	-0.0318
Frequency 25%	-0.3190	**0.8703**	-0.3155	-0.0133
Frequency 75%	-0.3568	**0.8460**	0.3335	-0.0549
Frequency 95%	-0.4155	**0.6136**	0.4707	-0.1146
Bandwidth 50%	0.0188	-0.2080	**0.8624**	-0.0484
Bandwidth 90%	-0.1267	-0.0406	**0.9260**	-0.0685
Eigenvalue	6.5846	4.4775	2.4975	1.0696
% of Variance	39.1324	26.6098	14.8425	6.3568
Cumulative %	39.1324	65.7422	80.5847	86.9415

Table A1. PCA loadings of song variables of the *S. ruficapilla* complex. The scores represent the correlation between the original variable and each PC.

	Kruskal-Wallis test			**p Holm-corrected Dun test**		
Component	**χ^2**	**ε^2**	***P*-value**	***cinerea* diamantina**	***cinerea ruficapilla***	***ruficapilla* diamantina**
PC1	55.68	0.57	***	***	***	0.68
PC2	43.57	0.44	***	***	0.002	***
PC3	1.35	0.01	0.5	0.47	0.98	0.56
PC4	27.69	0.28	***	***	0.001	0.003

Signif. code: *** = 0.001

Table A2. Results of Kruskal-Wallis tests comparing the distribution of song PCA scores among clades and *pos-hoc* paired tests with correction (Dunn test).

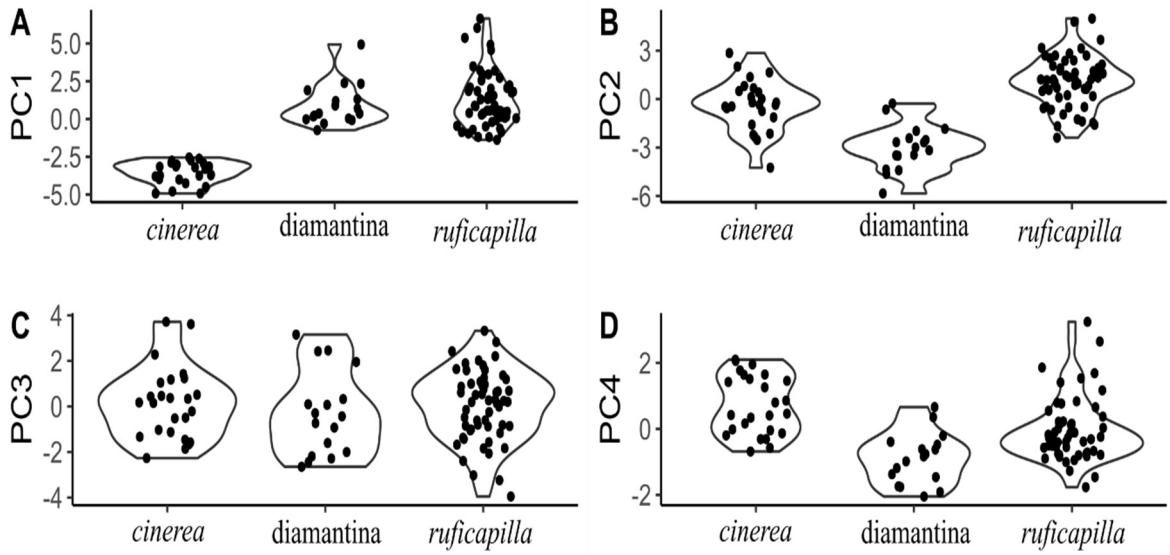

Figure A2. Violin plots comparing the distribution of song PCA scores among the clades of the *S. ruficapilla* complex. The sequence from A-D represents PCs 1-4.

		FDA					k-means					PAM					Hierarchical				
	clades	***cinerea***	**diamantina**	***ruficapilla***		**% accuracy**	***cinerea***	**diamantina**	***ruficapilla***		**% accuracy**	***cinerea***	**diamantina**	***ruficapilla***		**% accuracy**	***cinerea***	**diamantina**	***ruficapilla***		**% accuracy**
Predicted	***cinerea***	25	0	0		100	24	1	0		96	23	1	1		92	24	1	0		96
	diamantina	0	14	3	94	82.3	0	16	1	86	94	0	15	2	89	88	0	12	5	88.7	70
	ruficapilla	0	3	53		94.6	0	11	45		80.35	0	6	50		89	0	5	51		91

Table A3. Classification matrix of songs from discriminant analysis (Flexible Discriminant Analysis), K-means cluster, PAM (Partitioning Around Medoids) among clades of the S. *ruficapilla* complex. Only variables with $r < 0.70$ were included. Accuracy percentual shows the first general value of the method for each clade.

Response Variable	Parametric term	Estimate	SE	*t*-value	*P*-value	R^2	Deviance
PC1	NDVI	0.31	0.158	1.962	0.053	0.72	0.81
	temperature	-0.202	0.136	-1.484	0.141		
	clade *cinerea*	5.792	0.652	8.887	***		
	clade diamantina	10.996	0.933	11.789	***		
	clade *ruficapilla*	5.745	0.546	10.525	***		
	Smooth term	**edf**	**Ref. df**	***F*-value**	***P*-value**		
	s(latitude)	4.1	5.03	10.667	***		
Response Variable	**Parametric term**	**Estimate**	**Std Error**	***t*-value**	***P*-value**	R^2	**Deviance**
PC2	NDVI	0.462	0.228	2.02	0.046	0.47	0.5
	temperature	0.141	0.223	0.631	0.53		
	pluviosity	-0.226	0.232	-0.975	0.332		
	clade *cinerea*	6.576	0.534	12.319	***		
	clade diamantina	3.626	0.532	6.814	***		
	clade *ruficapilla*	7.95	0.417	19.043	***		
	Smooth term	**edf**	**Ref. df**	***F*-value**	***P*-value**		
	s(latitude)	1.002	1.004	0.083	0.78		

Table A4. Generalized additive models (GAM) of song PCA (PC1 and PC2). SE: standard error. Effective degrees of freedom (edf) larger than one suggests a non-linear relationship. Basis: thin plate spline smoother. Family: gaussian. Link: identity. Signif. codes: 0 <= '***' < 0.001.

Response Variable	Parametric term	Estimate	SE	*t*-value	*P*-value	R^2	Deviance
Max Frequency	NDVI	-0.014	0.029	-0.488	0.63	0.31	0.36
	temperature	-0.015	0.028	-0.539	0.59		
	pluviosity	0.022	0.029	0.745	0.46		
	clade *cinerea*	1.445	0.067	21.552	***		
	clade diamantina	1.007	0.081	12.471	***		
	clade *ruficapilla*	1.439	0.049	29.344	***		
	Smooth term	**edf**	**Ref. df**	***F*-value**			
	s(latitude)	1.086	1.666	0.009	0.97		
Bandwidth 90%	NDVI	0.185	0.137	1.347	0.18	0	0.071
	temperature	-0.186	0.131	-1.423	0.16		
	pluviosity	-0.099	0.133	-0.746	0.46		
	clade *cinerea*	4.018	0.554	7.253	***		
	clade diamantina	3.284	0.746	4.401	***		
	clade *ruficapilla*	3.609	0.452	7.979	***		
	Smooth term	**edf**	**Ref. df**	***F*-value**			
	s(latitude)	1.755	2.259	0.719	0.42		

Table A5. Generalized additive models (GAM) of Maximum Frequency and Bandwidth 90%. SE: standard error. Effective degrees of freedom (edf) larger than one suggests a non-linear relationship. Basis: thin plate spline smoother. Family: gaussian. Link: log (Maximum Frequency) and identity (Bandwidth 90%). Signif. codes: 0 <= '***'

Response Variable	Parametric term	Estimate	SE	*t*-value	*P*-value	R^2	Deviance
Song length (duration)	NDVI	0.085	0.023	3.624	***	0.72	0.82
	clade *cinerea*	0.604	0.093	6.498	***		
	clade diamantina	1.301	0.13	10.016	***		
	clade *ruficapilla*	0.901	0.079	11.47	***		
	Smooth term	**edf**	**Ref. df**	***F*-value**			
	s(latitude)	1.69	2.168	10.676	***		
Song Pace	NDVI	0.02	0.028	0.713	0.48	0.66	0.75
	Clade *cinerea*	1.234	0.065	18.998	***		
	Clade diamantina	0.345	0.081	4.27	***		
	Clade *ruficapilla*	0.468	0.049	9.573	***		
	Smooth term	**edf**	**Ref. df**	***F*-value**			
	s(latitude)	1	1	0.089	0.77		

Table A6. Generalized additive models (GAM) of song length and song pace. Standard error. EDF Effective derees of freedom (edf) larger than one suggests a non-linear relationship. Basis: thin plate spline smoother. Family: gaussian. Link: log.

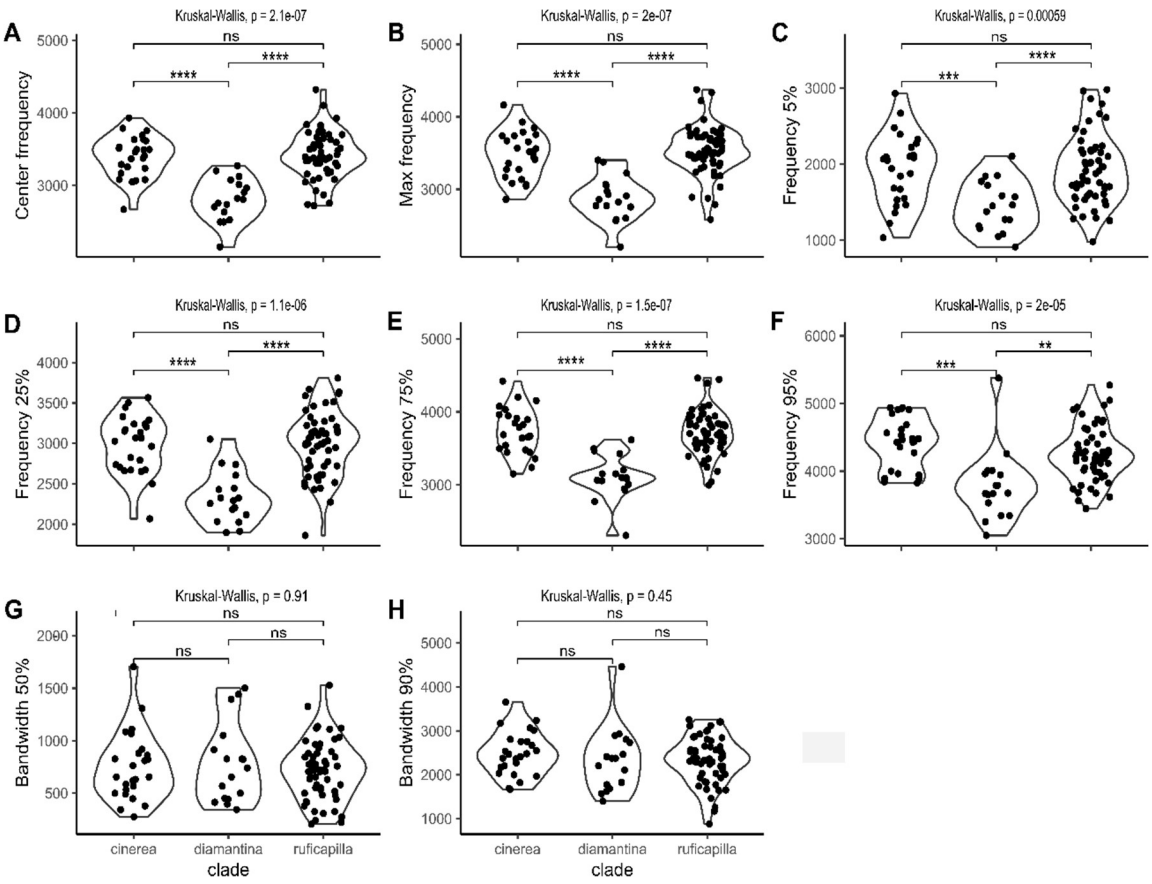

Figure A3. Violin plots comparing the distribution of raw spectral song variables among the clades of the *S. ruficapilla* complex. **** $P < 0.0001$; *** < 0.001; ** < 0.01; * < 0.05; ns > 0.05.

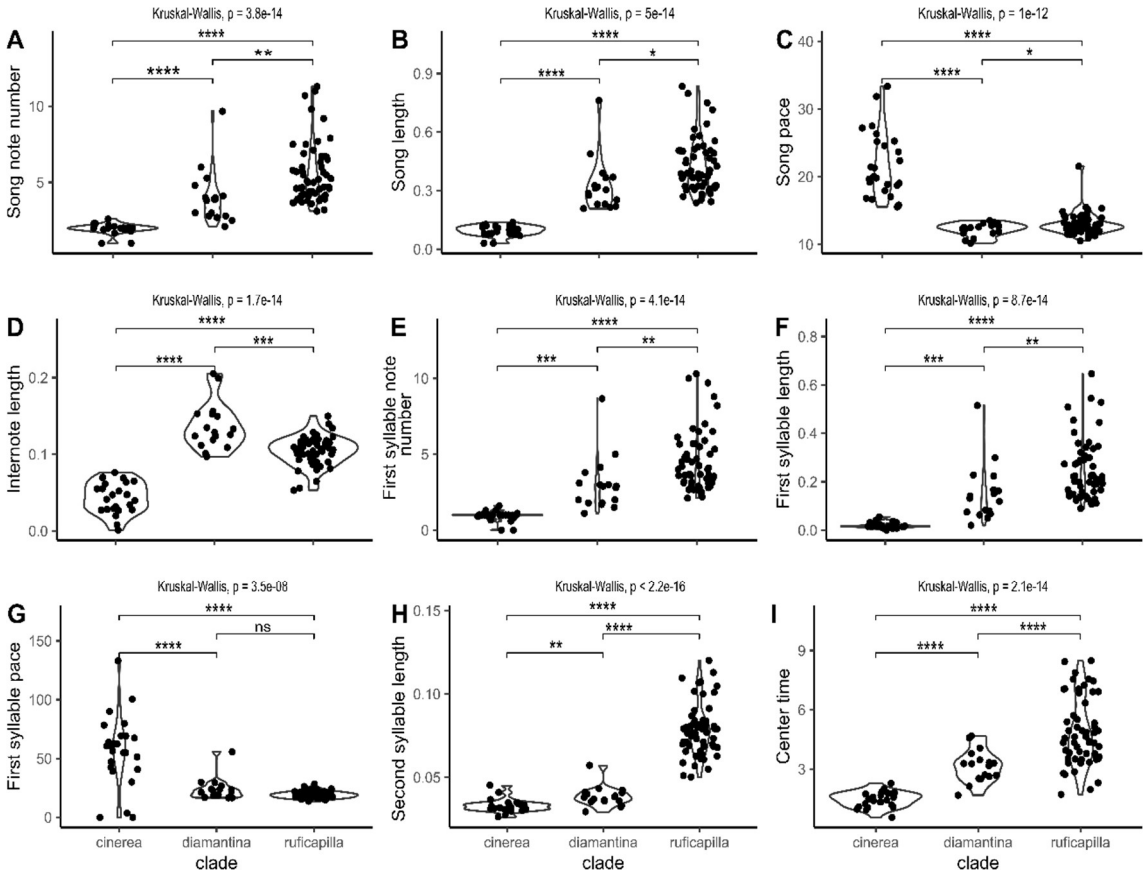

Figure A4. Violin plots comparing the distribution of raw temporal song variables among the clades of the *S. ruficapilla* complex.: **** $P < 0.0001$; *** < 0.001; ** < 0.01; * < 0.05; ns > 0.05.

Capítulo 2

The role of evolutionary history and acoustic similarity on song discrimination in the *Synallaxis ruficapilla* complex: fitting the other piece of the puzzle

Sidnei Sampaio dos Santos[1,3], Henrique Batalha Filho[1,3], and Marcos Maldonado-Coelho[2]

[1] Programa de Pós-Graduação em Biodiversidade e Evolução, Instituto de Biologia, Universidade Federal da Bahia, Brazil

[2] Smörbollsgatan 2, 24756 Dalby, Sweden

[3] National Institute of Science and Technology in Interdisciplinary and Transdisciplinary Studies in Ecology and Evolution (INCT IN-TREE), Instituto de Biologia, Universidade Federal da Bahia, Brazil

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Abstract

The mechanisms that govern the discrimination of divergent signals might have important consequences to the origin of barriers to gene flow and often play a crucial role in the process of speciation. Auditory discrimination can be influenced by the degree of signal divergence, which in turn can be constrained by phylogenetic history. That is, closely related species are expected to exhibit more similar signals. However, the relationship between evolutionary history and vocal variation and its effect on behavioral responses has been rarely studied in birds with innate vocalizations. In this study, we assessed the role of acoustic signal variation and evolutionary relatedness in behavior discrimination in suboscine passerine birds, a large radiation of tropical birds thought to develop songs without learning. Song playback experiments were performed in three localities along the South American Atlantic Forest representing three evolutionary lineages of the Synallaxis ruficapilla spinetail complex. We designed these experiments to test whether behavior discrimination was influenced by either acoustic similarity or common ancestry. Our results showed that all experimental subjects exhibited symmetric responses and that recognition of local songs was always stronger relative to divergent foreign songs. Vocal divergence was found to be biologically significant in eliciting responses but neither acoustic similarity nor common ancestry influenced significantly auditory discrimination. Finally, a non-significant heterogenous discrimination trend between foreign songs can provide insight into the impact of acoustic divergence on behavioral discrimination in recently diverged evolutionary lineages.

Keywords: playback; specific recognition; suboscines; vocal variation

The evolution of acoustic signal discrimination is a key process in speciation (Ryan et al., 2003; Ord and Stamps, 2009; Bradbury and Vehrencamp, 2011). Animals that rely on vocal communication must discriminate to recognize mates, intruders, and competitors in different forms and intensities (Ryan and Rand, 1993; Sherman et al., 1997; Hyman et al., 2004). Moreover, the receiver innate auditory template might evolve to maximize the acoustic discrimination between local and divergent foreign songs (Sherman et al., 1997). This is because discrimination errors can have severe costs on individual fitness such as wasting energy and hybridization (Gröning and Hochkirch, 2008; Grether et al., 2017). Thus, understanding how acoustic discrimination evolves provides valuable insights into the origin of barriers to gene flow (Jones, 1997; Wilkins et al., 2013).

A fundamental requisite for acoustic discrimination is signal variation and divergence (Freeman and Montgomery, 2017; Mejías et al., 2021). In this respect, higher acoustic similarity and perception is often related to the degree of common ancestry (Ryan and Rand, 1999; Price and Lanyon, 2002; Mejías et al., 2021) and the likelihood of behavior response decreases as signal divergence increases (Shepard, 1987). Hence, evolutionary history can constrain acoustic structure (Arato and Fitch, 2021) and perceptual mechanisms (Wilczynski et al., 2001). However, acoustic variation is not always concordant with evolutionary relationships (Salzburger et al., 2002; Nyári, 2007; Seneviratne et al., 2012) and sources of this discrepancy are the diversifying forces of selection (sexual and natural) and drift (genetic and cultural) (Slabbekoorn and Smith, 2002; Podos and Warren, 2007). For instance, populations and species inhabiting dissimilar/similar habitats can be under divergent/convergent social and ecological selection that result in vocal variation divorced from phylogeny (Haavie et al., 2004; Yandell et al., 2018). In addition, closed-related sympatric species can

evolve songs that are more divergent or convergent than expected by their degree of evolutionary relatedness because of selection against hybridization or interspecific resource competition (i.e. character displacement, Noor, 1999; Seddon, 2005; Kirschel et al., 2009). That is, distinct evolutionary scenarios might underly the divergence of signals and perceptual mechanisms that decouple them from phylogeny (Ryan et al., 2003; Bernal et al., 2007; Ryan et al., 2007; Sandoval et al., 2013).

The relationship between evolutionary history and acoustic variation and its impact on behavioral responses have rarely been considered (Kort and ten Cate, 2001). If acoustic discrimination is influenced by common ancestry, the behavioral response would be related to the degree of evolutionary relatedness, independent of vocal similarity. Alternatively, if acoustic discrimination is influenced mostly by vocal similarity, the intensity of discrimination will be independent of phylogeny. In fact, some studies have demonstrated that distantly related species exhibiting acoustic similarities still recognize each other songs (Wolfenden et al., 2015; Louder et al., 2019).

Acoustic discrimination in birds has been extensively investigated during the last decades but there is a clear bias towards systems of vocal learners (Ratcliffe and Grant, 1985; Parker et al., 2018; Demko et al., 2019). In this regard, our understanding on the mechanisms governing acoustic discrimination in birds with innate songs remains poorly known (Seddon and Tobias, 2007; Tobias and Seddon, 2009; Macedo et al., 2019). A good model to address these questions is the suboscine passerine birds, a tropical avian radiation with ca. 1400 species (del Hoyo and Collar, 2016). For most suboscines, song is thought to develop without the influence of learning (Kroodsma and

Konishi, 1991; Kroodsma et al., 2013;Touchton et al., 2014; but see ten Cate, 2021) and acoustic discrimination in this group seems to evolve at a faster rate relative to song learners (i.e. oscine passerine birds; Freeman et al., 2017, 2022).

Here, we unravel these questions using a suboscine model system endemic to the South American Atlantic Forest, the spinetails of the *Synallaxis ruficapilla* complex. This complex encompasses two species, the Bahia spinetail *Synallaxis cinerea* and the Rufous-capped spinetail *S. ruficapilla*, which exhibit a strong phylogeographic structure and a complex pattern of song geographic variation (Batalha Filho et al., 2013, 2019, (Santos et al., in review). There are two clades in both *S. ruficapilla* and *S. cinerea* (Batalha Filho et al., 2013, 2019; Figure 1A and B). One clade in *S. cinerea* is isolated geographically from the other clades in the Chapada Diamantina mountains (Batalha-Filho et al., 2019). In this study, we selected the latter and the coastal clade in *S. cinerea* (hereafter diamantina and *cinerea* clades, respectively) and the southern clade in *S. ruficapilla* (hereafter *ruficapilla* clade). These clades exhibit distinct vocal signatures in temporal and spectral aspects and song variation is incongruent with phylogeny (Santos et al., 2023). Specifically, songs of the diamantina clade are structurally more similar to songs of the non-sister *ruficapilla* clade (Santos et al., 2023; Figure 1A and B). Thus, this is a good system to disentangle the influences of ancestry and acoustic similarity on auditory discrimination. To our knowledge, this is the first study that explicitly test the role of these factors on behavioral discrimination in suboscine passerine birds.

We aimed to answer the following questions: (i) Do divergent populations and species discriminate between local and divergent foreign songs (hereafter local and foreign songs)? If vocal divergence has meaningful evolutionary consequences, behavioral recognition would be stronger towards local relative to foreign songs; (ii) Is

acoustic discrimination influenced more by evolutionary relationships or by acoustic similarity? If acoustic discrimination evolves in concert with evolutionary relatedness, discrimination would be stronger towards more distant related populations and species, independent of vocal similarity. In this context, the diamantina clade should exhibit a stronger response to the songs of the sister clade *cinerea* relative to the non-sister *ruficapilla* clade (Figure 1A and B). Alternatively, assuming that song perception is driven by factors such as individual experience (i.e., learning familiar intraspecific acoustic signals), stronger discrimination would be elicited towards structurally less similar songs. Thus, the reciprocal behavioral response would be stronger between individuals of the non-sister *ruficapilla* and diamantina clades than each would be towards individuals of the *cinerea* clade. Here, we used playback experiments to answer the questions outlined above and our results show that (i) song discrimination is stronger towards foreign songs relative to local songs in all clades and (ii) contrary to predictions, song discrimination is seemingly not influenced by either evolutionary relationships or acoustic similarity.

Methods

Study model – The Rufous-capped spinetail occurs from Argentina and east of Paraguay to the southeast of Brazil (states of Minas Gerais and Espírito Santo), from sea level to 1400 m (Remsen Jr., 2000) (Figure 1). The Bahia spinetail is restricted to Brazil from the northeastern state of Minas Gerais into the state of Bahia, from sea level to 1000 m (Remsen Jr., 2000) (Figure 1). Both species are fairly common in undergrowth and understory of humid and dry forests (Ridgely and Tudor, 1994; Pacheco and Gonzaga, 1995). This species complex constitutes a good model for answering the questions

outlined above because: (i) their songs are supposedly innate (Kroodsma 1984; Kroodsma and Konishi 1991; Touchton et al. 2014) and hence allow testing hypotheses without the influence of cultural evolution and enable us to gain insights into the factors underlying vocal evolution and acoustic discrimination in other suboscine birds as well as in other avian groups with innate songs; (ii) they belong to the Furnariidae family, a radiation with dull and conservative plumage color (Marcondes and Brumfield, 2019). Therefore, vocalizations are likely essential in mediating intra and inter-specific recognition in species inhabiting the dimly lit forest understory. The *cinerea* and the *ruficapilla* clades are syntopic and hybridize in a narrow region at the northeastern state of Minas Gerais (Batalha-Filho et al., 2019). We performed our playback experiments outside this hybrid zone to avoid any possible homogenizing influence of hybridization in vocal variation and, consequently, any confounding effect on acoustic discrimination, which was beyond the scope of our study.

General field methods – We conducted our playback experiments in three localities, each corresponding to one population of the three clades: 1) southern clade of *S. ruficapilla* – humid forest areas at RPPN Santuário do Caraça, Catas Altas, Minas Gerais (20° 06’ S, 43° 48’ W), from October 15 to 21, 2020; 2) coastal clade of *S. cinerea* – humid forests at the Plantações Michelin, Igrapiúna, Bahia (13° 83’ S, 39° 2’ W), from January 8 to 18, 2021; 3) diamantina clade of *S. cinerea* – dry forest areas in Ibicoara, Bahia (13° 41’ S, 41° 32’ W), from January 9 to 17, 2020. The distances among these three sites are 250 km - coastal *cinerea* clade to diamantina clade; 840 km - coastal *cinerea* clade to *ruficapilla* clade; and 780 km - diamantina clade to *ruficapilla* clade (Figure 1C). All three experimental sites are outside the hybrid zone reported for the *ruficapilla* complex (Batalha-Filho et al., 2019).

Playback design – We assumed that the behavior response to playback experiments were representative of each of the three clades. Our experiments consisted in three treatments: first, local songs were played back to the original population; second, songs of other clades were broadcasted in each population (Figure 1C). That is, each population was exposed to its own song (local song) and to the songs of the other two clades (foreign song). This design enabled each population to be exposed to acoustic stimuli representative of either clades that are closely related or clades with high vocal similarity. Given the discordance between evolutionary relationships and acoustic variation in the *ruficapilla* complex (Santos et al., 2023; Figure 1), we exposed each population to all possible playback combinations. The third treatment represented a random stimulus effect. Here, the songs of the white-shouldered fire eye (*Pyriglena leucoptera*), an understory antbird species that co-occur with the *ruficapilla* species complex, were broadcasted in all experimental sites. This random treatment allowed us to evaluate if the behavioral response was associated with the presence of the experimenters or solely by an exposure to an experimental set of local and foreign songs.

Playback stimuli – We used records obtained in the field and from public repositories, including Xeno-canto (www.xeno-canto.org) and Macaulay Library (www.macaulaylibrary.org). Each stimulus consisted in one minute recording from different individuals, keeping the natural silence intervals. We used one cut by individual with a high signal-to-noise ratio. Only one single recording per individual maximizes the independence among stimuli (McGregor, 1992). We applied a high-pass filter at 300 Hz and a low-pass filter at 10 kHz to eliminate background noise.

Additionally, we normalized each recording to -1 dB to preserve a stable amplitude in all individual replicates using Audacity(R) version 2.3.3 (Audacity Team - https://audacityteam.org/).

Playback procedure – We started the experiments with 25 territories in each experimental site. However, we failed to find some subjects during playback sessions. Thus, we used only behavioral responses from 15 territories in our analyses. The minimal distance between each territory was 250 m. In each territory, we used three sessions to expose individuals to local, foreign, and random playback stimuli. We broadcasted songs with a Pignose Legendary 7-100 loudspeaker (Las Vegas, Nevada, USA) positioned at 1m height. Before each playback stimulus was broadcasted, the maximum amplitude level was standardized at -70 dB at the distance of 1 m using a sound pressure level meter (SKDEC–02, Skill-Tec TM - São Paulo, São Paulo, Brazil). In each trial, we played one minute of a song stimulus, followed by five minutes of observations. Before starting each session, we waited three minutes after arriving at the territory. If any bird was already singing upon our arrival, we waited until the bird stopped singing. We then started the playback session three minutes later. The following response parameters to playback trials were recorded: (1) response latency - onset of vocalizations and flights in seconds after the onset of playbacks; (2) approach - the nearest distance to the playback source; (3) time spent within < 3m from the playback source; (4) number of calls – number of calls emitted after the onset of playbacks; and (5) number of songs – number of songs emitted after the onset of playbacks. To account for the high correlation among response variables, we applied a principal component analysis (PCA) separately in each population (McGregor1992;

Dingle et al. 2010). We used only eigenvalues greater than 1 (PCA response, hereafter PCAR). The lower overall Kaiser-Meyer-Olkin (KMO) calculated was 0.58 (*cinerea* clade – Table 1), which is close to the cut-off suggested for behavioral studies (≤ 0.6, Budaev, 2010). We excluded the variable "number of calls" from the PCA analysis in the subject diamantina because no individuals emitted calls after the stimuli were broadcasted. We used an electronic distance measurer (Vonder VD30) to measure subject approach. Playback trials were performed with an interval of at least 24h from each other in each territory. This minimizes the possibility of habituation to stimuli from previous playback sessions. Finally, we randomized the order of song playback in each territory where distinct stimuli categories were broadcasted. Since we did not record any response of subjects to white-shouldered fire-eye songs, the random control was excluded from downstream analyses.

Statistical analyses – To assess the response to playback trials we used Generalized Linear Mixed Models (GLMMs). PCARs (PC1 and PC2) were included in all models as response variables. We performed two sets of analyses. In the first set, models were built to contrast the discrimination between local and divergent foreign songs whereas the second set of models aimed to test the relative influence of evolutionary history and acoustic similarity on auditory discrimination.

Set 1: the influence of vocal divergence on acoustic discrimination – We combined all songs of the two clades broadcasted in each experimental site as a single foreign group. In each model, the class of stimulus (local and foreign) and the order of the broadcasted stimuli were included as fixed factors. The identity of individuals in each territory was included as a random factor. We used the Fisher exact test to test the proportional

responses to each clade. Here, we compared the proportional frequency response to distinct clades using two classes: (i) yes, if subjects responded to foreign; (ii) no, if subjects exhibited no response. A given individual subject received an "yes" if it elicited any vocal behavior, approximation, or movements to an acoustic stimulus. In the absence of any vocal behavior or if the subject maintained the natural behavior after the onset of playback stimuli, a "no" was recorded. Song category (local or foreign) and broadcast order were included as fixed factors.

Set 2: the influence of evolutionary history and acoustic similarity on acoustic discrimination – We run two additional GLMMs, one including the response of *ruficapilla* individuals and the other including the response of diamantina individuals to playback trials. Because the songs of the *ruficapilla* clade are structurally more similar to songs of the non-sister diamantina clade (Figure 1A and B), contrasting the response of *ruficapilla* individuals to vocal stimuli of both diamantina and *cinerea* sister clades allows testing the influence of acoustic similarity on acoustic discrimination when accounting for evolutionary relatedness (Figure 1C). On the other hand, comparing the response of diamantina individuals to vocal stimuli from both the sister *cinerea* clade and the non-sister *ruficapilla* clade allows one to assess whether acoustic similarity (higher with the *ruficapilla* clade) or common ancestry (sister to *cinerea*) influence acoustic discrimination (Figure 1C). As above, we included clade and the playback stimuli order as fixed factors while individual identity in each territory was included as a random factor.

Ethical note – Our playback experimental procedures were approved by the Biology Institute Ethical Committee (No. 07/2018-UFBA).

Results

The influence of vocal divergence on acoustic discrimination – The GLMMs results indicated that the three clades of the *ruficapilla* complex exhibited a similar pattern, with stronger response to local relative to foreign songs (*cinerea* PC1: $\chi2 = 23.90$, df = 1, $P < 0.001$; *cinerea* PC2: $\chi2 = 7.82$, df = 1, $P < 0.008$; diamantina PC1: $\chi2 = 38.10$, df = 1, $P < 0.001$; *ruficapilla* PC1: $\chi2 = 97.55$, df = 1, $P < 0.001$) (Figures 2 and 3; Table 2). The response to local stimulus in all clades induced a shorter latency response, a higher number of songs emitted, and closer approaches to playback source (Table 2; Supplementary material - Figure S1).

Even though the stronger response was towards local songs, foreign songs also elicited a response in all clades of the *ruficapilla* complex (Figure 2). However, the intensity of response did not differ among clades (*cinerea*: Fisher's exact test, $P = 0.33$; diamantina: Fisher's exact test, $P = 1$; *ruficapilla*: Fisher's exact test, $P = 1$, Table S1). Yet, there was a trend suggesting that individuals of the diamantina clade respond to both *cinerea* and *ruficapilla* songs with similar intensity whereas the response to foreign song treatments was heterogenous in both *cinerea* and *ruficapilla* clades (Figure 2). In general, individuals exposed to foreign song stimuli exhibit longer response delay (higher latency), greater distance from the playback source, and fewer emitted songs relative to local songs (Figure S1). Finally, the sequence of broadcasted stimuli had a significant effect only in the *ruficapilla* clade (PC1 behavioral response; $\chi2 = 8.92$, df = 2, $P < 0.011$) (Table 2).

The influence of evolutionary history and acoustic similarity on acoustic discrimination – Acoustic discrimination could not be explained by acoustic similarity in the *ruficapilla* clade (Table 3). Likewise, neither common ancestry nor acoustic similarity

influenced the response in the diamantina clade. The order of broadcasted song stimuli (i.e. the third stimulus) had a significant effect on the behavioral response (Table 3).

Discussion

Our study tested whether Atlantic Forest spinetails discriminate local from foreign song stimuli and if either evolutionary history or acoustic similarity could predict the behavioral response. Local songs similarly induced a more intense response than foreign songs in all clades of the *ruficapilla* complex, suggesting that acoustic signals are potentially important discrimination cues of divergent populations. Notwithstanding, the behavioral response in our study system was not significant predicted by either common ancestry or acoustic similarity. Yet, the pattern of response to foreign songs can provide insights into mechanisms and pathways of acoustic evolution of both signal and discrimination in the complex. Below we discuss our results and suggest possible explanations for our findings.

Behavioral response to local and foreign song stimuli – All clades in the *ruficapilla* complex discriminated with higher intensity local songs relative to foreign ones. This pattern was symmetrical and suggests that individuals from the same population pose a larger competitive threat than foreign ones. A stronger behavioral response to conspecific/local stimulus has been supported by broad evidence and shows the importance of divergent acoustical signals in mediating social interactions (Hick et al., 2015). In fact, songs can be very important mating cues in the origin of barriers to gene flow in early stages of speciation (Uy et al., 2018). In the *ruficapilla* complex, it is possible that acoustic variation and the recognition system are coupled as a byproduct of selective pressures on high effectiveness in discriminating among local individuals (e.g., mates, neighbors). These finds are relevant for recently diverged lineages (Batalha-Filho

et al., 2019), especially in contact zones or isolated in allopatry, and suggest that vocal divergence is biologically meaningful in the complex, with songs being potentially relevant as pre-zygotic barriers.

Behavior response to foreign signals is widespread in many animal groups (Gröning and Hochkirch, 2008; Peiman and Robinson, 2010). Responses in all clades in the *ruficapilla* complex agree with this broad pattern even though we have not found a significant differences in responses between clades. Despite absence of significance, our results indicated an asymmetric trend in responses between clades: the diamantina clade showed the same response intensity to both foreign stimuli while the between-clade response was heterogenous in *ruficapilla* and *cinerea* clades. Territoriality in a competition context for resources other than mates is likely a mechanism that can underly the response to a foreign/divergent song in the group (Gröning and Hochkirch, 2008; Peiman and Robinson, 2010; Oliveira and Bshary, 2021). In this context, songs of members of the *ruficapilla* complex can elicit responses of congeneric species and the other way around. For example, *S. frontalis* and *S. spixi* forage in the same strata in the understory as individuals of the *cinerea* and diamantina clades and agonistic interactions were observed between individuals of the latter clade and the former two congeneric species. Agonistic interactions can often occur, and songs can be a clue to detect a potentially competing invader (Prescott, 1987; Grether et al., 2009). Pre-existing sensory bias can facilitate the competitor's recognition (Grether, 2011) and response to foreign and divergent songs in some *Synallaxis* species could be a consequence of this bias.

Influence of evolutionary history and acoustic similarity – Species discrimination results from intricate interactions and varies considerably based on context (Gröning

and Hochkirch, 2008; Ord et al., 2011). For the *ruficapilla* complex, it is likely that selection has shaped the recognition of local signals, but responses to foreign songs still persist. This suggests that foreign songs in all three clades can be used as cues to recognize potential mates or rivals. Discrimination against foreign cues is prevalent in many animals, and in a broader context it is considered independent of either acoustic similarity or prior familiarity (Ord et al., 2011), and can be more frequent in congeneric species (Gröning and Hochkirch, 2008). Our results reject a stronger influence of acoustic similarity in foreign song discrimination. The clades in the *ruficapilla* complex seem to align with a broader behavioral pattern; that is, in a given subject clade, the response did not differ between foreign songs, independently of their acoustic similarity.

Our results also reject the role of common ancestry in foreign song response. This contrasts with evidence suggesting that evolutionary relatedness influence the patterns of discrimination (Kort and Ten Cate, 2001, Ord et al., 2011). In our study, one potential explanation for the absence of a phylogenetic effect on acoustic discrimination is that the detection and identification of specific signals can be impacted by signal jamming, especially when signals of different species are similar in nature (Gröning and Hochkirch, 2008). This can lead to the misinterpretation of foreign signals as their own (Wolfenden et al., 2015). In the case of the *ruficapilla* complex, signal jamming is more likely to occur between diamantina and *ruficapilla* due to their higher song similarity (Santos et al., 2023). In this case, we expected random behavioral responses towards foreign signals due to jamming. However, our findings suggested a statistically non-significant trend (i.e., different from zero) instead of a random response. Additionally, it can be challenging to distinguish between the influence of signal jamming on the

behavioral response from the influence of the recognition system, when the latter is driven mostly or solely by vocal similarity.

In fact, the asymmetric response towards foreign songs in the *ruficapilla* complex is noteworthy, even if statistically non-significant. Beyond the territorial context, the retention of features from the ancestral recognition system could direct the behavioral response to foreign songs in a specific manner between members of the complex (Mendelson and Shaw, 2012). These tendencies may be perceived as a hypothesis that predicts the direction and potential outcome of the evolution of discrimination in the complex. The first picture is a generalizing recognition system with multiple cues driving similar responses to songs of the foreign diamantina clade (Candolin, 2003). The observed trend in responses from *cinerea* to diamantina may be related to individuals of these two clades sharing the same recognition system template due to shared ancestry (i.e., sister clades). On the other hand, the trend of *ruficapilla* individuals responding with higher intensity to songs of the diamantina clade could be due to a shared ancestral recognition system template associated with temporal cues. Indeed, the temporal song elements in the *ruficapilla* complex was the principal factor explaining a higher vocal similarity between the non-sister *ruficapilla* and diamantina clades (Santos et al., in review). This underscores the idea that different parts of the song can elicit specific behavioral responses (Shieh et al., 2013). Future playback studies should be designed to test if different parts of the song in the *ruficapilla* complex elicit distinct responses among clades. This is a gap in our understanding about the evolution of vocal discrimination in suboscine birds.

The role of sex in auditory response – Acoustic discrimination can be sex specific (Ord et al., 2011). In our study, we could not determine the sex of individuals during the

experiments as there is no sexual plumage dimorphism. However, it should be noted that we never observed a joint response of two individuals to any song stimuli in our playback experiments; only one individual responded in every trial. Males can be less discriminatory and more aggressive relative to females (Searcy and Brenowitz, 1988). Hence, if were only males responding to acoustic stimuli in our experiment, then it can be suggested that acoustic similarity and common ancestry does not explain male response in the *ruficapilla* complex.

Conclusions – The *ruficapilla* complex has a pattern of vocal divergence and acoustic discrimination that can be considered a puzzle. We found meaningful acoustic discrimination associated with vocal divergence in lineages recently divergent and likely in earlier stages of the speciation process. One piece of the puzzle that still needs to be tested in this system is the hybrid zone. A detailed research program in the hybrid zone, integrating genetic and vocal variation associated with acoustic discrimination experiments is warranted and should enhance our understanding of how acoustic signals determine the origin of barriers to gene flow and contribute to species cohesion.

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Manuscript title: The role of evolutionary history and acoustic similarity on song discrimination in the *Synallaxis ruficapilla* complex: fitting the other piece of the puzzle

Authors: Sidnei Sampaio, Henrique Batalha-Filho and Marcos Maldonado-Coelho

Figures and Tables

Table 1. Behavioral response variables, eigenvalues, and PCAR scores (PC1 and PC2). Response latency - time in seconds after starting the song playback; Approach - the closest distance to the song playback source; Time spent within < 3m - from the song playback source; Number of calls – number of calls emitted after starting the song playback; and Number of songs – number of songs emitted after starting the song playback.

Clade	Variables	Components		KMO (Overall)	Bartlett's Test of Sphericity
		PC1	PC2		
cinerea	Response latency	0.906	-	0.58	$P < .001$
	Approach	0.784	-0.527		
	Time spent within < 3m	-	0.903		
	Number of calls	-	0.545		
	Number of songs	-0.904	-		
	Eigenvalue	2.7342	1.1688		
	% Variance	54.685	23.377		
	Cumulative % variance	78.1			
diamantina	Response latency	0.944	-	0.61	$P < .001$
	Approach	0.946	-		
	Time spent within < 3m	-	-		
	Number of songs	-0.633	-		
	Eigenvalue	2.3309	-		
	% Variance	58.27	-		
	Cumulative % variance	58.27			
ruficapilla	Response latency	0.955	-	0.65	$P < .001$
	Approach	0.94	-		
	Time spent within < 3m	-0.526	-0.625		
	Number of calls	-	0.819		
	Number of songs	-0.867	-		
	Eigenvalue	2.9355	1.0684		
	% Variance	58.71	21.368		
	Cumulative % variance	80.1			

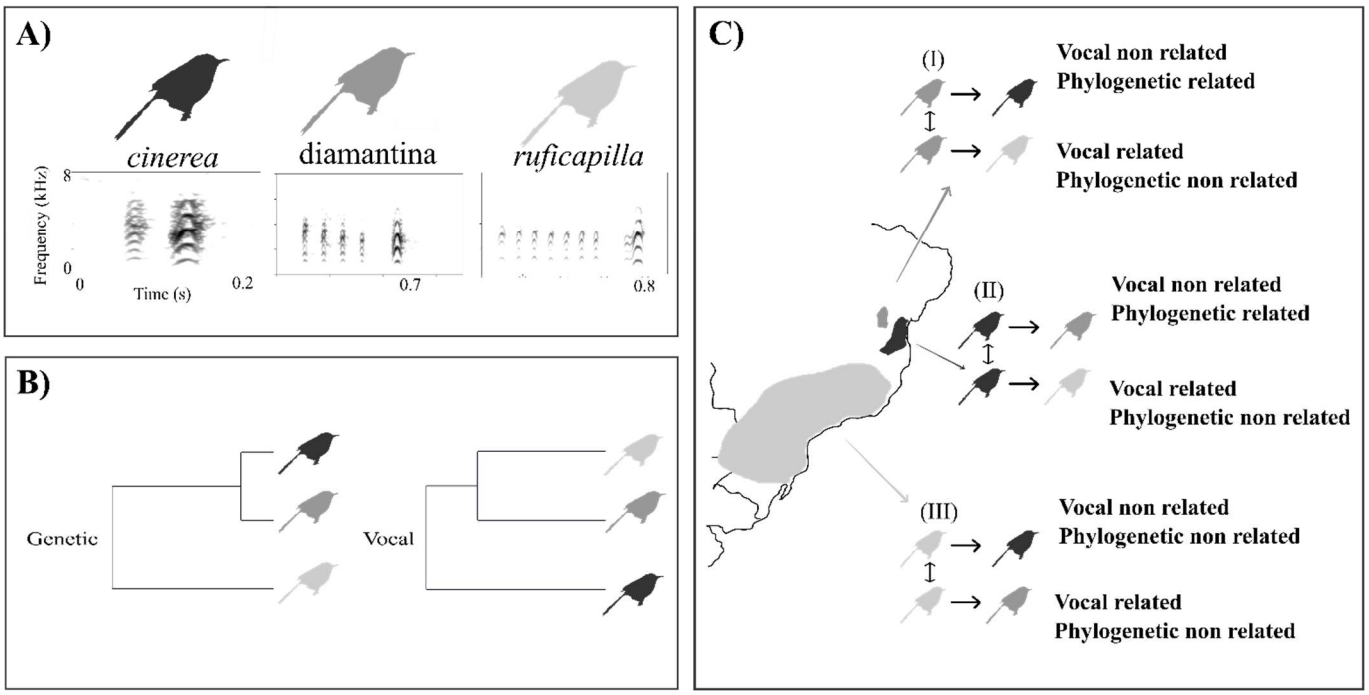

Figure 1. A) Sonograms of clades in the *ruficapilla* species complex (Santos et al., 2023). B) Dendrograms representing phylogenetic relationships (genetic; see details in Batalha-Filho et al., 2019) and acoustic similarity (vocal, see details in Santos et al., 2023) in the *ruficapilla* complex. C) Experimental design of this study.

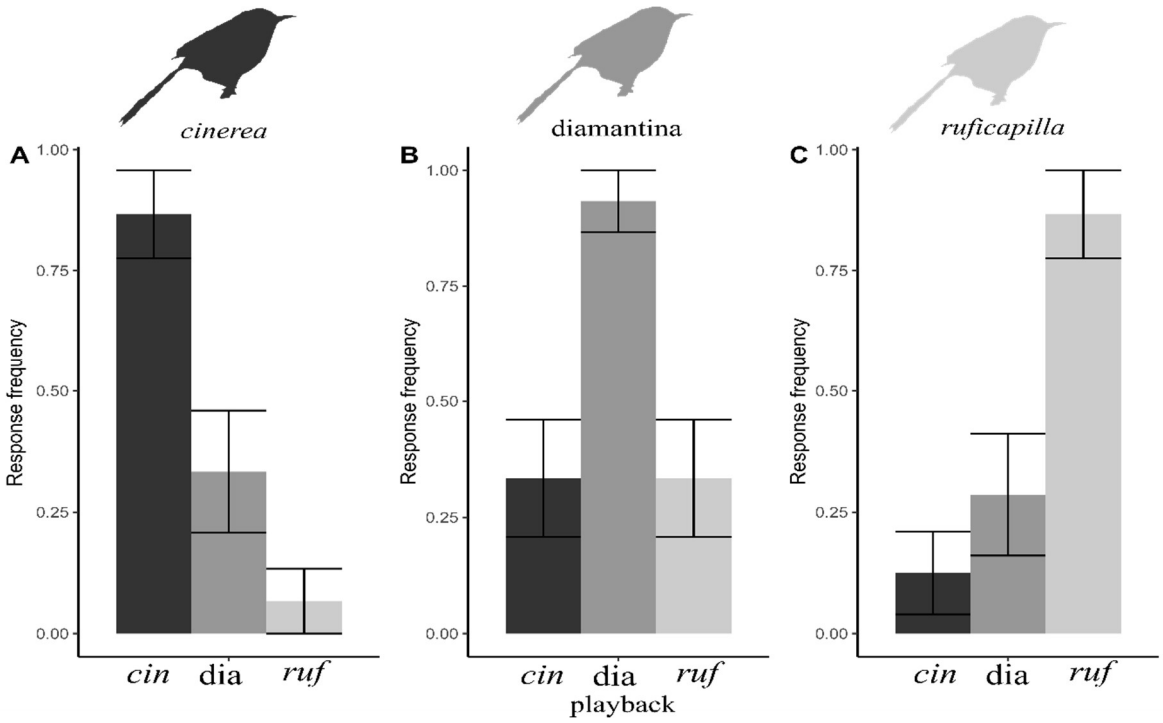

Figure 2. Behavioral response frequency (BRF) in the *ruficapilla* complex. Frequency represents the strength of a response (*yes* or *no*) to the distinct categories of broadcasted songs. A) BRF in the *cinerea* (*cin*) clade; B) BRF in the diamantina (dia) clade; C) BRF in the *ruficapilla* (*ruf*) clade.

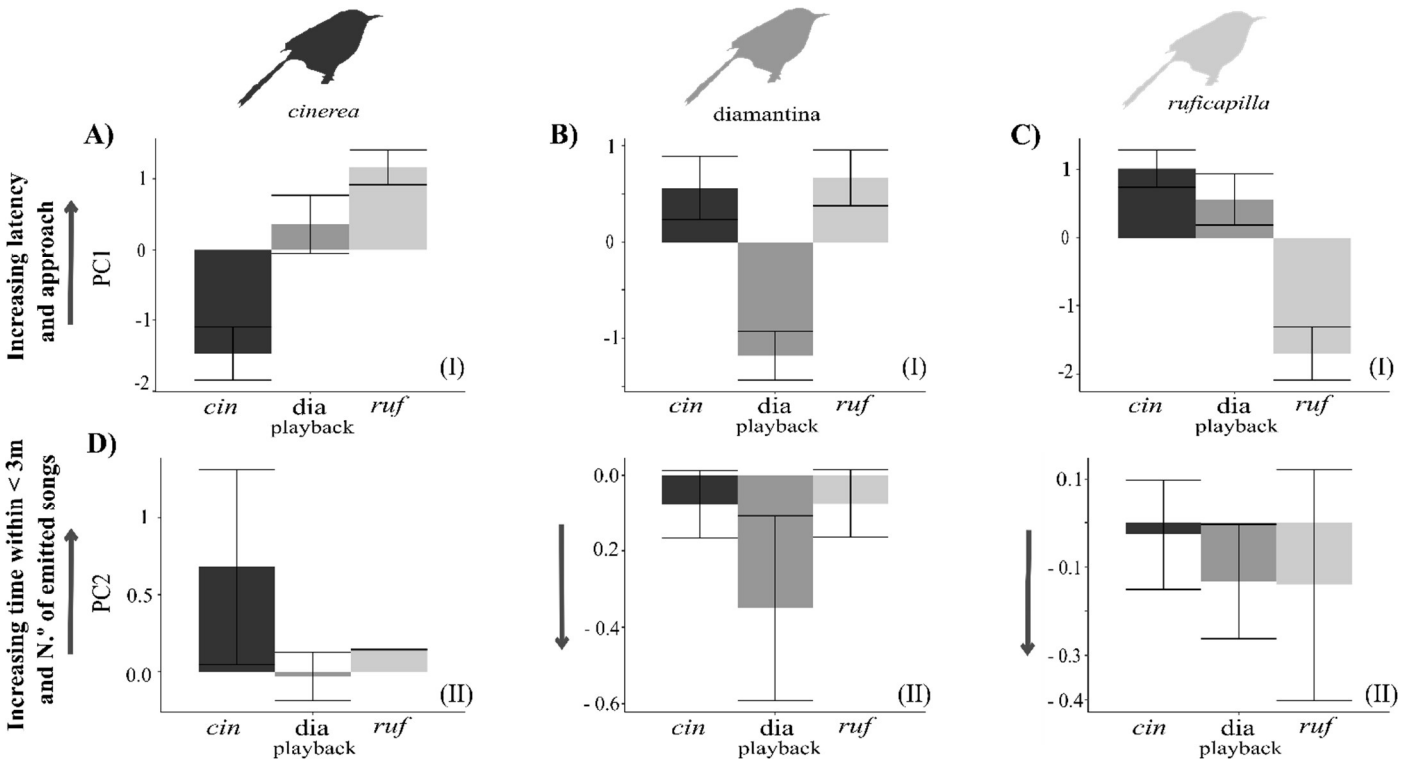

Figure 3. Behavioral response of clades in the *ruficapilla* complex to local songs and to foreign songs. A, B, and C depicts responses of *cinerea* (*cin*), diamantina (dia), and *ruficapilla* (*ruf)* clades, respectively. Box plots show the 25th to 75th percentiles.

Table 2. Generalized linear mixed model results of the behavioral response (PC1 and PC2) to playbacks of local and foreign songs. Seq play indicates the order in which a specific song stimulus was broadcasted during the playback experiments. CI = confidence interval. Family: Binomial, Link: log. Signif. codes: 0 <= '***' < 0.001

Subject	Fixed effect	Estimate	CI	*P*	$\chi 2$	Conditional R^2	Marginal R^2
cinerea clade (PC1)	(Intercept)	2.44	(1.84, 3.04)	**< .001**		0.89	0.45
	foreign song	0.99	(0.58, 1.41)	**< .001**	23.901		
	Seq play [second]	-0.04	(-0.58, 0.49)	0.87			
	Seq play [third]	0.21	(-0.33, 0.75)	0.439	1.1363		
cinerea clade (PC2)	(Intercept)	2.3	(1.84, 2.75)	**< .001**		0.59	33
	foreign song	-0.59	(-1.01, -0.16)	**0.008**	7.8163		
	Seq play [second]	-0.19	(-0.57, 0.18)	0.304			
	Seq play [third]	0.17	(-0.24, 0.57)	0.415	3.5785		
diamantina clade (PC1)	(Intercept)	1.89	(1.45, 2.33)	**< .001**		0.9	0.79
	foreign song	1.32	(0.89, 1.76)	**< .001**	38.089		
	Seq play [second]	0.5	(-0.06, 1.06)	0.077			
	Seq play [third]	0.25	(-0.26, 0.76)	0.332	3.3547		
ruficapilla clade (PC1)	(Intercept)	1.67	(1.18, 2.16)	< .001		0.94	0.72
	foreign song	1.65	(1.32, 1.99)	**< .001**	97.533		
	Seq play [second]	0.61	(0.17, 1.05)	**0.007**			
	Seq play [third]	0.44	(0.03, 0.86)	**0.037**	8.9162		
ruficapilla clade (PC2)	(Intercept)	3.29	(2.49, 4.09)	< .001		0.71	0.1
	foreign song	0.2	(-0.57, 0.97)	0.6	0.2788		
	Seq play [second]	0.14	(-0.60, 0.89)	0.696			
	Seq play [third]	-0.21	(-0.90, 0.49)	0.55	1.0022		

Table 3. Generalized linear mixed model results of the behavioral response (PC1) to playbacks in each clade testing the influence of evolutionary history and acoustic similarity. Seq play indicates the order in which a specific song stimulus was broadcasted during the playback experiments. CI = confidence interval. Family: Gamma, Link: identity. Signif. codes: 0 <= '***' < 0.001

Subject	Fixed effect	Estimate	CI	*P*	$\chi 2$	Conditional R^2	Marginal R^2
diamantina clade (PC1)	(Intercept)	3.65	(2.69, 4.61)	**< .001**		0.81	0.17
	ruficapilla song	0.24	(-0.68, 1.16)	0.593	0.2931		
	Seq play [second]	0.44	(-0.83, 1.71)	0.483			
	Seq play [third]	0.22	(-0.92, 1.36)	0.694	0.5633		
ruficapilla clade (PC1)	(Intercept)	2.81	(1.80, 3.81)	**< .001**		0.94	0.47
	diamantina song	-0.04	(-0.78, 0.70)	0.909	0.0134		
	Seq play [second]	0.81	(-0.26, 1.87)	0.13			
	Seq play [third]	1.8	(0.95, 2.65)	**< .001**	19.548		

Supplementary Material

Manuscript title: The role of evolutionary history and acoustic similarity on song discrimination in the *Synallaxis ruficapilla* complex: fitting the other piece of the puzzle

Authors: Sidnei Sampaio, Henrique Batalha-Filho and Marcos Maldonado-Coelho

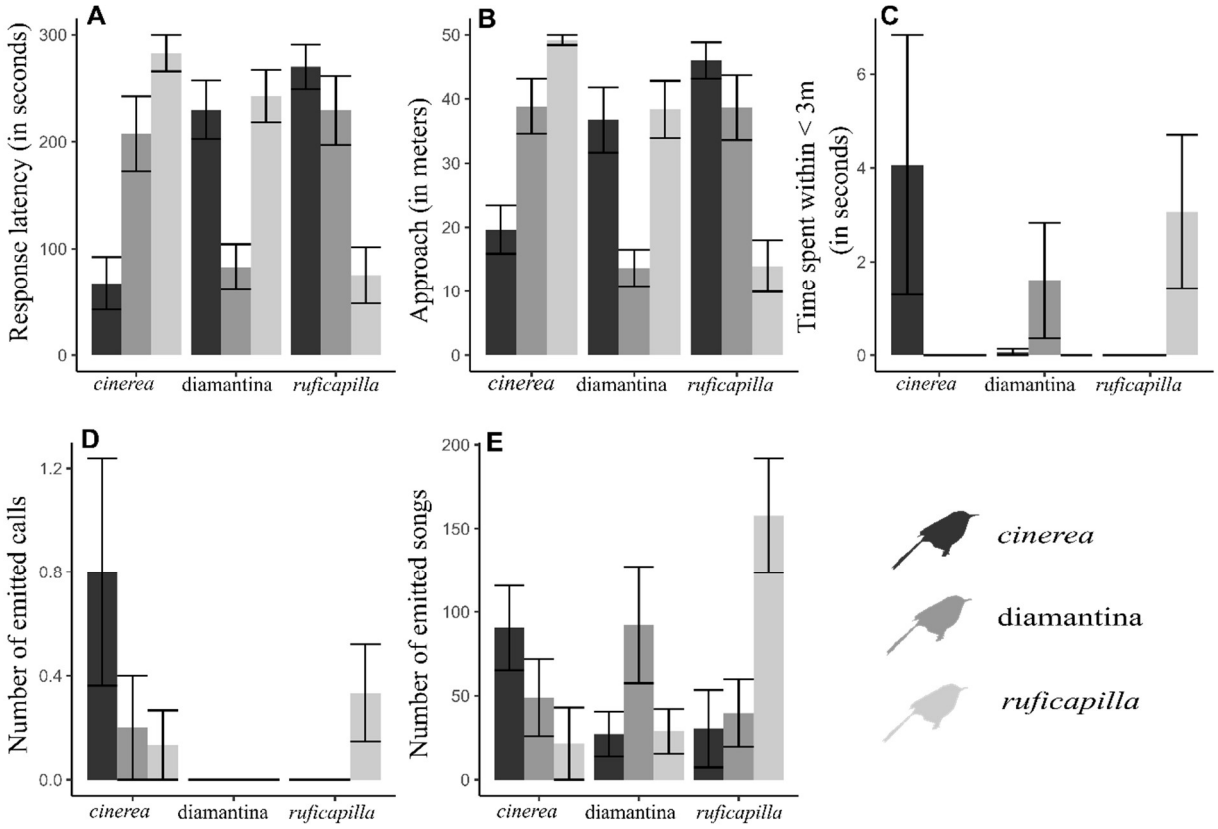

Figure S1. Raw behavioral variables recorded for each subject (clade) in our song playback experiments. Response latency - time in seconds after starting the song playback; Approach - the closest distance (seconds) to the song playback source; Time spent within < 3m – time spent within < 3m from the song playback source; Number of calls – number of calls emitted after starting the song playback; and Number of songs – number of songs emitted after starting the song playback.

Table S1. Contingency table showing the total number and percentage of behavioral response (yes/no) to foreign song stimuli in each clade.

			response to diamantina			
	response to *ruficapilla*		**no**	**yes**	**Total**	**Fisher's exact test**
cinerea clade	no		10	4	14	$P = 0.33$
		%	66.7	26.7	93.3	
	yes		0	1	1	
		%	0	6.7	6.7	
	Total		10	5	15	
		%	66.7	33.3	100	
			response to *ruficapilla*			
	response to *cinerea*		**no**	**yes**	**Total**	
diamantina clade	no		7	3	10	$P = 1$
		%	46.7	20	66.7	
	yes		3	2	5	
		%	20	13.3	33.3	
	Total		10	5	15	
		%	66.7	33.3	100	
			response to diamantina			
	response to *cinerea*		**no**	**yes**	**Total**	
ruficapilla clade	no		9	4	13	P = 1
		%	60	26.7	86.7	
	yes		2	0	2	
		%	13.3	0	13.3	
	Total		11	4	15	
		%	73.3	26.7	100	

Considerações Finais

A presente Tese investigou se (i) o papel de forças estocásticas e determinísticas moldaram a diversificação de sinais sonoros em aves e se (ii) a discriminação acústica pode ser explicada pela história filogenética ou pela similaridade vocal. Como modelo de estudo utilizamos aves suboscines, mais especificamente duas espécies endêmicas à Floresta Atlântica: *Synallaxis ruficapilla* e *S. cinerea*, que são tratadas de modo geral como complexo *ruficapilla*.

O padrão de variação vocal do complexo *ruficapilla* sugere que cada clado investigado possui assinatura vocal distinta. Aspectos temporais do canto foram mais influentes na variação do espaço multivariado vocal. Por outro lado, as variáveis espectrais foram as mais importantes na separação dos clados. Isso sugere que diferentes elementos do canto estariam sob forças de seleção distintas. A variação no espaço multivariado vocal mostrou ainda que não há congruência entre a variação vocal e as relações filogenéticas propostas para o grupo (Batalha-Filho et al., 2013; 2019). Este padrão é intrigante e inesperado, principalmente para aves com cantos inatos. A deriva genética, seleção sexual e heterogeneidade na cobertura florestal ao longo da distribuição do complexo surgiram como candidatos para explicar um verdadeiro quebra cabeças em que consiste a variação vocal do grupo.

A resposta comportamental de discriminação acústica no complexo *ruficapilla* indicou reconhecimento significativo e simétrico para os sons de todos os clados, ou seja, cada clado responde com maior intensidade ao próprio canto do que aos cantos de outros clados. Isso sugere que a divergência vocal deve ter relevância biológica significante no complexo e pode ter papel fundamental durante o processo de especiação no grupo. Em especial, possui potencial de atuar no reconhecimento especifico e servir como barreira ao fluxo gênico. Ainda que não significativa, todos os clados do complexo exibiram resposta comportamental ao canto de outros clados. Isso sugere que o canto de populações geneticamente e vocalmente divergentes ainda pode servir como pistas para potenciais parceiros ou competidores. Essa resposta não exibiu nenhuma relação com as relações filogenéticas nem com a similaridade vocal entre os clados. Ao considerar que a divergência dentro do complexo é recente, não descartamos a possibilidade de retenção ancestral de similaridade no sistema sensorial de reconhecimento que ainda pode

influenciar a resposta comportamental, ainda que de forma não significativa. A influência de ruídos sonoros do ambiente também surgiu como uma provável explicação nessa resposta ao canto de outros clados, principalmente naqueles com maior similaridade vocal (*ruficapilla*/diamantina). Nesse caso, ruídos do ambiente poderiam mascarar ou diminuir a detecção e/ou fidelidade dos sinais acústicos e apenas parte deles seriam reconhecidos, o que levaria a erros de reconhecimento.

Em conclusão, variação vocal e reconhecimento específico podem ser mais complexas do que previamente assumido em aves suboscines. Os resultados encontrados para o complexo *ruficapilla* são um exemplo desse argumento. Outros estudos com suboscines podem trazer novos *insights* de como a divergência vocal é moldada por diferentes forças e o quanto pode influenciar na resposta de discriminação, mesmo em linhagens que evoluíram recentemente.

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