

ESCOLA DE CIÊNCIAS DA SAÚDE E DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E EVOLUÇÃO DA BIODIVERSIDADE
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REPRODUCTIVE BIOLOGY AND PHYLOGENETIC RELATIONSHIPS OF *Vitreorana baliomma* (ANURA: CENTROLENIDAE)

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2020

PÓS-GRADUAÇÃO - STRICTO SENSU



Pontifícia Universidade Católica
do Rio Grande do Sul

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baliomma (ANURA: CENTROLENIDAE)**

VICTOR MORAES ZUCCHETTI

DISSERTAÇÃO DE MESTRADO

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**Reproductive biology and phylogenetic relationships of *Vitreorana baliomma* (Anura:
Centrolenidae)**

Dissertação apresentada como requisito para a obtenção do grau de Mestre pelo Programa de Pós-Graduação em Ecologia e Evolução da Biodiversidade da Escola de Ciências da Saúde e da Vida da Pontifícia Universidade Católica do Rio Grande do Sul.

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Sumário

AGRADECIMENTOS.....	7
RESUMO	9
ABSTRACT	10
1. INTRODUÇÃO GERAL	11
Objetivos	17
Referências.....	18
2. CAPÍTULO 1: Manuscrito formatado para artigo	23
Abstract.....	25
Introduction	26
Material and methods	29
Results.....	36
Discussion	40
Conclusions	48
Acknowledgments	49
References	49
Supporting information	59
Tables	63
Figure captions.....	65

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RESUMO

Informações sobre comportamentos e ecologia são escassas para as espécies de *Vitreorana*, especialmente as que ocorrem na Mata Atlântica. Traços reprodutivos são importantes em centrolenídeos e o conhecimento de aspectos comportamentais podem nos ajudar a compreender as complexas relações entre os clados. Neste trabalho, reportamos os comportamentos reprodutivos de *V. baliomma*, um pequeno centrolenídeo descrito somente com base em características morfológicas. Descrevemos também o uso de micro-habitat, canto de anúncio, e a influência do ambiente sobre a atividade de adultos e espessura das posturas de ovos. Além disso, analisamos a posição filogenética da espécie em Centrolenidae, a fim de confirmar sua presença dentro do gênero *Vitreorana*. Nós realizamos amostragens em dois córregos no sul da Bahia, entre novembro de 2018 e abril de 2019. Quando em amplexo, indivíduos se movimentaram subindo e descendo pela vegetação, realizando rotações e mudando de superfície foliar. Após a oviposição, as fêmeas de *V. baliomma* realizaram comportamento de “brooding” de curto período. Pela primeira vez, confirmamos a presença de “brooding” realizado pela fêmea em nesta espécie. O local de oviposição reflete alguns dos micro-habitats dos adultos. Os cantos de *V. baliomma* podem possuir uma ou duas notas e os parâmetros são similares aos de outras espécies de *Vitreorana*. As análises filogenéticas e do canto confirmaram a permanência de *V. baliomma* dentro do gênero, mas as relações em *Vitreorana* permanecem nebulosas. O número de machos vocalizando teve uma correlação positiva com a umidade do ar, o que provavelmente mostra um efeito da precipitação diária no comportamento reprodutivo da espécie. Nosso estudo expande o conhecimento da biologia reprodutiva e elucida os comportamentos reprodutivos da espécie, registrando a ocorrência de “brooding” realizado pelas fêmeas. Nossos resultados sugerem que este é um comportamento generalizado em *Vitreorana*. Além do mais, nossos resultados reforçam o padrão de evolução do cuidado parental em Centrolenidae.

Palavras-chave: Cuidado parental; Canto de anúncio; Mata Atlântica; Padrões de acasalamento; Uso de micro-habitat

ABSTRACT

Behavioral and population ecology information are lacking for all *Vitreorana* species inhabiting the Atlantic Forest. Glassfrogs exhibit a diversity of interesting reproductive behaviors, and knowledge of behavioral aspects can help elucidate the complex relationships between clades. In this study, we report on the reproductive behaviors of *V. baliomma*, a small species of Centrolenidae known only by morphological features of collection specimens. We also describe microhabitat use, advertisement call and the influence of environment on activity of adults and on clutches thickness. Moreover, we conducted phylogenetic analyses using molecular data to assess *V. baliomma* position in Centrolenidae and confirm their presence within the genus *Vitreorana*. We sampled two streams in southern state of Bahia, Brazil, between November 2018 and April 2019. For the first time, we confirm the presence of female-only egg-brooding in this species. Oviposition site reflected some of the adult microhabitats. However, clutches were found at lower height and horizontal distances, on both surfaces of the leaves and on larger leaves than those used by adults. Calls of *V. baliomma* males have one or two notes, and parameter values are similar to other *Vitreorana* species. Call and phylogenetic analyses validated *V. baliomma* and confirmed the phylogenetic position within the genus, but the relations in *Vitreorana* are still nebulous. The number of calling males was positively correlated with air humidity, which probably shows an effect of daily rainfall on reproductive behavior of this species. Our study expands the knowledge on the reproductive biology and elucidate the reproductive behaviors, describing the occurrence of maternal egg-brooding for the species. Our results suggest that egg-brooding is a widespread behavior in *Vitreorana*. Moreover, this find reinforces the pattern of care evolution in Centrolenidae.

Keywords: Advertisement call; Atlantic Forest; Mating patterns; Microhabitat use; Parental care

1. INTRODUÇÃO GERAL

Anuros possuem uma grande diversidade de traços relacionados à sua biologia reprodutiva (Vitt e Caldwell 2014), sendo um dos grupos com maior variedade de modos reprodutivos entre os vertebrados (Haddad e Prado 2005; Crump 2015). Aspectos reprodutivos têm importância na sistemática, evolução, ecologia e conservação de espécies (Hoffmann et al. 2008; Guayasamin et al. 2009; Vargas-Salinas et al. 2014; Delia et al. 2017; Escalona-Sulbarán et al. 2019). Contudo, informações a respeito da biologia reprodutiva de muitas espécies é escassa ou inexistente (Wells 2007; Delia et al. 2017).

O modo reprodutivo em anuros normalmente é determinado por um conjunto de características que incluem local de reprodução, local de deposição dos ovos (oviposição), estrutura da postura, local de desenvolvimento larval e cuidado parental, quando presente (Vitt e Caldwell 2014). Essas estratégias reprodutivas podem ser vistas como uma combinação de fatores como comportamento, fisiologia e morfologia, que exercem função na produção da prole sobre certas condições ambientais e sociais (seleção natural e sexual). Assim, essas condições em que a espécie ocorre podem selecionar características através de adaptações, como as ligadas à reprodução, para aumentar o fitness (Trivers 1972, 1974; Duellman e Trueb 1994). Algumas espécies podem apresentar variabilidade no comportamento reprodutivo, os ajustando a mudanças abióticas do ambiente (Delia et al. 2013; Lehtinen et al. 2014; Crump 2015; Ospina-L et al. 2019). Por exemplo, a atividade reprodutiva de anuros neotropicais é diretamente correlacionada com a precipitação diária (Cardoso e Haddad 1992; Canelas e Bertoluci 2007; Vockenhuber et al. 2008; Bastos-Riascos et al. 2017; Rios-Soto et al. 2017). Entre os comportamentos reprodutivos importantes para maximizar o fitness das espécies, o cuidado parental atua aumentando a sobrevivência ou crescimento da prole,

após a fertilização, e pode ser realizada por um ou ambos parentais (Trivers 1972; McDiarmid 1978; Clutton-Brock 1991). Este é um comportamento raro em anuros (10–20 % das espécies), sendo o atendimento aos ovos a forma mais comum (Crump 1995; Lehtinen e Nussbaum 2003; Wells 2007).

As vocalizações de anuros são um traço importante no reconhecimento de espécies e isolamento reprodutivo. Os machos emitem cantos para indicar sua localização, defender territórios e atrair parceiras, enquanto, no geral, fêmeas selecionam os machos preferidos através de parâmetros do canto (Duellman e Trueb 1994; Wells 2007). A variabilidade dos cantos entre espécies de anuros é em grande parte explicado pelo sinal filogenético. Os cantos de anúncio são uma ferramenta útil em trabalhos de sistemática, uma vez que eles são, no geral, espécie-específicos, estão sobre forte seleção sexual e são constantemente emitidos durante o período reprodutivo (Bosch e De la Riva 2004; Kohler et al. 2017).

A seleção de micro-habitat é um importante componente para o sucesso reprodutivo em anuros (Murphy 2003). Espera-se que indivíduos selecionem o melhor local em uma composição complexa de opção que variam de acordo com características bióticas e abióticas (Warner et al. 1993). O micro-habitat influencia na variabilidade do canto (Bosch e De la Riva 2004) e o local de vocalização pode aumentar a eficácia do sinal acústico (Greer e Wells 1980). Além disso, o local de oviposição tem grande importância na sobrevivência da prole e os indivíduos selecionam o local baseado em sinais ambientais, como a não presença de predadores e potencial risco de dessecação, historicamente associado com o fitness reprodutivo (Murphy 2003; Touchon e Warkentin 2009; Touchon e Worley 2015; Dodd e Buchholz 2018).

Centrolenidae é um grupo interessante devido a seus traços morfológicos únicos, e diversidade de características ecológicas e comportamentais, bem como sua complexidade filogenética e biogeográfica (Cisneros-Heredia e McDiarmid 2007; Guayasamin et al. 2009; Delia et al. 2010; Castroviejo-Fisher et al. 2014). Traços reprodutivos são importantes em centrolenídeos, apresentando variação no local de canto, local de oviposição, sexo que realiza e tempo despendido no cuidado parental (Guayasamin et al. 2009; Delia et al. 2017). Seleção sexual pode ser importante na diversificação de Centrolenidae, e traços acústicos (frequência dominante e faixa de banda) tem um alto sinal filogenético (Escalona-Sulbarán et al. 2019).

Esta família é popularmente conhecida como pererecas-de-vidro, sendo endêmicos das Américas do Sul e Central. Habitam riachos e cachoeiras florestadas, empoleirando-se e depositando seus ovos em folhas, ramos, musgos e rochas sobre a água (Cisneros-Heredia e McDiarmid 2007; Guayasamin et al. 2009; Twomey et al. 2014). Após a eclosão, os girinos caem na água e continuam seu desenvolvimento enterrados na areia e detritos no fundo dos córregos (Kubicki 2007; Guayasamin et al. 2009). O cuidado parental era considerado um comportamento raro em pererecas-de-vidro e exclusivamente realizado por machos, sendo associado a territorialidade dos mesmos, onde indivíduos acumulam e permanecem próximos das posturas durante o desenvolvimento dos embriões (McDiarmid 1978). O cuidado dos ovos pelos machos é conhecido em espécies de *Hyalinobatrachium* (Greer e Wells 1980; Hayes 1991; Vockenhuber et al. 2008, 2009; Valencia-Aguilar et al. 2012) e algumas de *Centrolene* (Lynch et al. 1983; Vargas-Salinas et al. 2007), onde foi hipotetizado como tendo evoluído de um estado ancestral sem cuidado (McDiarmid 1978; Delia et al. 2013). O comportamento de “brooding” é a forma de cuidado dos ovos encontrada em

Centrolenidae, se resumindo a um contato ventral onde o parental posiciona o seu corpo sobre a postura (Hayes 1991; Guayasamin et al. 2009). Muitos estudos têm investigado a influência do “brooding” nas posturas, descrevendo que este comportamento aumenta a sobrevivência dos embriões, protegendo contra dessecação, predadores e infecções fúngicas. A influência e os níveis do cuidado podem variar devido a fatores abióticos como o período chuvoso e os níveis de umidade do ar (Hayes 1991; Crump 1995; Vockenhuber et al. 2008, 2009; Delia et al. 2010, 2013; Lehtinen et al. 2014; Vargas-Salinas et al. 2014).

Cuidado maternal já foi detectado em centrolenídeos (Jacobson 1985), mas recentemente, muitos autores tem descrito eventos reprodutivos, notando a ocorrência do comportamento de “brooding” realizado pelas fêmeas (Guevara-Molina e Vargas-Salinas 2014; Bravo-Valencia e Delia 2016; Díaz-Ricaurte et al. 2016, 2019; Bastos-Riascos et al. 2017; Arcila-Pérez et al. 2017). Delia et al. (2017) mostrou que o cuidado parental é um traço marcante na família. Este comportamento pode variar em relação ao sexo que realiza o cuidado e o tempo investido no cuidado, e o cuidado maternal prolongado foi inferido como sendo o estado ancestral em Centrolenidae. Além disso, *Cochranella granulosa* e *Teratohyla pulverata* exibem comportamento de “brooding” de curto período que aumenta a espessura da postura em até quatro vezes, e posturas que não receberam o cuidado, metade morreram. O “brooding” de curto período hidrata as posturas, sendo um comportamento importante na sobrevivência dos embriões, onde o aumento da espessura da postura serve como uma proteção contra dessecação e predação. *Vitreorana ritae* foi uma das espécies observadas com cuidado maternal (anteriormente como sem cuidado), porém, somente um indivíduo foi observado e

ainda não tem definição se este comportamento é amplamente distribuído no gênero (Delia et al. 2017).

O gênero *Vitreorana* possui 10 espécies, distribuídas ao longo da Cordillera de la Costa, Escuda das Guianas, Amazônia e um grupo vicariante encontrado na Mata Atlântica e florestas de galeria do Cerrado (Guayasamin et al. 2009; Pontes et al. 2014; Santana et al. 2015; Frost 2019). *Vitreorana baliomma* é uma pequena espécie (16.4–21.2 mm) encontrada em florestas Ombrófilas associadas a córregos e riachos na porção norte da Mata Atlântica, desde 98 até 620 metros acima do nível do mar. Esta espécie foi descrita baseada somente em características morfológicas de espécimes coletados e permanece sem informações a respeito de sua posição filogenética, morfologia dos girinos e descrição do canto (Gouveia et al. 2012; Pontes et al. 2014). Indivíduos usam a vegetação presente nas margens, ao longo do córrego, se empoleirando em folhas e troncos, em alturas de 0,3 até 4 m. A postura dos ovos ocorre preferencialmente na superfície de cima (adaxial) das folhas, porém, indivíduos também utilizam a superfície de baixo (abaxial), em alturas de 0,5 até 2 m (Guayasamin et al. 2009; Gouveia et al. 2012; Lisboa et al. 2019). Machos ativos normalmente formam coros, onde um primeiro macho vocaliza e os outros replicam, comportamento conhecido como “lek behavior” (Wells 2007; Gouveia et al. 2012). Dois estudos avaliaram aspectos reprodutivos de *V. baliomma* e nenhum detectou a ocorrência de cuidado parental (Gouveia et al. 2012; Lisboa et al. 2019).

As pererecas-de-vidro possuem muitas homoplasias morfológicas (Guayasamin et al. 2008). Pontes et al. (2014) incluiu *V. baliomma* no gênero *Vitreorana* baseando-se em afinidades biogeográficas (todas as espécies de Centrolenidae ocorrentes na Mata Atlântica são deste gênero), mas traços morfológicos podem suportar sua inclusão nos

gêneros *Teratohyla* ou *Chimerella*. Por isso, é difícil determinar a sua afinidade filogenética sem o uso de sequências de DNA (Guayasamin et al. 2009; Pontes et al. 2014). Apesar de *Vitreorana* ser considerado monofilético, as relações dentro do gênero permanecem controversas, possuindo muitos nós com pouco suporte (Guayasamin et al. 2009; Twomey et al. 2014; Santana et al. 2015). Paz et al. (2018) conduziu um estudo filogeográfico utilizando *V. uranoscopa* and *V. eurygnatha*, incluindo uma amostragem extensa de indivíduos coletados ao longo da distribuição de ambas espécies. Eles identificaram quatro linhagens divergentes de *V. eurygnatha* e 12 de *V. uranoscopa*, apontando um baixo suporte para a monofilia de ambas as espécies.

Objetivos

- Descrever os padrões de comportamento reprodutivo em *Vitreorana baliomma*
- Investigar a ocorrência de cuidado parental em *Vitreorana baliomma*
- Caracterizar os parâmetros físicos dos locais onde se encontravam indivíduos adultos e posturas de ovos de *Vitreorana baliomma*
- Caracterizar as posturas de ovos de *Vitreorana baliomma*
- Quantificar as características temporais e espectrais do canto de anúncio de *Vitreorana baliomma*
- Inferir as relações filogenéticas de *Vitreorana baliomma* em Centrolenidae usando sequencias de DNA e análises filogenéticas
- Avaliar a influência de variáveis abióticas no número de indivíduos de *Vitreorana baliomma* ativos
- Avaliar a influência do micro-habitat na espessura das posturas de ovos de *Vitreorana baliomma*

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2. CAPÍTULO 1: Manuscrito formatado para artigo

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1 **Reproductive Biology and Phylogenetic Relationships of *Vitreorana baliomma* (Anura:**
2 **Centrolenidae)**

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27 **Abstract**

28 Behavioral and population ecology information are lacking for all *Vitreorana* species inhabiting
29 the Atlantic Forest. Glassfrogs exhibit a diversity of interesting reproductive behaviors, and
30 knowledge of behavioral aspects can help elucidate the complex relationships between clades.
31 In this study, we report on the reproductive behaviors of *V. baliomma*, a small species of
32 Centrolenidae known only by morphological features of collection specimens. We also describe
33 microhabitat use, advertisement call and the influence of environment on activity of adults and
34 on clutches thickness. Moreover, we conducted phylogenetic analyses using molecular data to
35 assess *V. baliomma* position in Centrolenidae and confirm their presence within the genus
36 *Vitreorana*. We sampled two streams in southern state of Bahia, Brazil, between November
37 2018 and April 2019. For the first time, we confirm the presence of female-only egg-brooding
38 in this species. Oviposition site reflected some of the adult microhabitats. However, clutches
39 were found at lower height and horizontal distances, on both surfaces of the leaves and on larger
40 leaves than those used by adults. Calls of *V. baliomma* males have one or two notes, and
41 parameter values are similar to other *Vitreorana* species. Call and phylogenetic analyses
42 validated *V. baliomma* and confirmed the phylogenetic position within the genus, but the
43 relations in *Vitreorana* are still nebulous. The number of calling males was positively correlated
44 with air humidity, which probably shows an effect of daily rainfall on reproductive behavior of
45 this species. Our study expands the knowledge on the reproductive biology and elucidate the
46 reproductive behaviors, describing the occurrence of maternal egg-brooding for the species.
47 Our results suggest that egg-brooding is a widespread behavior in *Vitreorana*. Moreover, this
48 find reinforces the pattern of care evolution in Centrolenidae.

49

50 **Keywords:** Advertisement call; Atlantic Forest; Mating patterns; Microhabitat use; Parental
51 care

52 **Introduction**

53 One of the main difficulties to address challenges of the current biodiversity crisis are
54 the gaps in our knowledge of some of the most basic characteristics of organisms (Hortal et al.
55 2015). These knowledge shortfalls imply that scientists must work with incomplete and often
56 unrepresentative data on a limited number of organisms and their characteristics (Hortal et al.
57 2015). Although technology may offer partial solutions to these shortfalls, fieldwork is still the
58 most important way to fill these gaps (Wilson 2017).

59 Species in the family Centrolenidae, known as glassfrogs, perfectly illustrate this
60 situation. Before the 90s, they were a poorly known group and most species were known from
61 just a cursory description of their external morphology based on preserved material (e.g., Taylor
62 1949; Rivero 1968; Lynch and Duellmann 1973; Duellman 1976; Rivero 1985). Ruiz-Carranza
63 and Lynch (e.g., 1991, 1995a, b, c, 1998) and later, Cisneros-Heredia and McDiarmid (2007)
64 and Guayasamin et al. (2008, 2009) changed this by describing many species, improving
65 taxonomic characters and building a detailed evolutionary framework through phylogenetic
66 studies of DNA sequences, respectively. We can now say that centrolenids are emerging as a
67 model system in ecology and evolution (e.g., Gouveia et al. 2012; Hutter et al. 2013;
68 Castroviejo-Fisher et al. 2014; Delia et al. 2013, 2017; Escalona-Sulbarán et al. 2019). This
69 family is restricted to the American tropics, where species inhabit forested waterfalls and
70 streams, perching and laying their eggs on leaves, branches, mosses or rocks overhanging the
71 water (Cisneros-Heredia and McDiarmid 2007; Guayasamin et al. 2009; Twomey et al. 2014).
72 After hatching, tadpoles fall into the water and continue their development buried in sand and
73 debris at the bottom of water bodies (Kubicki 2007; Guayasamin et al. 2009). Despite their
74 relevance in evolutionary studies, important gaps in our basic knowledge of the diversity of
75 glassfrogs may lead to misidentification of ecological and evolutionary patterns and processes,
76 and inefficient use of limited resources for conservation purposes. In this study, we identified

77 *Vitreorana baliomma* as a glassfrog of primary interest to overcome important knowledge gaps
78 in the phylogenetic relationships (Darwinian shortfall) and biological traits (Raunkiaeran
79 shortfall) of glassfrogs.

80 *Vitreorana baliomma* is a small species (snout-to-vent length = 16.4–21.2 mm)
81 described only on the basis of morphological characteristics of preserved specimens (Pontes et
82 al. 2014). It occurs in forests associated with streams and creeks in the northern portion of the
83 Atlantic Forest of Brazil, from 100 to 600 meters a.s.l. (Gouveia et al. 2012; Pontes et al. 2014;
84 Figure 1). Individuals use marginal vegetation along streams, perching on leaves and trunks,
85 ranging in height from 0.3 to 4 m (Gouveia et al. 2012).

86 The phylogenetic affinities of this species are speculative and even its placement within
87 *Vitreorana* is somewhat tentative (Pontes et al. 2014). This placement in genus is due to the
88 presence of the following characters in *V. baliomma*: parietal and urinary bladder peritoneum
89 transparent, pericardium and visceral peritoneum white, green bones in life, bulbous liver, and
90 dorsal coloration in preservative lavender. Besides *Vitreorana*, this combination of characters
91 is present in species of genera *Chimerella*, *Hyalinobatrachium*, and *Teratohyla* within
92 Centrolenidae. Faced with this situation and in the absence of DNA sequences, Pontes et al.
93 (2014) opted for *Vitreorana* on the basis of biogeographic affinities because all previously
94 known species of glassfrogs from the Atlantic Forest form a clade within *Vitreorana*
95 (Guayasamin et al. 2009; Santana et al. 2015), which is the sister taxon of the Amazonian and
96 Guiana Shield species of the genus. However, all the Atlantic Forest species of *Vitreorana* have
97 the synapomorphy white urinary bladder peritoneum (Guayasamin et al. 2009), which is
98 conspicuously absent in all other species of *Vitreorana* (including *V. baliomma*). The
99 phylogenetic relationships of *V. baliomma* transcend the details of glassfrog systematics
100 because the relationships between the Atlantic Forest and Amazonia-Guiana Shield clades
101 constitutes a key example of the biogeographic connection between these two regions in the

102 late Miocene (Castroviejo-Fisher et al. 2014; Rodrigues et al. 2014; Prates et al. 2017). If *V.*
103 *baliomma* is more closely related to other glassfrogs than to the Atlantic Forest's *Vitreorana*
104 clade, this would imply a second biogeographic connection for glassfrogs between Amazonia
105 and Atlantic Forest.

106 Glassfrogs have become a recurrent focus of amphibian reproductive strategies, with
107 focus on issues such as parental care, acoustic communication, microhabitat selection, and the
108 effect of environmental variables on reproductive activity (e.g., Greer and Wells 1980;
109 Jacobson 1985; Hayes 1991; Vockenhuber et al. 2008; Valencia-Aguilar et al. 2012;
110 Lehtinen et al. 2014; Vargas-Salinas et al. 2014; Bravo-Valencia and Delia 2016; Díaz-
111 Ricaurte et al. 2016, 2019; Arcila-Pérez et al. 2017; Bastos-Riascos et al. 2017; Rios-Soto et
112 al. 2017; Ospina-L et al. 2019). Little is known about the reproductive biology of *V. baliomma*.
113 Gouveia et al. (2012) and Lisboa et al. (2019) reported that females lay their eggs, preferably,
114 but not exclusively, on the upper surface (adaxial) of leaves, at 0.5 to 2.0 m high. Males
115 normally form call choruses, where a first male calls and others reply, which is known as “lek
116 behavior” (Wells 2007; Gouveia et al. 2012). Parental care is so far never reported, and the
117 advertisement call has not been quantitatively described. Other aspects of reproduction, such as
118 microhabitat use or whether climatic conditions influence of on behavioral activity, are also
119 unknown. This lack of data is problematic if we consider the phylogenetic position of
120 *Vitreorana* as one of the earliest splitting clades within the tribe Cochranellini (Guayasamin et
121 al. 2008; Castroviejo-Fisher et al. 2014; Twomey et al. 2014) and that the aforementioned
122 characters are also unknown for most of the other species of *Vitreorana*. For example, Delia et
123 al. (2017) studied the evolution of parental care in glassfrogs, but knowledge about the
124 distribution of this behavior in the whole genus is based on the observation of a single female
125 of *Vitreorana ritae*.

126 Considering the aforementioned relevance of *V. baliomma* to advance our knowledge
127 on the systematics, evolution, and ecology of Centrolenidae, we conducted field expeditions to
128 collect data to address the following issues: (i) Describe the pattern of reproductive behaviors;
129 (ii) investigate the occurrence of parental care; (iii) characterize the physical parameters of the
130 sites used by adults and egg clutches; (iv) characterize egg clutches; (v) quantify the temporal
131 and spectral characteristics of the advertisement call; (vi) infer its phylogenetic relationships
132 within Centrolenidae using DNA sequences and phylogenetic analyses; (vii) evaluate the
133 influence of abiotic variables on the number of active adult individuals; and (viii) evaluate the
134 influence of microhabitat on clutch thickness.

135

136 **Material and methods**

137

138 *Study area*

139 We collected data between November 2018 and April 2019, in two streams with a
140 sand/rocky bed: (i) Fazenda Santo Antônio (15°30'8.65"S, 39°22'33.14"W; 105 m a.s.l.),
141 municipality of Mascote and (ii) at 3 km of Cachoeira do Tijuípe (14°23'17.61"S,
142 39°2'37.89"W; 89 m a.s.l.) in BA001 – Ilhéus-Itacaré road, municipality of Uruçuca. Both
143 streams are located in the Atlantic Forest in southern state of Bahia, Brazil. The stream in
144 Mascote occurs in a *cabruca* forest, which is an agroforestry system where some of the native
145 forest serves as shade for the cocoa trees (*Theobroma cocoa*). The locality near the road BA001
146 is formed by a well regenerated secondary forest. The sampled area is inserted in the
147 conservation unit Área de Proteção Ambiental (APA) Costa de Itacaré and within the buffer
148 zone of Parque Estadual Serra do Conduru (PESC). In southern Bahia, climate is characterized
149 as being hot and humid, without a dry season (Köppen 1936). Mean annual and daily rainfall

150 are between 1500–1700 mm and 50–100 mm, respectively, while mean annual temperature is
151 23–24°C and relative air humidity is $\geq 80\%$ (Roeder 1975).

152

153 *Field sampling*

154 We sampled at night, between 17:00 and 03:00 hours of the next day, with sporadic
155 diurnal visits to verify permanence of adults near clutches, which totalized 35 hours of sampling
156 in 16 days. We measured temperature and air humidity at the beginning and end of each
157 sampling period (period of time from arrival until leaving the stream), using a digital term-
158 hygrometer Instrutherm HT-260 (accuracy $\pm 2.5^\circ\text{C}$; $\pm 3.5\%$ RH). For each sampling period, we
159 used the sum of the start and end values divided by two. We conducted visual encounters for
160 adults and clutches and auditory searches for calling males. We marked encounter sites of adults
161 and clutches with a uniquely coded flagging tape. When possible, we differentiated males from
162 females by observing the vocal sac, present only in males, and by the presence of oocytes in the
163 oviduct, visible through the belly, of reproductively active females. We collected opportunely
164 representative voucher specimens of adults (females and males), embryos, and tadpoles. We
165 euthanized specimens in lab with a topical dose of the anesthetic benzocaine (50 mg/g) and
166 fixed them in 10 % formalin. After 24 h, we preserved adults in 70 % ethanol. We removed a
167 sample of muscle tissue from the thigh of some specimens and preserved it in 96 % ethanol for
168 DNA analyses. We deposited specimens and tissue samples in the Amphibian Collection of
169 Museu de Ciências e Tecnologia of Pontifícia Universidade Católica do Rio Grande do Sul
170 (MCP).

171

172 *Mating behaviors*

173 We monitored pairs in amplexus until oviposition and recorded other intraspecific
174 interaction events throughout the amplexus duration. We used a headlamp with a red-light filter

175 to reduce the disturbance caused by white light (Vargas-Salinas et al. 2014; Touzot et al. 2020).
176 We visited egg clutches in the following day and night to evaluate the presence of adults and
177 their potential interactions with egg clutches after oviposition (monitored by VMZ).

178

179 *Microhabitat data*

180 We recorded the following variables for each encounter site of adults, pairs and clutches:
181 (i) vertical height to substrate, (ii) horizontal distance from streambed, (iii) type of structure
182 used as perch (e.g. leaf, branch, moss, rock), (iv) perch size, (v) side (e.g. adaxial or abaxial)
183 and (vi) type of leaf surface (e.g. glabrous or pilose), (vii) position relative to the streambed
184 (e.g. streambed, bank of stream, outside bank of stream), (viii) side to the stream flow (e.g. left
185 or right), (ix) canopy cover. We measure vertical height from the encounter site to the substrate,
186 and horizontal distance from location of the adult, pair or clutch on the perch to the streambed
187 margin. We quantified both variables using a 5 m millimetric tape. We quantified perch size
188 using a 2 m millimetric tape to measure the length (L) and width (W) of the leaf, and after
189 calculating the ellipse area (A) with the formula $A = (L/2) \times (W/2) \times \pi$. We quantified
190 canopy cover using photos taken during daytime visits, with a fisheye lens attached to the phone
191 camera. We took all photos with the phone positioned 5 cm above and over the center of the
192 encounter site and leveled at 180°. For each photo, we calculated the percentage canopy free
193 pixels using the software Gap Light Analyzer (Frazer et al. 1999).

194

195 *Egg clutch characterization*

196 We recorded the diameter and thickness of entire egg capsule, number of eggs, stage of
197 development, and embryo coloration of each observed clutch. We measured length (L) and
198 width (W) using a digital caliper (accuracy ± 0.02 mm), and then calculated the diameter (D)
199 by the formula $D = 2\sqrt{A/\pi}$, where we defined the ellipse area (A) for clutches too. To measure

200 clutch thickness, we used small wooden rods marked in millimeters (mm). We used Gosner
201 (1960) to determine the embryonic stage of clutches.

202

203 *Bioacoustics*

204 We recorded the calls of 37 males using a Marantz PMD660 digital recorder connected
205 to a Sennheiser K6/ME66 unidirectional microphone on April 7, 11, 12 and 14, 2019, between
206 21:10 and 00:15 (air temperature between 26.1°C and 24.3°C; one voucher specimen VMZ
207 018) from the site near Cachoeira do Tijuípe, BA001 (recorded by VMZ); and five individuals
208 using a Tascam DR40 digital recorder with an internal microphone on January 9, 2018, between
209 19:00 and 20:00 (air temperature between 22.0°C and 21.0°C; one specimen collected under
210 label MZUESC 20012) at Fazenda Santo Antônio (recorded by ORP). We recorded, in an
211 additional locality at RPPN Ararauna, municipality of Una, Bahia (15°18'08.31"S,
212 39°09'42.34"W), seven individuals using a Marantz PMD660 digital recorder connected to a
213 Sennheiser K6/ME66 unidirectional microphone on October 7, 2015, around 21:00 (air
214 temperature = 21.9°C; two voucher specimens MZUESC 18531 and 18534; recorded by IRD).
215 We used calls of males nearby the original recorded male for analyses.

216 We measured the following variables of each advertisement call: (1) call duration (s);
217 (2) dominant frequency (Hz); (3) maximum frequency (Hz); (4) minimum frequency (Hz); (5)
218 bandwidth (Hz); (6) number of pulses; (7) pulse duration (s); and (8) inter-pulse interval (s).
219 We measured minimum and maximum frequency 6 dB below the peak intensity of the dominant
220 frequency. We followed the terminology of Köhler et al. (2017) and reported all values as mean
221 \pm standard deviation (minimum–maximum values). We measured temporal and spectral
222 variables from oscillograms and power spectra, respectively. We measured all variables with
223 the software Raven Pro 1.3 (Center for Conservation Bioacoustics 2008) using the following

224 settings: Blackman with window size 5 ms, overlap 80 %, and DFT size of 1024 samples. We
225 used R 3.6.1 (R Core Team 2019) with the packages seewave and tuneR to make all figures.

226 To compare *V. baliomma* calls with those of other species of the genus, we analyzed
227 recordings of all *Vitreorana* species (except for *V. parvula*). We obtained the recordings used
228 for comparison from personal collections (from the authors and other contributors) and online
229 databases (Fonoteca Neotropical Jacques Vielliard [<https://www2.ib.unicamp.br/fnjv/>] and
230 Fonoteca Zoológica [<http://www.fonozoo.com/>]). Due to the limited quality of some of these
231 recordings, we only measured the first six aforementioned acoustic variables.

232

233 *Molecular methods and phylogenetic analyses*

234 We extracted Genomic DNA from ethanol-preserved muscle tissue using the Wizard
235 Genomic DNA Purification Kit (Promega, Madison-WI, USA) following instructions indicated
236 by the manufacturer. We performed polymerase chain reactions (PCR) in 25 µL volumes with
237 2.0 µL of genomic DNA to amplify ~ 600 bp of the 16S rRNA gene (primers 16Sar and 16Sbr;
238 Palumbi et al.1991), ~ 600 bp of the 12S rRNA gene (primers t-Phe-frog and 12S-frogR; Wiens
239 et al. 2005), and ~540 bp of *POMC* (primers POMC-1 and POMC-2; Wiens et al. 2005). We
240 checked amplifications in agarose gels (1–2 %) and sequenced fragments in both directions.
241 We used Sequencher v4.1.4. to assemble and edit chromatograms.

242 Additionally, we downloaded homologous sequences of other *Vitreorana* available
243 from GenBank (as of 30 December 2019). As outgroups, we used homologous sequences from
244 GenBank of one or two terminals of all other centrolenid genera. We rooted all trees with
245 sequences of *Allophryne ruthveni*, once this genus is widely accepted to be the sister taxon to
246 all Centrolenidae (Guayasamin et al. 2009). If genetic distances were < 1 %, we assigned
247 sequences from different specimens to a single composite terminal (Appendix S1), in order to
248 reduce the amount of missing entries per terminal and search space during phylogenetic

249 analyses (Wilkinson, 1995; Kearney, 2002; Brower, 2018). We list specimens and DNA
250 sequences used in phylogenetic analysis in Appendix S1.

251 We individually aligned sequences of each marker using MUSCLE (Edgar 2004) in
252 AliView (Larsson 2014) under default parameters. We combined the alignments mentioned
253 above into a single matrix using SequenceMatrix 1.8 (Vaidya et al. 2011). We used parsimony
254 and maximum likelihood (ML) phylogenetic analyses. To search for the most parsimonious
255 tree, we used TNT v1.5 (Goloboff and Catalano 2016) with new technology search (Sectorial
256 Search, Ratchet, Drift and Tree Fusing) at level 70 and a minimum of 100 hits of the best tree
257 (equal cost for all transformations, gaps treated as fifth state). We assessed clade support with
258 1000 jackknife pseudoreplicates (JK), with removal probability of 0.36 ($\sim e^{-1}$), which
259 purportedly renders jackknife and bootstrap (BS) values comparable (Farris et al. 1996).

260 For ML analyses, we determined the most appropriate model of nucleotide substitution
261 and data partition using the software PartitionFinder v2.1.1 (Lanfear et al. 2016). We divided
262 the dataset by gene and by codon positions. We used the options “*branchlengths = linked*”,
263 “*models = all*”, “*model_selection = AICc*” and “*search = greedy*”. We performed 500
264 independent searches in Garli v2.01 under default settings and using the models and partitions
265 derived from the results of PartitionFinder. We assessed clade support with 1000 BS
266 pseudoreplicates using Garli with the same aforementioned settings. We used SumTrees
267 package in DendroPy v4.4.0 (Sukumaran and Holder 2010) to add BS frequencies onto the best
268 ML tree.

269

270 *Statistical analyses*

271 We tested the influence of abiotic variables on the number of calling males and sampled
272 individuals (males and females), and the influence of clutch structure and microhabitat variables
273 on clutch thickness (see details below). We considered number of calling males and sampled

274 individuals for each sampling period. We performed all analyses in R 3.6.1 (R Core Team,
275 2019), with packages *nlme* for the Linear Model (LM), Linear Mixed Model (LMM) and
276 Generalized Linear Mixed Model (GLMM) analyses and *ggplot2* to generate the figures. We
277 ran Shapiro-Wilk tests (function: *shapiro.test*) and found that nightly number of calling males
278 and clutch thickness followed a normal distribution ($W = 0.83706$, $p = 0.1945$; $W = 0.96809$, p
279 $= 0.7377$), but not sampled individuals ($W = 0.66706$, $p = 0.0002649$). Consequently, we
280 performed an LMM analysis (function: *lme*) to test if average temperature and humidity during
281 sampling periods are a predictor of number of calling males, and a GLMM analysis (function:
282 *glmer*) to test if these same variables are a predictor of number of sampled individuals. We used
283 the sampling locality as a random variable, while considering different abundances in each area,
284 in both analyses.

285 To test if the variables summarizing clutch structure (i.e., number of eggs, diameter and
286 developmental stage) are a predictor of clutch thickness, we performed a LM analysis (function:
287 *lm*), and a LMM to test if microhabitat variables (i.e., height, distance, leaf surface and size,
288 canopy openness, position and stream margin) are a predictor of clutch thickness. Prior to the
289 analysis, we transformed leaf size values into square root to decrease the amplitude of data, and
290 leaf surface, position and stream margin into numeric values (function: *as.numeric*). We added
291 developmental stage as a random effect to test if microhabitat variables are predictor of clutch
292 thickness in LMM, given that it is an intrinsic feature of clutches (see Results). We used the
293 statistics AIC and R^2 to select additive or simple models to test if abiotic variables predict
294 number of calling males and sampled individuals, and if both clutch structure and microhabitat
295 variables predict clutch thickness. We used the ML function and a Poisson distribution to fit
296 LMM and GLMM, respectively. We considered results as statistically significant if P -values
297 were ≤ 0.05 .

298

299 **Results**

300 We found a total of 24 individuals (14 males, six females and four individuals without
301 sex confirmed) and 19 clutches (one clutch with some embryos hatched, disregarded for number
302 of eggs, diameter and thickness). During sampling, temperature varied from 23.7–29.1°C and
303 humidity from 77–93.2%. The study area experienced abnormally low rainfall between January
304 and March 2019.

305

306 *Mating behaviors*

307 We observed four mating events. On the days of the observations, temperature varied
308 from 24.3–26°C and humidity from 90.7–93.2 %. At approximately 22:20 on April 11, 2019,
309 we located a female 30 cm away from a male vocalizing from a bush. The male was calling on
310 the upper side of a leaf ~ 100 cm above the ground. The female moved along the leaves, circling
311 the male's calling site. At 22:25, the female jumped to the leaf positioned in front of the calling
312 male. The male, still calling, turned to face the female, which then jumped to the same leaf. The
313 male then climbed on the female's back and initiated axillary amplexus. The amplexant pair
314 moved along the bush by jumping to leaves above and below the initial site (Figure A) and
315 switching between the upper and lower surfaces of the leaves (Figure B); while on leaves, they
316 constantly performed 360° rotation movements (Figure C). After 102 min in amplexus, the
317 female laid a clutch with 13 eggs, 1.30 cm diameter and 3 mm thickness. Right after oviposition,
318 the female moved backwards, positioning its body above the clutch in a brooding posture. The
319 male released the female, just after she laid the clutch, jumped to a leaf approximately 10 cm
320 above the oviposition site, and began to vocalize again. The female remained for 110 min
321 positioned above the clutch, raising the posterior part of her body at intervals of 1 to 10 minutes
322 (Figure D). At 01:58, the female left the clutch, remained next to it for a few seconds, then
323 jumped to other leaves and left the oviposition site.

324 In the other three mating events, we observed the same behaviors exhibited by the pair
325 described above, with differences described as follows. In one case, at 21:10, also on April 11,
326 2019, we found the individuals already in amplexus. After 25 min, oviposition occurred and
327 few seconds later the male released the female, ending amplexus. The clutch had 14 eggs, 1.40
328 cm diameter and 5 mm thickness. Afterwards, the female remained with the belly above the
329 clutch for 99 min, executing the movements with the posterior part of its body. In the third
330 mating event, we found individuals in amplexus at 23:14 on April 14, 2019. While in amplexus,
331 the female performed movements with its front limbs, rubbing them on her face. After 56 min
332 in amplexus, it started to rain and, the pair became more active, moving more frequently in the
333 vegetation. At 01:08, while attempting to jump to an adjacent leaf, the pair fell into a spider
334 web. Individuals continued to be observed for 60 min. They remained in amplexus as the female
335 tried to break free from the web, unsuccessfully. After 60 min in the spider web, we collected
336 the individuals and placed them in a transparent plastic bag with humid leaves from the
337 surrounding vegetation. On the next day, we found an egg clutch deposited inside the collection
338 bag and attached to the upper side of a leaf. Finally, in the fourth event on December 3, 2018,
339 we collected an amplexant pair that we placed in a transparent plastic bag and made direct
340 observations on oviposition behaviors. The pair remained in amplexus for ~100 min until
341 oviposition. After oviposition, the female performed brooding behavior for ~120 min, with up
342 and down movements.

343 Although we observed some males calling during amplexus, we were not able to record
344 them. To the ear, the calls emitted during amplexus appeared identical to the advertisement call.
345 We did not observe individuals close to the clutches in the following visits, either at day or
346 night. We did not observe aggressive behaviors between conspecific males or predation events
347 involving individuals or clutches.

348

349 *Microhabitat of adults and egg clutches*

350 We found all adults on the upper surface of green glabrous leaves (N = 24). The size of
351 used leaves ranged from 6.6 x 2.2 to 39.9 x 8.5 cm. Most adults used the left side of streams (n
352 = 23), in leaves positioned above streambed (n = 14) (Table 1). We found all clutches in green
353 glabrous leaves (N = 19), ranging in size from 6 x 2.4 to 61.3 x 28.6 cm, more frequently on
354 the adaxial surface (n = 15) and on the left side of stream (n = 14), above the streambed (n =
355 15) (Table 1).

356

357 *Clutch characterization*

358 The clutch size averaged of 15 ± 4 eggs (7–22). The mean diameter and thickness of
359 egg capsule were of 2.49 ± 0.75 cm² (1.31–4.32) and 6.7 ± 2.8 mm (2–13), respectively (N =
360 18). Clutches had embryos between stages >10 to 25 according to Gosner (1960), where >10
361 was the stage immediately after oviposition and 25 the stage of hatching embryos. We found
362 two different aged clutches on the same leaf (19 and 21). All clutches exhibited a transparent
363 gelatinous matrix surrounding the embryos. In stages 10–16 we observed yellowish cream
364 embryos (slightly greenish immediately after deposition) (**Figure 3** Egg clutches of *Vitreorana*
365 *balionna* in early (A), intermediate (B) and late (C) stagesFigure A). In stages 19–22, we
366 observed whitish embryos or slightly grayish, but with a yellowish cream vitellus (Figure B).
367 In late-stage embryos (23–25), the tadpoles were slightly brown to gray with reddish hues and
368 a reduced yellowish cream vitellus (Figure C). In early stage clutches, eggs were in a monolayer
369 array (Figure A). However, this changed over time (Figure B and C), with most clutches
370 increasing in thickness during development.

371

372 *Bioacoustics*

373 We recorded two types of calls, which we labelled “A” and “B”, both presenting a
374 pulsed pattern. Call “A” was the most frequent recorded and consists of a single “preep” like
375 note. Call “B”, emitted only sporadically, has two notes and sounds like a “preep prep” to the
376 ear. We only recorded call “B” in the stream near BA001. In general, acoustic variables are
377 similar between calls “A” and “B”, differing slightly in call duration and number of pulses
378 (Table 2). In both call types, only the fundamental frequency is appreciable. Calls “A” and “B”
379 have amplitude modulation, reaching its peak in the middle of the call. Calls also have
380 frequency modulation, with a pattern similar to amplitude modulation. The first pulse has the
381 lowest frequency, which is followed by a slight and gradual increase in subsequent pulses
382 (Figure). In call “B”, the first note has 6 to 10 pulses and lasts 0.168–0.298 s. The second note
383 has 4 to 5 pulses and lasts 0.101–0.147 s. The interval between the first and second notes is
384 0.194–0.232 s. We did not observe frequency modulation between call “B” notes.

385

386 *Phylogenetic analyses*

387 Final dataset included 2468 characters (12S: 980, 40 % of terminals; 16S: 884, 36 %;
388 POMC: 604, 24 %). PartitionFinder selected the following data partitions and models of
389 nucleotide evolution: The models used were GRT+I+G for 12S and 16S, TRNEF+G for POMC
390 first codon, and HKY+I for POMC second and third codons. ML searches found a single most
391 likely tree ($L_n = -11423.87158$), which includes a polytomy involving the Atlantic Forest
392 species of *Vitreorana* (Figure). Parsimony searches found 54 trees of 1894 transformations and
393 the strict consensus is well-resolved except for a basal polytomy for *Vitreorana* (Figure).

394 All phylogenetic analyses recovered *Vitreorana* as monophyletic (BS = 75; JK = 51)
395 within Centroleninae (BS = 65; JK = 50). The ML analysis recovered an Atlantic Forest +
396 Cerrado clade (BS < 50), with *V. uranoscopa* as the sister taxon of *V. eurygnatha* (BS < 50)
397 and forming a polytomy with *V. baliomma* and *V. franciscana* (Figure). This east Brazilian

398 clade is the sister taxon of a clade (BS < 50) composed by the two Cordillera de la Costa species
399 (*V. antisthenesi* and *V. castroviejo*; BS = 86) and the Guiana Shield + Amazonia species (BS
400 = 67). Parsimony recovered *V. franciscana* and *V. baliomma* as part of a polytomy. The groups
401 of the Cordillera de la Costa (JK = 89), Guyana Shield + Amazonia (JK < 50), and *V.*
402 *uranoscopa* + *V. eurygnatha* (JK < 50) are also monophyletic, but as part of a polytomy within
403 *Vitreorana*.

404

405 *Linear models*

406 The AIC selected the additive models above the simple models to relate weather
407 variables with number of calling males (AIC = 90.16924) or number of sampled individuals
408 (AIC = 60.2). In all other cases simple models were selected. Number of calling males was
409 positively correlated with air humidity (value = 0.07965956, SE = 0.02928244, t = 2.7203865,
410 p = 0.0297), but not with temperature (Figure 2). The number of sampled individuals were not
411 correlated with relative humidity or temperature. Clutch thickness was positively correlated
412 with developmental stage (estimate = 0.3391, SE = 0.1153, t = 2.942, P = 0.00957; F = 8,655,
413 R² = 0.3511, p = 0.009568) (Figure 3). However, clutch thickness was not related to the number
414 of eggs in the clutch (F = 2.24, R² = 0.1228, p = 0.1539) or clutch diameter (F = 0.756, R² =
415 0.04512, p = 0.3974). Clutch thickness was positively correlated with leaf size (value =
416 0.125764, SE = 0.0465318, t = 2.702763, p = 0.0354; AIC = 85.5833) (Figure 4). All other
417 microhabitat variables did not influence clutch thickness (leaf height, AIC = 91.97329;
418 horizontal distance, AIC = 90.6665; leaf surface, AIC = 91.21294; canopy openness, AIC =
419 58.7324; position in stream, AIC = 90.48317; side in stream, AIC = 91.22174).

420

421 **Discussion**

422

423 *Reproductive behavior and parental care*

424 The reproduction of *Vitreorana baliomma* involves the following sequence of
425 behaviors: males vocalize from leaves (always from the upper side), females probably choose
426 for males using cues from their sounds, axillary amplexus occurs, females move around the
427 vegetation while in amplexus (purportedly scouting for selection of oviposition site),
428 oviposition of a single, mono-layer egg clutch on a leaf (more frequently on the upper side),
429 termination of amplexus, females perform brooding behavior (apparently to hydrate the eggs),
430 and development of the embryos within the egg capsule until stage 25 without further
431 interactions with adults. This general sequence of behaviors is shared with all other species of
432 glassfrogs, with variation associated with details regarding aspects such as call and oviposition
433 sites, sex of the caring parent, and extension and type of parental care (Greer and Wells 1980;
434 Jacobson 1985; Vockenhuber et al. 2008; Vargas-Salinas et al. 2014; Arcila-Pérez et al. 2017;
435 Bastos-Riascos et al. 2017; Rios-Soto et al. 2017; Díaz-Ricaurte et al. 2019). However, ours is
436 the first study recording parental care in an Atlantic Forest glassfrogs. *Vitreorana baliomma*
437 females perform brief brooding behavior for 99-120 min just after oviposition. Although we
438 have not quantified the effects of this behavior on embryos' survival, it is likely to have a similar
439 impact to what Delia et al. (2017) found for *C. granulosa* and *T. pulverate* — two species of
440 closely related genera (Guayasamin et al. 2009) — where brief brooding behavior functions to
441 hydrate eggs, protecting embryos from dehydration and predation. Our discovery of female
442 brooding behavior in *V. baliomma* contrasts with the only two studies by Gouveia et al. (2012)
443 and Lisboa et al. (2019) that provided data on reproductive aspects of *V. baliomma*, not
444 documenting parental care in this species. Lisboa's et al. (2019) study did not observe
445 oviposition events, which significantly reduces the chances of observing female brooding
446 behavior. Gouveia et al. (2012), based on a single amplexant pair, described that the female
447 stayed near the clutch until dawn, a behavior we did not observe, without mentioning

448 interactions between the female and its egg clutch. This could indicate that *V. baliomma*
449 regulates its maternal care based on environmental factors such as moisture, daily rainfall or
450 both. This would be similar to the variation in paternal care observed in *Hyalinobatrachium*
451 *fleischmanni* (Delia et al. 2013). Another possibility is that maternal brooding only occurs in
452 certain populations so that the character has phylogeographic signal. Finally, we cannot rule
453 out that these authors disturbed female behavior when taking measurements of the eggs
454 immediately after oviposition as stated in Gouveia et al. (2012: 18).

455 We found that females brooded their eggs for an average of 99 min, and during this time
456 they elevated the posterior part of their body. This movement may serve to capture air moisture,
457 considering that the belly of anurans has greater absorption capacity, or to adjust her body above
458 the clutch. Other centrolenid species with brief maternal care brooded for a similar period of
459 time. For example, the female of *V. ritae* brooded for 80 min (Delia et al. 2017). However,
460 variation is large, from 10–180 min including species of the genera *Centrolene*, *Espadarana*,
461 *Nymphargus*, and *Teratohyla* (Jacobson 1985; Guevara-Molina and Vargas-Salinas 2014; Diaz-
462 Ricaurte et al. 2016; Arcila-Pérez et al. 2017; Bastos-Riascos et al. 2017; Rios-Soto et al. 2017).

463 The behaviors observed during the amplexus — lasting on average duration of 107 min,
464 which is similar to the duration reported by Gouveia et al. (2012) — suggest that the female is
465 selecting the best place for deposition of the egg clutch. While in amplexus, the female does
466 not only move through leaves of the plant in which the amplexus started, but also in those of
467 other nearby plants and frequently changing leaf surfaces. This surface change behavior
468 explains why we found 21 % of clutches on the abaxial surface of leaves. This can be a random
469 variation, but we argue that might results from a trade-off. Eggs on the upper surface will have
470 greater contact with rainwater to maintain hydration, while those on the lower surface may be
471 better protected from UV rays and predators. Leaf-side use for oviposition is considered a
472 taxonomic informative trait in Centrolenidae (e.g., Ruiz-Carranza and Lynch 1991;

473 Guayasamin et al. 2009). However, field observations indicate that this character is more
474 variable intraspecifically than previously thought (Delia et al. 2010; Bravo-Valencia and Delia
475 2016; Arcila-Perez et al. 2017). Taking this variation into account may have an important
476 impact on our understanding of the evolution of this behavior.

477 Amplectant pairs moved for more than an hour before oviposition. This is compatible
478 with the observation that species with maternal care move greater distances, involving more
479 time, than those with paternal care when in amplexus (Jacobson 1985). This might also be
480 related to territoriality. In species with just maternal care, males might choose the best site to
481 enhance their acoustic (or other) signals, while females must choose a good place for embryo
482 development. On the other hand, in species with paternal care, males might choose a place that
483 is good for both signaling for mates and for embryos development. As these sites are probably
484 limited, males are expected to display more aggressive territorial behaviors (McDiarmid and
485 Adler 1974; Restrepo and Naranjo 1999; Cardozo-Urdaneta and Señaris 2012; but see Noronha
486 and Rodrigues 2018).

487

488 *Microhabitat of adults and egg clutches*

489 Our results on variables associated with oviposition site indicate a clear preference for
490 the adaxial side of large glabrous green leaves, relatively close to the substrate, above water,
491 and on the left side of streams (Table 1). Some of these preferences clearly contrast with those
492 used by adults outside of oviposition, which are more often found at higher heights, larger
493 horizontal distances, and on smaller leaves. We suspect that the glabrous surface facilitates that
494 the tadpoles slip and fall into the stream, while, as previously discussed, the presence of egg
495 clutches in both the adaxial and abaxial sides of leaves may be related to a trade-off between
496 hydration from rainwater in the upper side and protection from predators and UV light on the
497 lower side. Oviposition sites located at lower heights can have higher survival probabilities

498 (Jacobson 1985), perhaps because due to higher humidity close to the ground (Cardoso and
499 Haddad 1992), which could decrease dehydration. Furthermore, these sites could benefit
500 tadpole hatching by decreasing their drop distance to water and wind influence. On the other
501 hand, because most adults that we observed were calling males, we hypothesize that their higher
502 height, compared to clutches, enhances the propagation of acoustic signal (Greer and Wells
503 1980; but see Valencia-Aguilar et al. 2012; Vargas-Salinas et al. 2014). We found all clutches
504 either directly above the streambed or, less frequently, within stream bank (flooded area after
505 strong rainfall). Considering that tadpoles need to fall into the water after hatching to continue
506 development, oviposition over the streams should be strongly selected for females (Cardozo-
507 Urdaneta and Señaris 2012). Meanwhile, we argue that selection over distance from the water
508 of calling site is milder, allowing males to vocalize from places outside the flooding area, which
509 are more covered by vegetation and could reduce predation risk. The choose of males for higher
510 heights could be a response too for this places more covered, once dense vegetation reduce
511 propagation of calls (Wells and Schwartz 1982, Vockenhuber et al. 2008).

512 Use of large leaves might be associated with male territoriality and prolonged care in
513 some *Hyalinobatrachium*, because males can attend many clutches at same time (Vockenhuber
514 et al. 2008; Valencia-Aguilar et al. 2012). We have not observed male territoriality and
515 prolonged care in this species, but our linear model shows a correlation of leaf size with clutch
516 thickness. We hypothesize that larger leaves may accumulate more water, aiding in clutch
517 hydration. Globular clutches (like in species of *Cochranella*, *Espadarana*, *Nymphargus* and
518 *Vitreorana*; Delia and Bravo-Valencia unpublished data) can retain larger amounts of water,
519 requiring an initial bout of maternal brooding and subsequent rainfall to maintain hydration
520 levels (Delia et al. 2017). Thus, water on leaves after rainfall might help to ensure embryo
521 survival. The preference for the left side of the stream could be related to the slope level, which,

522 by pure chance, is similar in the two sampled streams, causing the left side to accumulate more
523 water.

524

525 *Egg clutches*

526 Egg and embryo color, number of eggs, and early stages of clutches are similar to the
527 descriptions of Gouveia et al. (2012). Although here we relate temporal modifications of the
528 egg clutches along development. In early stages clutches are deposited in a monolayer array,
529 however throughout embryogenesis they gain a more complex tridimensional structure due to
530 increases in egg and egg-jelly hydration (Figure). We also found a relationship between
531 developmental stage and clutch thickness (Figure 3). As well as clutch structure, this probably
532 results from the addition of water to the jelly, as also suggested by Delia et al. (2013).

533

534 *Bioacoustics*

535 The presence of two types (“A” and “B”) of calls in *Vitreorana baliomma* is shared with
536 reports of multiple other species of anurans, including glassfrogs (Boul et al. 2006; Santana et
537 al. 2015). Different types of vocalizations might have different functions, such as mating,
538 territorial, antagonistic, and distress (Toledo et al. 2015). The function is normally inferred
539 through the context in which the individual emits the call. We did not observe any details during
540 fieldwork that could help us to interpret the potential different functions of calls “A” and “B”,
541 although call “A” was more frequent. Other species of *Vitreorana* emit advertisement calls with
542 a variable number of notes, such as *V. eurygnatha* (1–3), *V. helenae* (1–2), *V. ritae* (2–3), and
543 *V. uranoscopa* (1–4). Furthermore, the first note of call “B” seems to be equivalent to the only
544 note of call “A” and the parameter values of each note in call “B” overlap those of the call “A”.
545 Taking all this information into account, we consider both call types as variations of the
546 advertisement call, which can have one or two notes. Although we describe both call types

547 separately (Table 2), we focus our comparisons on call type “A” because it was more frequently
548 emitted.

549 The advertisement call of *V. baliomma* is different from those of other species of the
550 genus (Table 2). This reinforces the potential use of these vocalizations in systematics and
551 acoustic monitoring. *Vitreorana baliomma* emits longer calls than all other species except *V.*
552 *ritae*. The number of pulses is larger than in *V. eurygnatha*, *V. uranoscopa*, *V. castroviejoi*, *V.*
553 *gorzulae* and *V. anthistenesi*, smaller than in *V. ritae*, and similar to *V. franciscana* and *V.*
554 *helenae*. Dominant frequency is only different from *V. franciscana*, *V. castroviejoi* and *V.*
555 *anthistenesi*, smaller in the first, and larger in last two species, respectively. Bandwidth is only
556 different in *V. ritae* (larger bandwidth than the other species).

557 The general structure of *Vitreorana* advertisement calls is conserved (Figure 5), with all
558 species sharing similar structural features such as calls composed by one or few short pulsed
559 notes, with harmonics and frequency modulation (Wen et al. 2012). Our analysis confirms this
560 pattern. However, we did not detect harmonics in species analyzed—*V. uranoscopa* with three
561 (Haga et al. 2014); *V. gorzulae* with one, *V. castroviejoi* with two, *V. helenae* with one (Señaris
562 and Ayarzagüena 2005); *V. franciscana* with one harmonic (Santana et al. 2015). The detection
563 of harmonics is probably related to parameter values used to construct spectrograms.

564

565 *Phylogenetic relationships*

566 We present the first phylogenetic analyses including data of *Vitreorana baliomma*. Maximum
567 likelihood and parsimony analyses corroborate the monophyly of *Vitreorana*, although with
568 moderate (BS) or low (JK) values of resampling indices. The phylogenetic relationships within
569 *Vitreorana* are poorly resolved, with basal (although different) polytomies in both parsimony
570 and ML analyses (Figure and Figure). Both results indicate that the position of *V. franciscana*
571 is particularly problematic. The dataset of this species is just represented by DNA sequences of

572 *POMC* (Santana et al. 2015), a nuclear protein coding gene without sufficient variation to
573 inform about the relationships among species of *Vitreorana*. Thus, an important next step to
574 improve our knowledge of the evolutionary relationships of *Vitreorana* implies gathering more
575 phylogenetically informative data for *V. franciscana*, such as mitochondrial genes and faster
576 evolving nuclear markers. Morphologically, *V. baliomma* is most similar to *V. antisthenesi* and
577 *V. gorzulae*, from the Venezuelan Cordillera de la Costa and the Guiana Shield, respectively.
578 All three species share a completely transparent parietal and urinary bladder peritona, white
579 peritona covering the heart, liver, stomach, and intestines, green bones in life, and dorsum
580 lavender in preservative (Guayasamin et al. 2009, Santana et al. 2015). If we consider that all
581 other species of Atlantic Forest *Vitreorana* (i.e., *V. eurygnatha*, *V. franciscana*, *V. parvula*, and
582 *V. uranoscopa*) share the presence of white pigment on the urinary bladder peritoneum, which
583 is absent from all other known glassfrogs, and the ML topology, which is more resolved, we
584 hypothesize that *V. baliomma* may be the sister taxon of a clade containing all other Atlantic
585 Forest species of *Vitreorana*. This topology would be consistent with a single glassfrog
586 dispersal into the Atlantic Forest from the Amazon (Castroviejo-Fisher et al. 2014), but it should
587 push back its date. Nonetheless, this scenario needs to be tested with the addition of more
588 characters. For example, Twomey et al. (2014), who used more genes but less species, found
589 that the Atlantic Forest *Vitreorana* were not monophyletic.

590

591 *Weather variables and sampled adults*

592 As expected, we found a relationship between number of calling males and average
593 relative air humidity. This relationship points to the importance of rainfall for breeding activity
594 in *V. baliomma*, inasmuch as precipitation is highly correlated with humidity. Unlike other
595 studies (e. g., Bastos-Riascos et al. 2017; Rios-Soto et al. 2017), we did not detected a
596 relationship between number of calling males and temperature.

597 Precipitation is a good predictor of calling activity in Centrolenidae (Vockenhuber et al.
598 2008; Bastos-Riascos et al. 2017; Rios-Soto et al. 2017), and *Vitreorana* species call, almost
599 exclusively, in and after days with rain (Cardoso and Haddad 1992; Canelas and Bertoluci
600 2007). Vocalizing is energetically demanding for anurans, causing a significant increase of their
601 metabolic rate (Gerhardt 1994). Glassfrogs are very small, with a thin and delicate integument,
602 and arboreal behavior. This combination of characteristics might make them very prone to rapid
603 water loss while vocalizing unless the air is saturated with water. In fact, during the unusual dry
604 long periods (i.e., more than three continuous weeks of almost zero precipitation) of our
605 sampling, the entire population became inactive and no males were heard, or individuals
606 observed.

607

608 **Conclusions**

609 The short time of brief female brooding after amplexus is probably key to understanding
610 why this behavior was basically unknown in the family. Through detailed field studies and
611 careful observations during oviposition, our study detected parental care and besides that we
612 were able to broaden the knowledge about the reproductive biology and microhabitat use by *V.*
613 *baliomma*. Our phylogenetic and call analyses corroborate that this species is within
614 *Vitreorana*, but the internal relationships within the genus remain partially unresolved. We
615 recommend future *Vitreorana* datasets analyses should use more DNA sequences and genes,
616 particularly for *V. franciscana*. Our discovery of brooding by females in *V. baliomma* reinforces
617 the pattern of parental care evolution hypothesized for centrolenids. Nevertheless, more studies
618 and experiments are necessary to determine if *V. baliomma* exhibits variation in parental care
619 and which variables could be associate with it. In addition, it is crucial to test the impact of
620 brooding and oviposition site (i.e., upper vs. lower side of leaves, large vs. small leaves) on
621 offspring survival.

622

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632

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845 **Supporting information**

846 **Appendix S1** List of species, voucher codes, localities, and GenBank accession numbers of the sequences analyzed in this study. In bold are
 847 specimens sequenced for this work

Genus	Species	Voucher	Locality	Genes		
				12S (~980bp)	16S (884bp)	POMC (604bp)
<i>Allophryne</i>	<i>ruthveni</i>	MAD 1512 / MAD 1852	Guyana: Kabocali Camp / Guyana: Pakatau Creek Camp	AY843564	AY843564	AY819077
<i>Celsiella</i>	<i>vozmediano</i>	MHNLS 17877	Venezuela: Estado Sucre: Península de Paria, Cerro Humo	EU663385	EU663025	EU663247
<i>Centrolene</i>	<i>daidalea</i>	MHUA 3271	Colombia: Dep. Cesar: Municipio González: Vereda San Cayetano	EU663366	EU663007	EU663192
<i>Chimerella</i>	<i>mariaelenae</i>	QCAZ 31729	Ecuador: Prov. Tungurahua: Stream on the Río Negro–Río Verde road	EU663350	EU662991	EU663179
<i>Cochranella</i>	<i>granulosa</i>	USNM 559082	Honduras: Dep. Gracias a Dios: Rus Rus	EU663370	EU663010	EU663195
<i>Espadarana</i>	<i>andina</i>	JMG 366	Venezuela: Estado Mérida: Quebrada Azul, on the road between La Azulita and El Hato	EU663335	EU662976	EU663166
<i>Hyalionobatrachium</i>	<i>tatayoi</i>	MHNLS 17174 / MHNLS 17183	Venezuela: Estado Zulia: stream near Tokuko	EU663419	GQ142047	EU663234
<i>Ikagogi</i>	<i>tayrona</i>	MAR 544	Colombia: Dep. Magdalena, Sierra Nevada de Santa Marta: road to San Lorenzo	EU663356	EU662997	EU663183
<i>Nymphargus</i>	<i>bejaranoi</i>	CBG 1488	Bolivia: Dep. Cochabamba: Chaquisacha	EU663422	EU663059	EU663239
<i>Rulyrana</i>	<i>flavopunctata</i>	QCAZ 32365	Ecuador: Prov. Morona Santiago:7.6	EU663368	EU663009	EU663194

Genus	Species	Voucher	Locality	Genes		
				12S (~980bp)	16S (884bp)	POMC (604bp)
<i>Sachatamia</i>	<i>ilex</i>	UCR 16861	W of 9 de Octubre, on the 9 de Octubre – Guamate road Costa Rica: Prov. de Limón: Finca	EU663347	EU662988	EU663176
<i>Teratohyla</i>	<i>spinosa</i>	USNM 538863	Honduras: Dep. Olancho: Quebrada El Guasimo	EU663383	EU663023	EU663208
<i>Vitreorana</i>	<i>antisthenesi</i>	MHNLS 17050 / MHNLS 17909	Venezuela: Estado Aragua: Parque Nacional Henri Pittier, Estación Biológica Rancho Grande	EU663390	EU447287	EU663213
<i>Vitreorana</i>	<i>baliomma</i>	MCP 14114 (163)	Brazil: BA: Parque Estadual Serra do Conduru, Uruçuca	-	576bp	-
<i>Vitreorana</i>	<i>baliomma</i>	MCP 14120 (1069)	Brazil: BA: RPPN Ararauna, Una	-	548bp	-
<i>Vitreorana</i>	<i>baliomma</i>	MCP 14121 (1070)	Brazil: BA: RPPN Ararauna, Una	-	576bp	-
<i>Vitreorana</i>	<i>baliomma</i>	MCP 14122 (1071)	Brazil: BA: RPPN Ararauna, Una	562bp	576bp	492bp
<i>Vitreorana</i>	<i>baliomma</i>	MCP 14123 (1072)	Brazil: BA: RPPN Ararauna, Una	-	576bp	-
<i>Vitreorana</i>	<i>castroviejoi</i>	MHNLS 16446	Venezuela: Estado Sucre: Península de Paría, 2.5 km W and 3.2 km N of Macuro	EU663363	EU663004	EU663189
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 10533	Brazil: SP: PESM Núcleo Curucutú, Itanhaém	-	KU495611	MH987838
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 15374	Brazil: ES: Reserva Augusto Ruschii, Nova Lombardia	-	KU495606	MH987862
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 2671	Santa Teresa Brazil: MG: Itatiaia, Itamonte (Brejo da Lapa)	-	KU495608	MH987811

Genus	Species	Voucher	Locality	Genes		
				12S (~980bp)	16S (884bp)	POMC (604bp)
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 343	Brazil: MG: Itatiaia, Itamonte (Brejo da Lapa)	-	KU495609	MH987792
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 369	Brazil: MG: Morro do Ferro, Poços de Caldas	-	KU495610	MH987793
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 5121	Brazil: SP: Campos do Jordão	-	KU495607	MH987817
<i>Vitreorana</i>	<i>eurygnatha</i>	CFBH 5729	Brazil: MG: Itamontes	EU663404	EU663043	-
<i>Vitreorana</i>	<i>eurygnatha</i>	MCP 14115 (382)	Brazil: BA: Macarani	562bp	576bp	486bp
<i>Vitreorana</i>	<i>franciscana</i>	MZUFV 9970	Brazil: MG: PARNA Serra da Canastra	-	-	KR921744
<i>Vitreorana</i>	<i>franciscana</i>	MZUFV 10046	Brazil: MG: PARNA Serra da Canastra	-	-	KR921743
<i>Vitreorana</i>	<i>gorzulae</i>	KU 181128 / MHNLS 16036 / MHNLS 17142	Venezuela: Estado Bolívar: km 127 on the El Dorado-Santa Elena de Uairén road / Venezuela: Estado Bolívar: Parque Nacional Canaima, Cuenca alta del río Cucurital, Atapare	EU663348	EU266749	EU663173
<i>Vitreorana</i>	<i>helenae</i>	MHNLS 17127 / MHNLS 17128	Venezuela: Estado Bolívar: San Ignacio de Yurani, Quebrada de Jaspe	EU663371	EU266753	EU663196
<i>Vitreorana</i>	<i>ritae</i>	MB 165 / MB 292	French Guiana: Terrain Comté / French Guiana: Cayenne: Aya, Trinit	EU663377	EU266755	EU663202
<i>Vitreorana</i>	<i>uranoscopa</i>	CFBH 11709	Brazil: SC: Barragem do Rio São Bento, Siderópolis	-	KU495615	MH987849
<i>Vitreorana</i>	<i>uranoscopa</i>	CFBH 12320 / MAR 180	Brazil: SC: Pequena Central Hidrelétrica de Arvoredo, Arvoredo Xaxim	KF639775	KF639775	MH987851

Genus	Species	Voucher	Locality	Genes		
				12S (~980bp)	16S (884bp)	POMC (604bp)
<i>Vitreorana</i>	<i>uranoscopa</i>	CFBH 1257	Brazil: SP: PESH Santa Virgínia, São Luis do Paraitinga	KY202834	-	MH987801
<i>Vitreorana</i>	<i>uranoscopa</i>	CFBH 13098	Brazil: RJ: Parque Nacional da Tijuca	-	KU495612	MH987855

849 **Tables**

850 **Table 1** Measurements of microhabitat used by adults and oviposition sites of *Vitreorana baliomma* in two streams in the Atlantic Forest, southern
851 Bahia, Brazil

	Height (cm)	Horizontal distance (cm)	Leaf surface		Leaf area (cm ²)	Stream side			Position at stream			Canopy openness (%)
			Adaxial (%)	Abaxial (%)		Left (%)	Right (%)	Center (%)	Streambed (%)	Bank (%)	Outside bank (%)	
Adults (N=24)	142 ± 43.5 (60.7–231.4)	12.3 ± 21 (0–72)	100	0	121 ± 76.2 (11.4–266.2)	95	5	0	62	33	5	10.5 ± 3.11 (3.72–15.24)*
Clutches (N=19)	79 ± 52 (6–219)	3.2 ± 12.9 (0–58)	79	21	236 ± 350.5 (11.3–1376.2)	74	21	5	79	21	0	13.75 ± 8.13 (6.58–37.89)**

852 * 20 adults measured

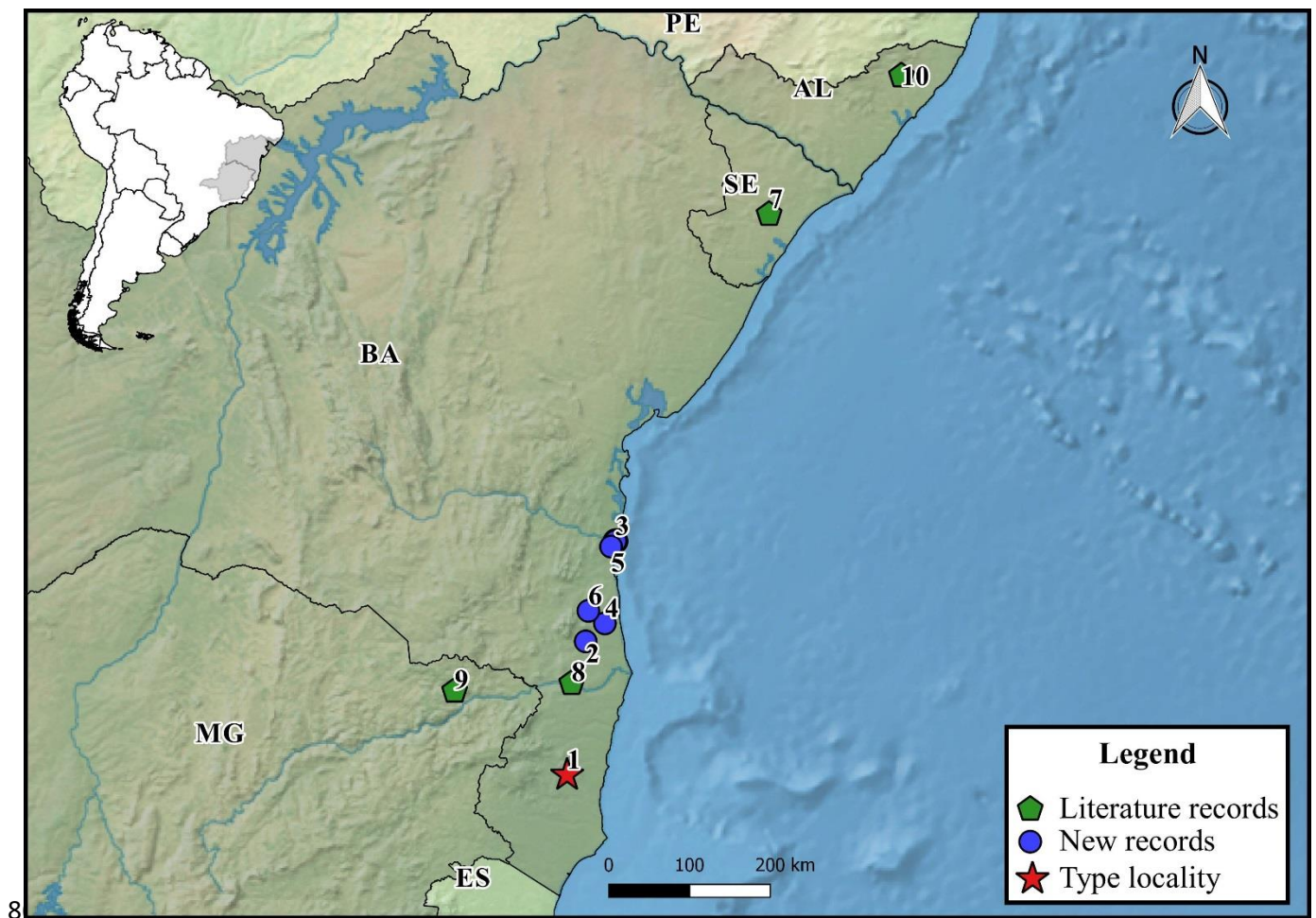
853 ** 12 clutches measured

854

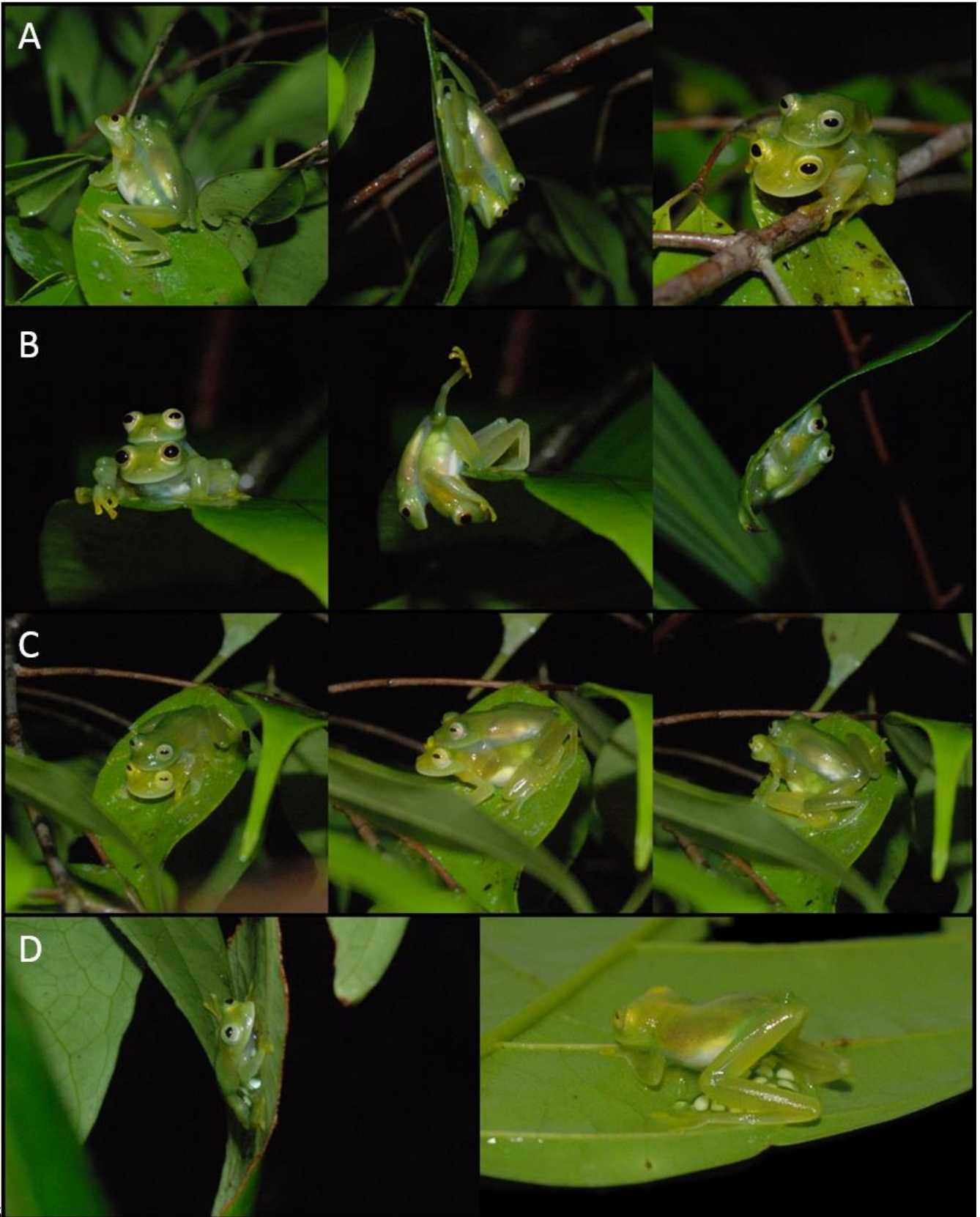
855 **Table 2** Parameter values of advertisement call for *Vitreorana* species. Variation values are determined in parentheses. n represents the number of
 856 calls analyzed for each species

Parameters	<i>V. eurygnatha</i> (n=12)	<i>V. uranoscopa</i> (n=34)	<i>V. franciscana</i> (n=17)	<i>V. ritae</i> (n=6)	<i>V. castroviejoi</i> (n=11)	<i>V. helenae</i> (n=4)	<i>V. gorzulae</i> (n=2)	<i>V. anthistenesi</i> (n=4)	<i>V. baliomma</i> ^A (n=65)	<i>V. baliomma</i> ^B (n=9)
Call duration (s)	0.131 ± 0.035 (0.099–0.186)	0.023 ± 0.005 (0.014–0.034)	0.049 ± 0.006 (0.029–0.062)	0.345 ± 0.105 (0.282–0.579)	0.018 ± 0.004 (0.012–0.029)	0.047 ± 0.021 (0.016–0.071)	0.022 ± 0.003 (0.019–0.026)	0.031 ± 0.003 (0.028–0.035)	0.211 ± 0.042 (0.101–0.313)	0.585 ± 0.055 (0.488–0.661)
Dominant frequency (Hz)	4441.4 ± 191.5 (4218.8–4875)	4657.5 ± 163.5 (4435.8–4909.6)	4264.1 ± 59.8 (3962.1–4565)	4732.9 ± 158.9 (4406.2–4875)	5254.1 ± 143.4 (4952.6–5469.4)	4586.6 ± 37.3 (4522–4608.1)	4888.5 ± 150.7 (4737.3–5038.8)	5469.4 ± 43 (5426.4–5512.5)	4788 ± 65.3 (4500–5156.2)	4713.5 ± 96.6 (4593.8–4921.9)
Maximum frequency (Hz)	4749.8 ± 207.2 (4512.1–5266.7)	4961.3 ± 138.2 (4702.2–5177.4)	4583.3 ± 111.6 (4412.3–4856)	5156.7 ± 113.9 (4914.6–5276.2)	5557.1 ± 145.7 (5322.9–5821.5)	4989.1 ± 26.3 (4964–5029.6)	5169.3 ± 121.4 (5047.9–5290.8)	5785 ± 27.7 (5756.2–5819.7)	5148.4 ± 180 (4766.1–5601.1)	5069.6 ± 104.5 (4919.5–5268.5)
Minimum frequency (Hz)	4084.3 ± 243.3 (3714.5–4553)	4375.4 ± 161.7 (4094.6–4579.7)	3973.8 ± 171.6 (3653–4299.8)	4154 ± 259.1 (3867–4582.5)	4935.6 ± 126.7 (4613.3–5055.3)	4186.4 ± 78.5 (4120.5–4316.5)	4527.9 ± 196.6 (4331.3–4724.6)	5155.8 ± 3.4 (5151–5160.4)	4443.5 ± 156.6 (4123.2–4800.6)	4331.6 ± 114.2 (4220.3–4609.7)
Bandwidth (Hz)	665.5 ± 86 (572.2–871.3)	586.1 ± 61.8 (509.9–772)	609.5 ± 84.8 (453.4–803.8)	1002.8 ± 256.8 (597.5–1409.2)	621.6 ± 104.8 (498.2–815.4)	802.7 ± 97.7 (651.1–900.9)	641.4 ± 75.2 (566.2–716.6)	629.2 ± 28.5 (601.5–668.7)	704.9 ± 90.5 (551.5–994)	738 ± 56.9 (658.8–833.2)
N° of pulses	3.3 ± 0.6 (2–4)	2.9 ± 0.7 (2–4)	5.9 ± 1 (3–8)	26.3 ± 8.2 (18–44)	2.9 ± 0.9 (2–5)	7 ± 2.9 (3–11)	3.5 ± 0.5 (3–4)	4.5 ± 0.5 (4–5)	6.6 ± 1.1 (4–9)	13 ± 1.7 (10–15)
Pulse duration (s)	-	-	-	-	-	-	-	-	0.010 ± 0.008 (0.003–0.017)	0.007 ± 0.002 (0.002–0.014)
Inter-pulse interval (s)	-	-	-	-	-	-	-	-	0.026 ± 0.006 (0.004–0.056)	0.025 ± 0.005 (0.009–0.052)

857 A - Call "A"
 858 B - Call "B"

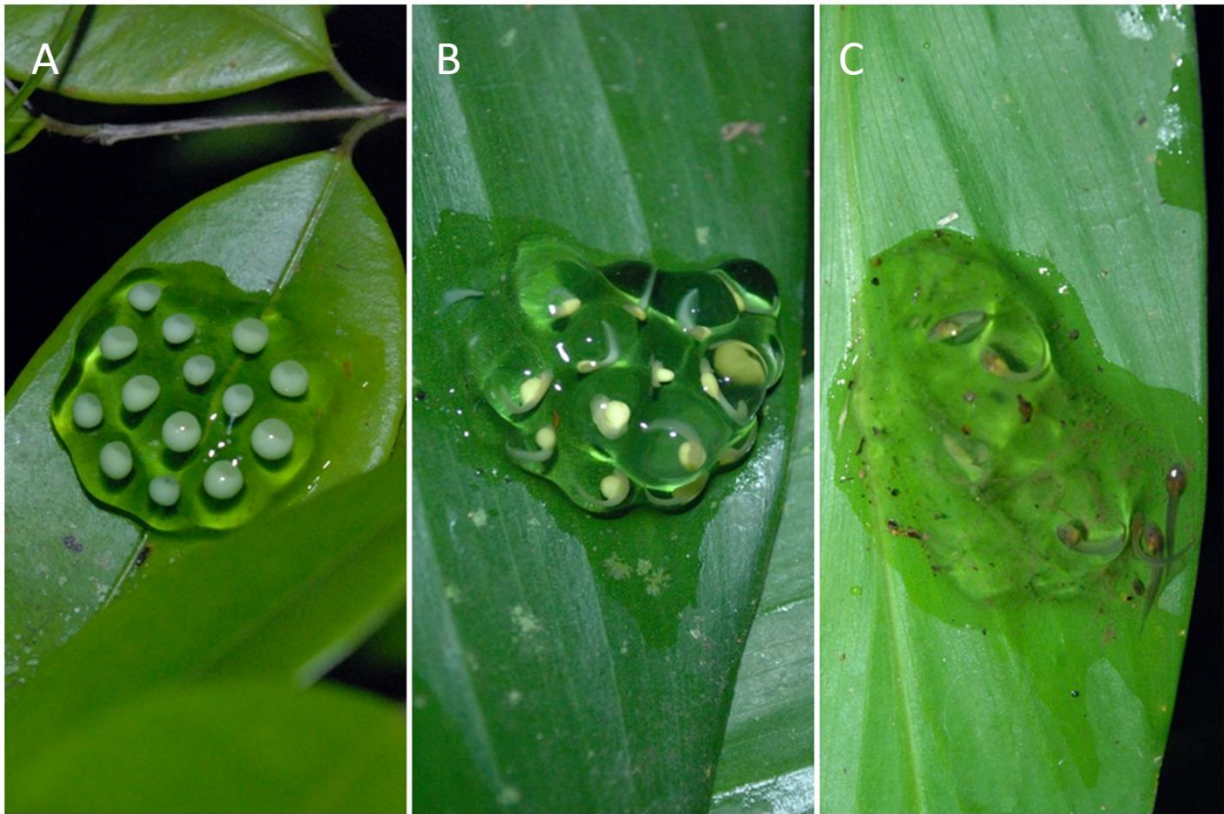


861 **Figure 1** Geographic distribution map of *Vitreorana baliomma*. 1 – Fazenda Novo Pau Brasil,
 862 municipality of Itamaraju; 2 – Fazenda Santo Antônio, municipality of Mascote; 3 – BA001
 863 (Ilhéus-Itacaré road), municipality of Uruçuca; 4 – RPPN Ararauna, municipality of Una; 5 –
 864 Parque Estadual Serra do Conduru, municipality of Uruçuca; 6 – PARNA Serra das Lontras,
 865 municipality of Arataca; 7 – PARNA Serra de Itabaiana, municipality of Areia Branca; 8 –
 866 Fazenda Palmeiras, municipality of Itapebi; 9 – Fazenda Limoeiro, municipality of Almenara;
 867 10 – Murici Ecological Station, municipality of Murici



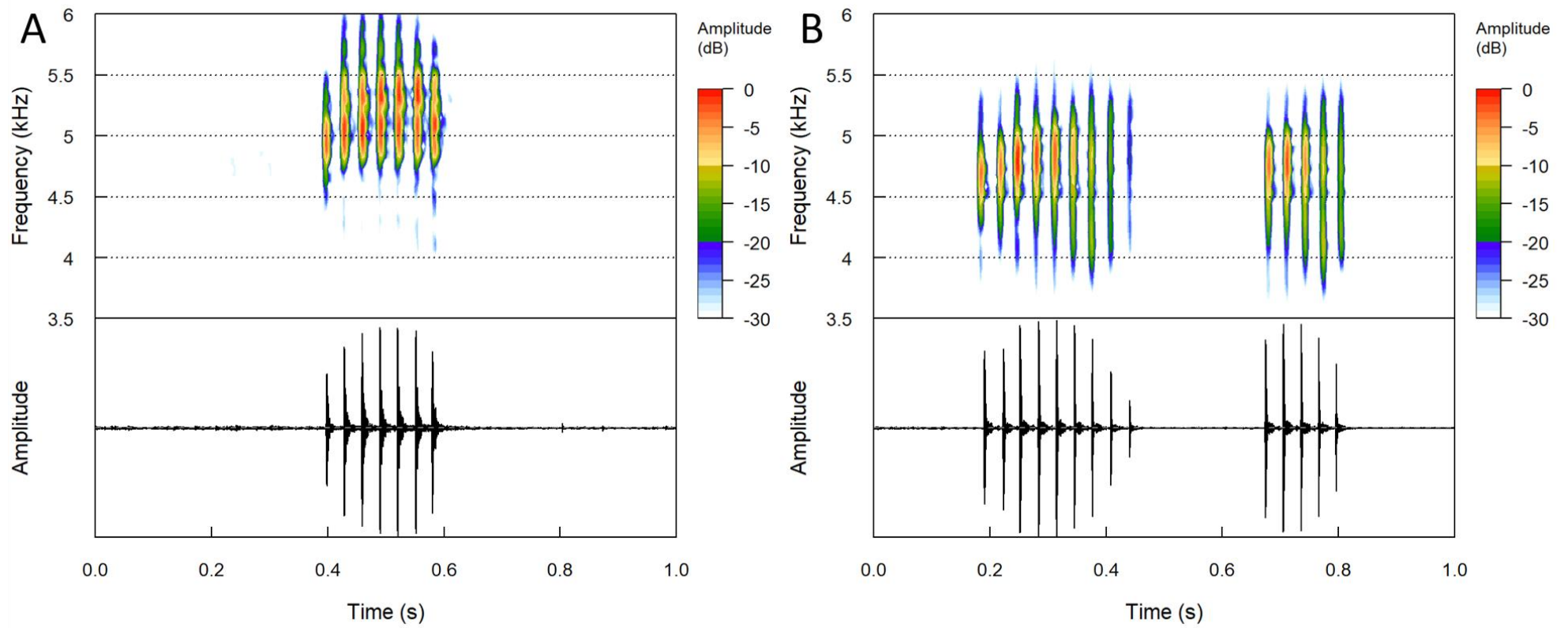
86

869 **Figure 2** Reproductive behavior in *Vitreorana baliomma*. While in amplexus, pairs move along
 870 leaves and branches (A), change leaf surface (B), and perform 360° and 180° rotations in leaves
 871 (C). After oviposition, females brood the egg clutch, elevating the posterior part of her body
 872 (D). Photographs are from multiple pairs



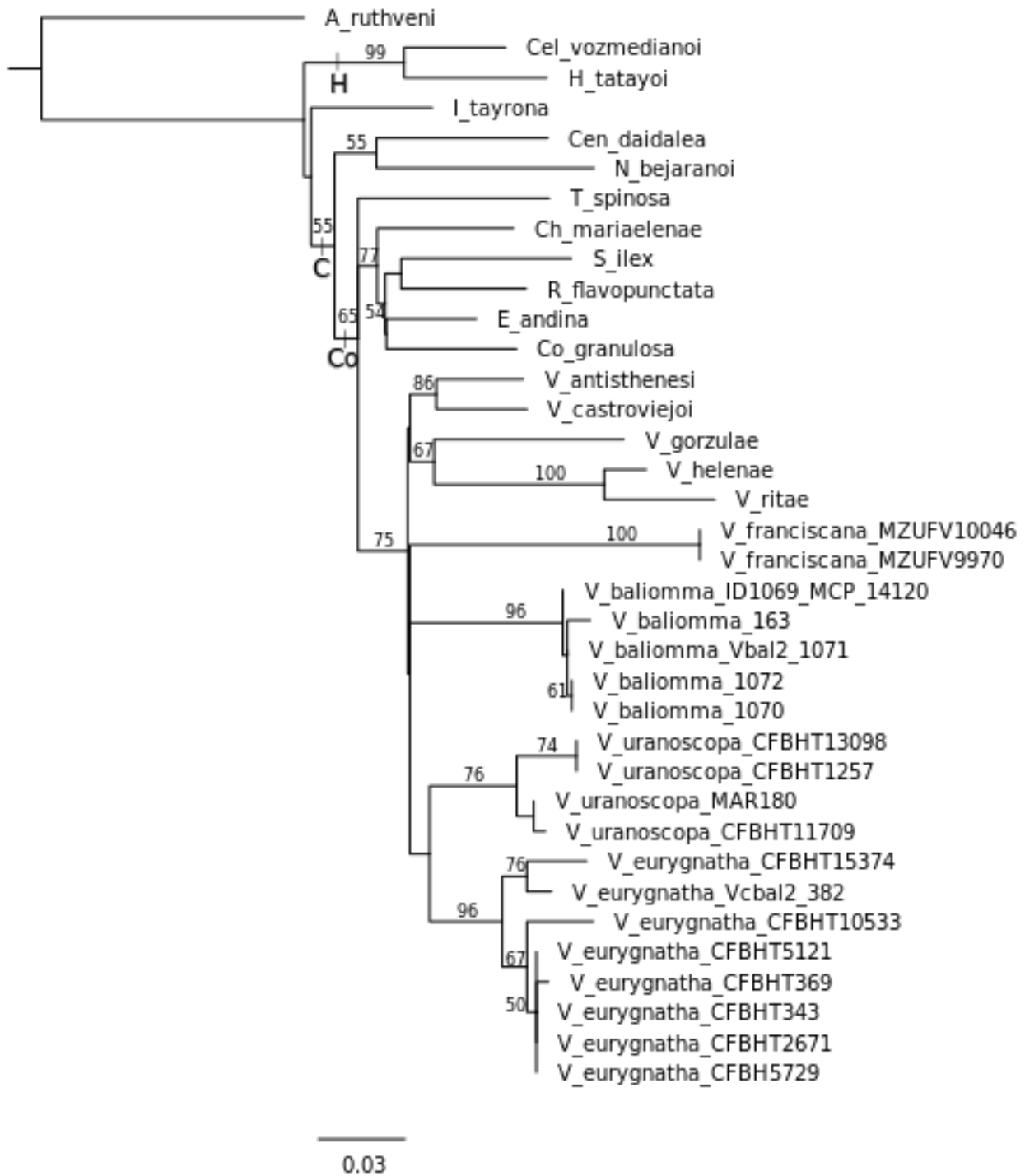
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874 **Figure 3** Egg clutches of *Vitreorana baliomma* in early (A), intermediate (B) and late (C) stages
875 of development



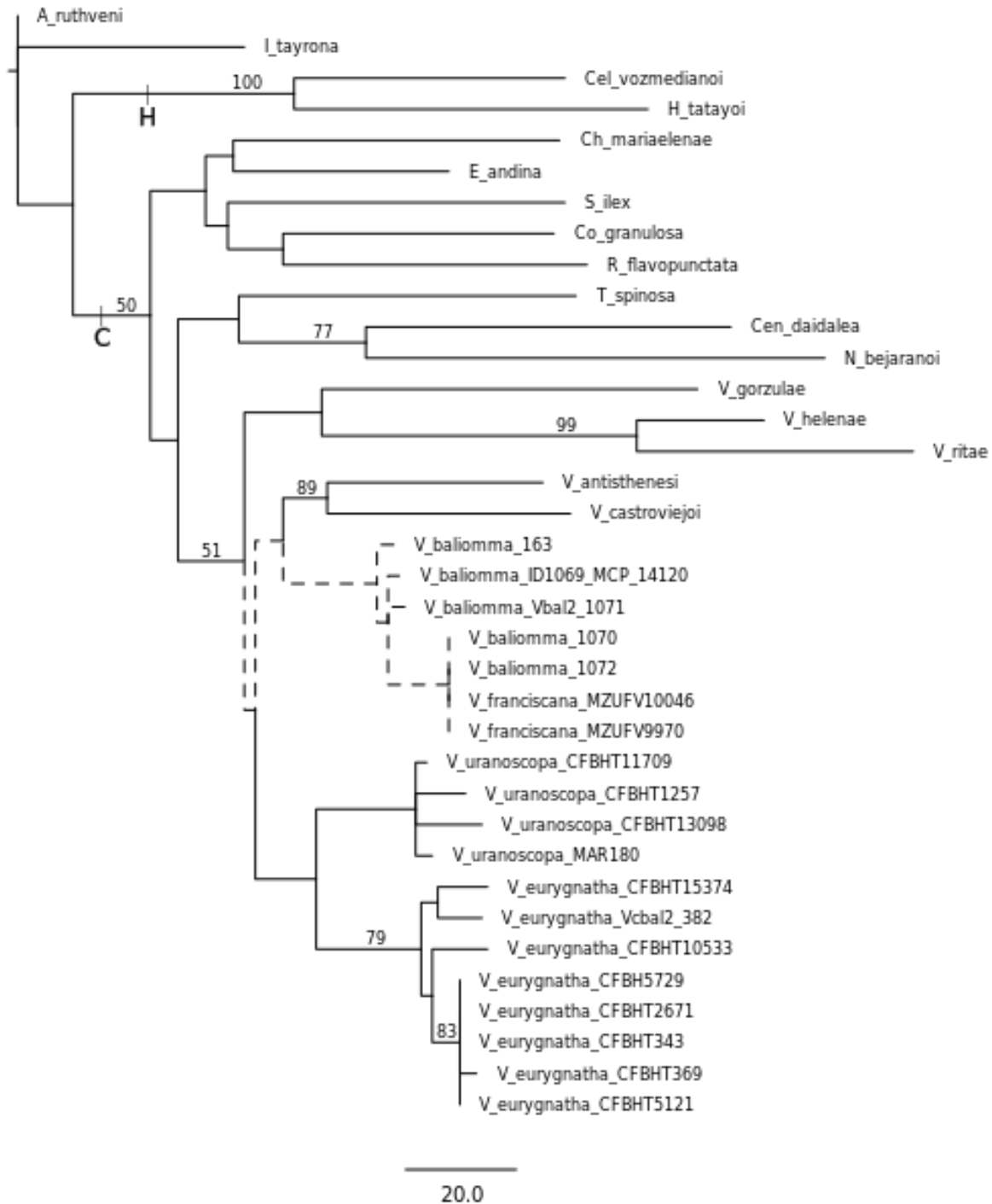
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877 **Figure 4** Audiospectrogram (above) and oscillogram (below) from call “A” and “B” of *Vitreorana baliomma*



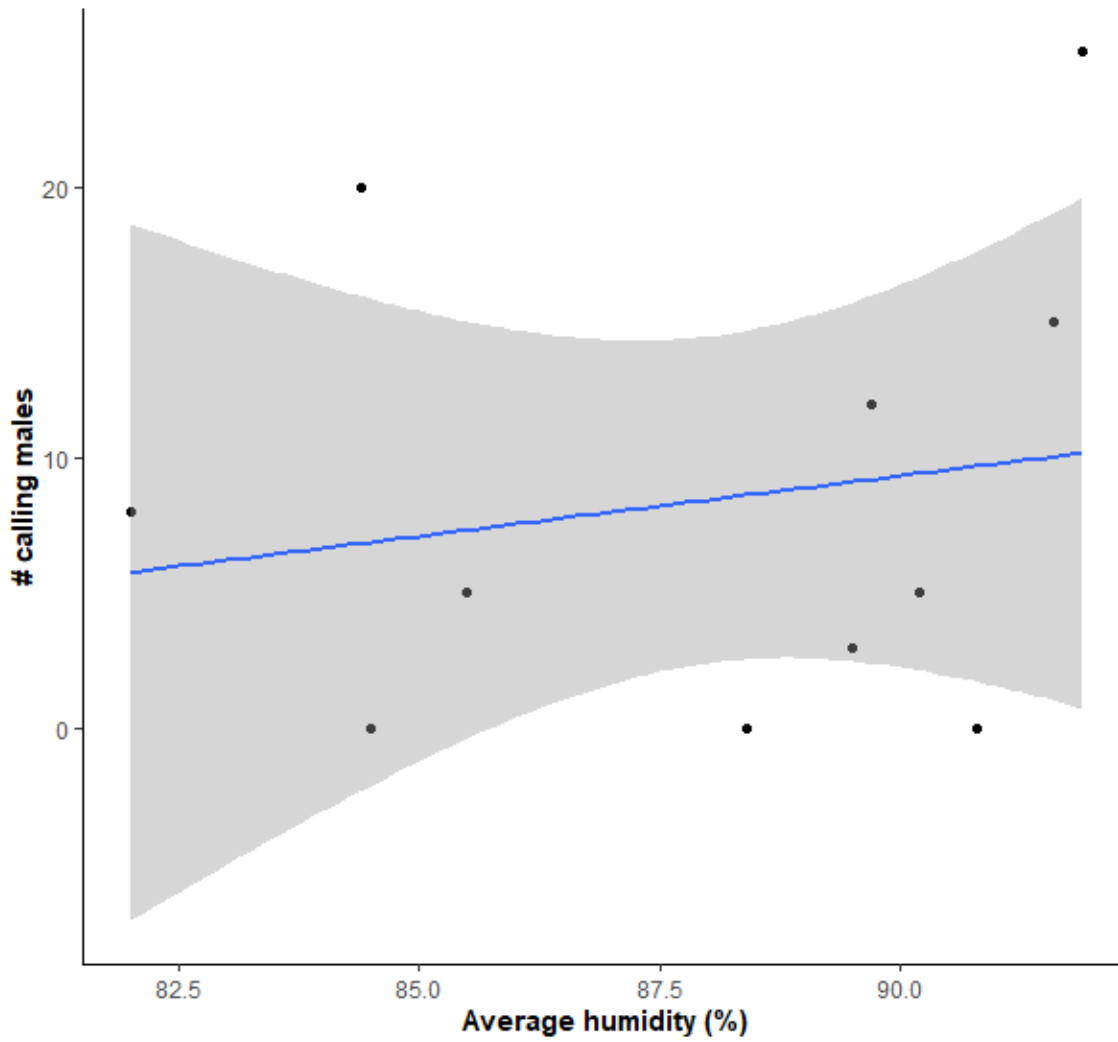
878

879 **Figure 5** Maximum Likelihood tree inferred by Garli based on concatenated 12S, 16S and
 880 POMC sequences ($L_n = -11423.87158$). Values above branches represent percentages ($\geq 50\%$)
 881 of 1000 bootstrap pseudoreplicates. Scale bar indicates expected substitutions per site. The
 882 subfamilies Hyalionobatrachinae (H) and Centroleninae (C), and the tribe Cochranellini (Co)
 883 are indicated



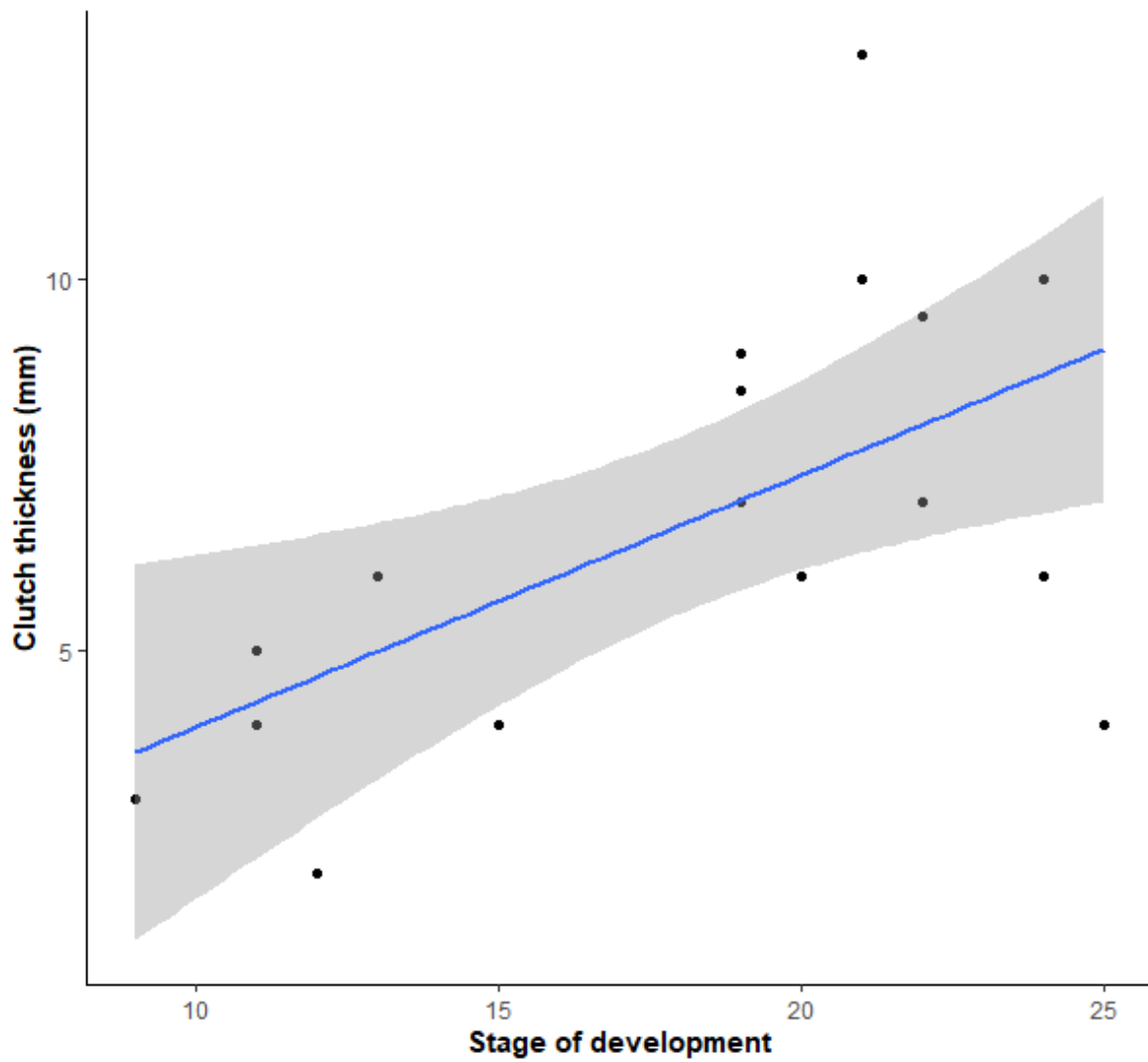
884

885 **Figure 6** One of the 54 most parsimonious trees (steps = 1894) resulting from parsimony
 886 analysis on TNT of concatenated 12S, 16S and POMC sequences. Collapsed clades in the strict
 887 consensus are indicated by dashed lines. Values above branches represents percentages (≥ 50
 888 %) of 1000 jackknife replicates. Scale bar indicates number of transformations. The subfamilies
 889 Hyalionobatrachinae (H) and Centroleninae (C) are indicated



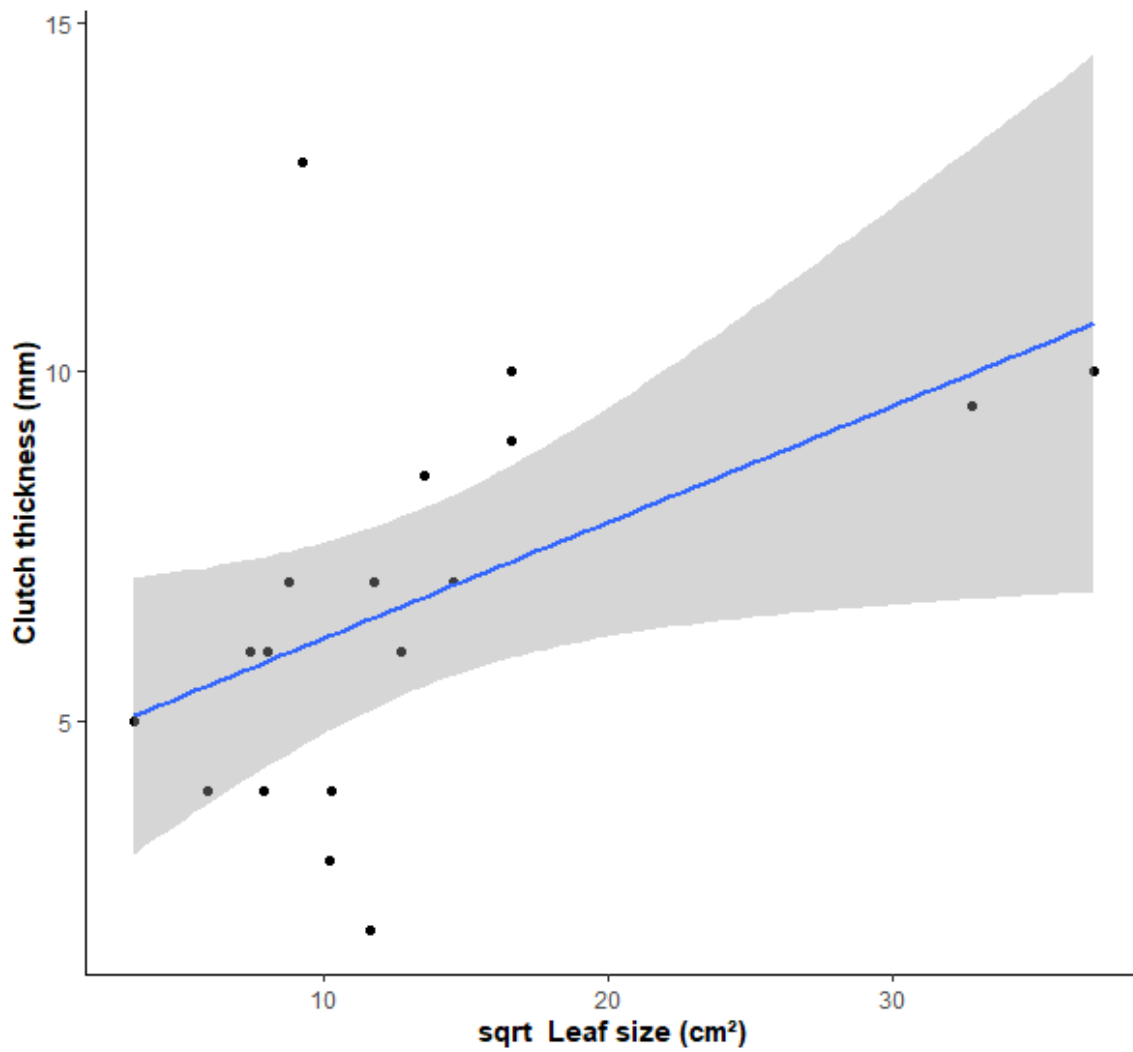
890

891 **Figure 2** Correlation between number of calling males of *Vitreorana baliomma* and air
 892 humidity. Gray area represents 95 % confidence interval



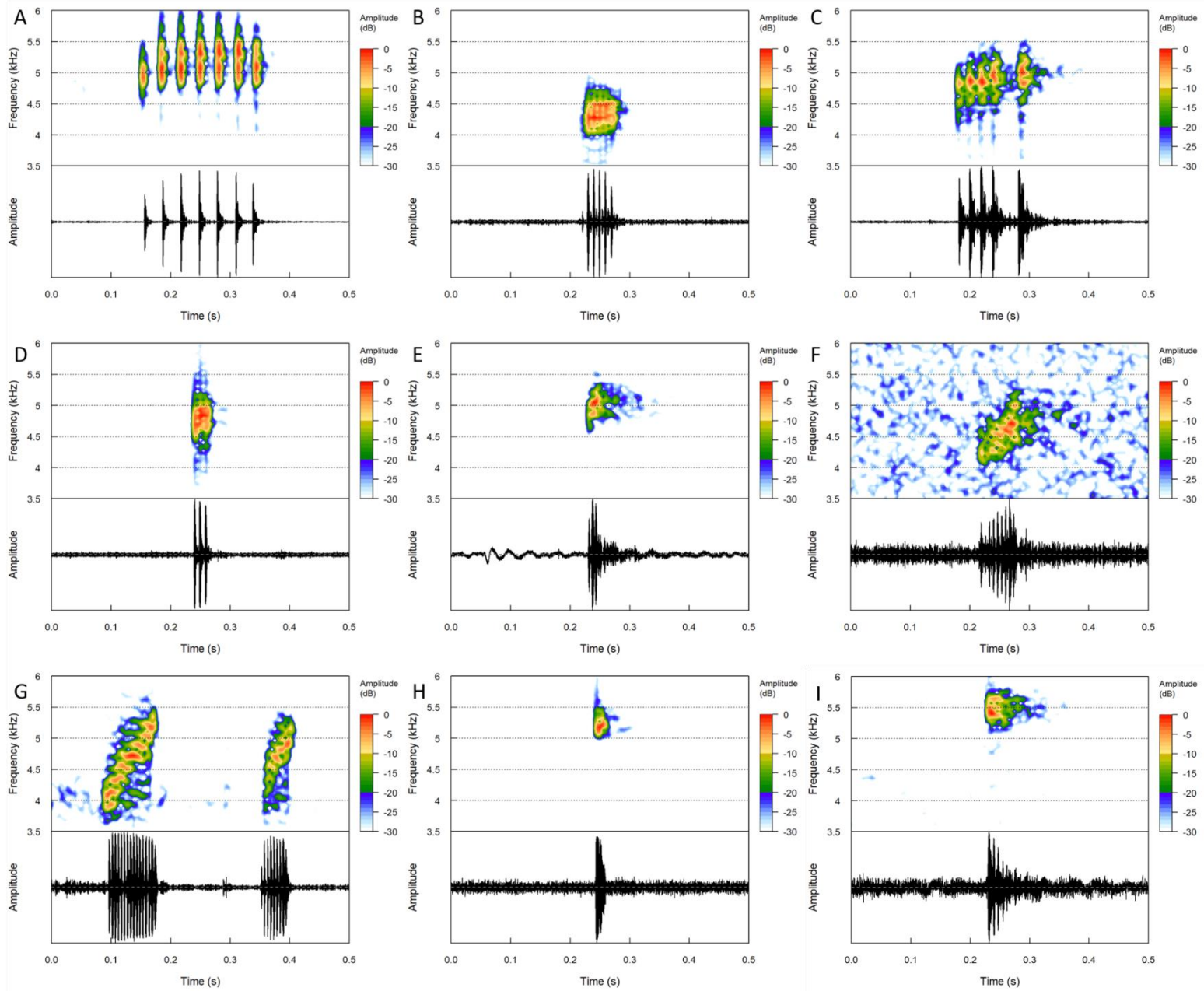
893

894 **Figure 3** Correlation between *Vitreorana baliomma* embryo developmental stage and clutch
 895 thickness. Gray area represents 95 % confidence interval



896

897 **Figure 4** Correlation between clutch thickness of *Vitreorana baliomma* and leaf size. Gray area
 898 represents 95 % confidence interval



900 **Figure 5** Audiospectrogram (above) and oscillogram (below) of advertisement calls of
 901 *Vitreorana baliomma* (A), *V. franciscana* (B), *V. eurygnatha* (C), *V. uranoscopa* (D), *V.*
 902 *gorzulae* (E), *V. helenae* (F), *V. ritae* (G), *V. castroviejoi* (H), and *V. antisthenesi* (I)



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