



## The rediscovery of *Hylodes regius*: new information about a rare and microendemic Atlantic rainforest Torrent frog

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Manuscript received: 18 February 2020

Accepted: 4 July 2020 by EDGAR LEHR

**Abstract.** The genus *Hylodes* (Torrent frogs) is endemic to the montane Atlantic forests of Brazil, comprising species that are mostly locally distributed and face severe threats to their continued existence. *Hylodes regius* was described in 1979 from the mist forests on the highlands of the Parque Nacional do Itatiaia in southeastern Brazil, and was then not found for 35 years before we recently discovered what is presently the only known population of this species. We document this find and provide fundamental information that has been lacking for this species, including data on its morphology, male coloration in life, descriptions of its advertisement and territorial calls, a description of the tadpole, natural history notes, and its currently known geographic distribution limits. We provide a phylogeny based on molecular data for the species of the *Hylodes lateristrigatus* species group, of which *H. regius* forms part. Additional studies and continuous monitoring coupled with conservation measures are essential for ensuring the persistence of the distinct species of *Hylodes* in the Parque Nacional do Itatiaia, in particular because we have indirectly detected the presence of the fungus *Batrachochytrium dendrobatidis*.

**Key words.** Amphibia, Anura, *Bd*, Brazilian Torrent frog, disappeared species, Hylodidae, Mantiqueira mountain range, polymorphism, tadpole, vocalisation.

### Introduction

*Hylodes* is a rheophilic Neotropical frog genus currently comprising 26 species. Also known as Torrent frogs, these inhabit fast-flowing streams in the montane Atlantic forest, ranging from the state of Espírito Santo to that of Rio Grande do Sul in eastern Brazil (SAZIMA & BOKERMANN 1982, DE SÁ et al. 2015, MALAGOLI et al. 2017). Knowledge of the actual distribution ranges of, and population threats to, *Hylodes* spp. is still sketchy, but around 58% of them (15 species) are endemic to small forested areas, which renders them vulnerable to ecological and anthropogenic pressures like disease outbreaks and habitat degradation. *Hylodes* spp. today face a variety of serious threats, which are not documented in their entirety, however. In general, searching for populations and species that have, or seem to have, disappeared improves our understanding of threats and helps with the mitigation of amphibian declines (BOLAÑOS et al. 2008, GONZÁLEZ-MAYA et al. 2013).

The highlands of the montane mist forests of the Parque Nacional do Itatiaia (PARNA Itatiaia; located within the Mantiqueira mountain range) are home to three endemic *Hylodes* spp. with restricted distributions: *Hylodes gla-*

*ber*, *H. ornatus*, and *H. regius*. For decades, *H. ornatus* was thought of as missing from the park until a small breeding population was discovered recently, whereas *H. glaber* is still missing (BILATE et al. 2012, DE SÁ et al. 2015; C. F. B. HADDAD pers. obs.). For its part, *Hylodes regius* was known only from its type-locality at between 1,850 and 2,100 m above sea level (a.s.l.) in the park, in the municipality of Itamonte, state of Minas Gerais, at the state boundary with Rio de Janeiro, southeastern Brazil (GOUVÊA 1979). The original description of *H. regius* is brief with some black-and-white figures, only characterising the general morphology of seven adult males and describing their colour pattern, but not even providing an etymology (GOUVÊA 1979). The specific epithet, *regius*, is Latin for “king” or “royal” and likely makes reference to the colourful pattern of *H. regius*, which sets it clearly apart from the typically brownish colours observed in its congeners and consists of golden yellow blotches and vivid purple-red sections (HADDAD et al. 2013), colours that are commonly associated with royalty. Except for the specimens of *H. regius* collected in 1977 and 1978, and described in 1979, there are no other records of this species’ populations in the PARNA Itatiaia. Therefore, the current geographic distribution, conservation status,

molecular data, and most taxonomic and phylogenetic relationships, details of its unusual colour pattern, advertisement and territorial calls, tadpoles, and natural history are still unknown for *H. regius*. Even regular visits to the type locality and surrounding areas in the park from 2005 onwards failed to produce fresh records of *H. regius* (BILATE et al. 2012). Being the first Brazilian national park, PARNA Itatiaia has been a fully protected area since 1937 and, thus, the apparent absence of any *Hylodes* species in its well-preserved forests, and that of another two frog species, *Holoaden bradei* and *Paratelmatobius lutzii* (HADDAD et al. 2016, MARTINELLI & TOLEDO 2016), is perplexing.

Motivated to gain a deeper understanding of the diversity and the contemporary disappearance of Brazilian Torrent frogs, we continuously and regularly visited the PARNA Itatiaia until we were fortunate enough to eventually find and collect some calling males of *H. regius* in 2012 and 2013, and a single tadpole at its type locality in 2016. As important as documenting this rediscovery itself is that we now have an opportunity to fill various gaps relevant to the taxonomy, evolution, and conservation that have been persisting for the species thus far. We therefore here provide morphological information, redescribe the colour pattern of males in life, describe their advertisement and territorial calls, the tadpole, provide natural history notes, and redraw the currently known geographic distribution of this species. We furthermore provide a phylogeny based on molecular data for the species of the *Hylodes lateristrigatus* species group of which *H. regius* forms part (HEYER 1982). Furthermore, we outline that additional studies and continuous monitoring coupled with conservation measures are necessary for ensuring the persistence of the distinct species of *Hylodes* in the PARNA Itatiaia.

## Material and methods

### Field sampling

We carried out five expeditions to the PARNA Itatiaia, all during the rainy seasons. The first expedition took place between 1 and 9 November 2011, the second between 7 and 10 February 2012, the third between 26 and 28 February 2012, the fourth between 30 January and 2 February 2013, and the fifth between 15 and 18 February 2016, totalling 24 days in the field. We collected adult male specimens by hand. For tadpoles, we tried different methods, including visual search and underwater traps with baits, but only dip netting and examining the sandy bottom of the stream facilitated our detecting and collecting one individual. Adults and the tadpole were euthanised with an overdose of anaesthetic (5% lidocaine). Immediately afterwards, we took tissue samples from the thigh muscles of the males and from the tail muscle of the tadpole, and preserved them in 100% ethyl alcohol. We then fixed adult males in 10% formalin and preserved them in 70% ethyl alcohol. The tadpole was fixed and preserved in 5% formalin. Samples collected for morphological and molecular analyses followed protocols approved by the ethics committee on animal use of the Uni-

versidade Estadual Paulista (UNESP), Rio Claro, São Paulo, Brazil. Voucher specimens and tissue samples were deposited in the Célio F. B. Haddad collection (CFBH) in the Departamento de Biodiversidade, Instituto de Biociências of UNESP, Rio Claro, São Paulo, Brazil.

### Molecular data and phylogenetic analysis

We conducted a molecular analysis to properly place *Hylodes regius* among its congeners in the *Hylodes lateristrigatus* species group (HEYER 1982). We used sequences generated by DE SÁ et al. (2015) and MALAGOLI et al. (2017), complementing them with new genetic data obtained in the present study (Table 1). All tissue samples are now stored in the tissue collections of the Museu Nacional (MNRJ), Departamento de Vertebrados of the Universidade Federal do Rio de Janeiro (UFRJ), and CFBH. We have focused here on *Hylodes* spp. that are associated with the Mantiqueira mountain range (*H. amnicola*, *H. japi*, *H. ornatus*, *H. perere*, *H. regius*, and *H. sazimai*) and with other mountain formations (*H. caete*, *H. fredii*, *H. phyllodes*, and *H. pipilans*). For *H. regius*, we included genetic data from all four individuals we collected, i.e., two adult males representing a darker colour morph, one adult male with a lighter complexion, and the tadpole. For each of the other *Hylodes* spp., we included genetic data from two individuals, except for *H. ornatus* and *H. fredii* for which we had only one sample available (DE SÁ et al. 2015, MALAGOLI et al. 2017). Based on GRANT et al. (2006) and PYRON & WIENS (2011), we also included in our molecular analysis *Hylodes nasus* (from the *Hylodes nasus* species group; HEYER 1982), *Crossodactylus caramaschii* and *Megaelosia boticariana* (as representatives of all other genera of Hylodidae), and *Cycloramphus boraceiensis* (Cycloramphidae) to root the trees. For all species of the *Hylodes lateristrigatus* and *H. nasus* species groups, we included samples of topotypes, except for an individual each of *H. caete* (CFBH 28968), *H. perere* (CFBH 22831), and *H. phyllodes* (CFBHT 03131), collected ca. 50, 65 and 45 km away from their type localities, respectively. Among the topotypes, we added samples from the holotypes of *H. caete* (CFBH 40524), *H. fredii* (MNRJ 36077), and *H. japi* (CFBH 33850), and samples from paratypes of *H. pipilans* (MNRJ 37307; MNRJ 39371).

We extracted total DNA from muscle tissues of the four new *Hylodes regius* individuals using a standard ammonium acetate precipitation protocol adopted from MANIATIS et al. (1982). We amplified a partial fragment of the mitochondrial 16S ribosomal RNA gene (16S, ~1450 base pairs) using the primers and protocols described in DE SÁ et al. (2015). The purified PCR fragments were sequenced by Macrogen Inc., Seoul, Republic of Korea, using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). We inspected and edited the chromatograms for quality using Geneious v11 (Biomatters, Auckland, New Zealand). New sequences and metadata generated in this study are deposited at GenBank (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA; Table 1).

Table 1. Specimens included in the genetic analysis to position the rare species *Hylodes regius* among its congeners in the *Hylodes lateristrigatus* species group (HEYER 1982). GenBank accession numbers and collection localities are also provided.

Species	Voucher number	Tissue number	GenBank	Type	Locality
<i>Hylodes regius</i>	CFBH 30970	CFBHT 15286	MN011544	Topotype	Parque Nacional do Itatiaia, Itamonte, MG
<i>Hylodes regius</i>	CFBH 34910	CFBHT 20218	MN011546	Topotype	Parque Nacional do Itatiaia, Itamonte, MG
<i>Hylodes regius</i>	CFBH 34911	CFBHT 20219	MN011547	Topotype	Parque Nacional do Itatiaia, Itamonte, MG
<i>Hylodes regius</i>	CFBH 40054	CFBHT 20156	MN011545	Topotype	Parque Nacional do Itatiaia, Itamonte, MG
<i>Hylodes amnicola</i>	CFBH 30971	CFBHT 15287	KJ961575	Topotype	Parque Estadual do Ibitipoca, Lima Duarte, MG
<i>Hylodes amnicola</i>	CFBH 30972	CFBHT 15290	KJ961576	Topotype	Parque Estadual do Ibitipoca, Lima Duarte, MG
<i>Hylodes japi</i>	CFBH 33850	CFBHT 16563	KJ961574	Holotype	Serra do Japi, Jundiá, SP
<i>Hylodes japi</i>	CFBH 25403	CFBHT 11971	KJ961571	Topotype	Serra do Japi, Jundiá, SP
<i>Hylodes ornatus</i>	CFBH 34905	CFBHT 18068	KJ961578	Topotype	Parque Nacional do Itatiaia, Itamonte, MG
<i>Hylodes perere</i>	CFBH 31106	CFBHT 15412	KJ961581	Topotype	Santa Bárbara do Monte Verde, MG
<i>Hylodes perere</i>	CFBH 22831	CFBHT 12651	KJ961580	–	Santa Rita do Jacutinga, MG
<i>Hylodes sazimai</i>	CFBH 29586	CFBHT 14632	KJ961584	Topotype	Serra das Cabras, Campinas, SP
<i>Hylodes sazimai</i>	–	CFBHT 14629	KJ961583	Topotype	Serra das Cabras, Campinas, SP
<i>Hylodes caete</i>	CFBH 40524	CFBHT 20491	KY627907	Holotype	Parque Estadual da Serra do Mar, Núcleo Curucutu, Itanhaém, SP
<i>Hylodes caete</i>	CFBH 28968	CFBHT 20490	KY627906	–	Parque Natural Municipal Nascentes de Paranapiacaba, Santo André, SP
<i>Hylodes fredii</i>	MNRJ 36077	MNRJ 36077	KY627913	Holotype	Parque Estadual de Ilha Grande, Angra dos Reis, RJ
<i>Hylodes phyllodes</i>	CFBH 39961	CFBHT 20497	KY627914	Topotype	Estação Biológica de Boracéia, Salesópolis, SP
<i>Hylodes phyllodes</i>	–	CFBHT 03131	KY627915	–	São Sebastião, SP
<i>Hylodes pipilans</i>	MNRJ 37307	MNRJ 37307	KJ961582	Paratopotype	Parque Nacional da Serra dos Órgãos, Teresópolis, RJ
<i>Hylodes pipilans</i>	MNRJ 39371	MNRJ 39371	KY627916	Paratopotype	Parque Nacional da Serra dos Órgãos, Teresópolis, RJ
<i>Hylodes nasus</i>	MNRJ 35435	MNRJ 35435	KJ961577	Topotype	Parque Nacional da Tijuca, RJ
<i>Megaalosia boticariana</i>	CFBH 06292	CFBHT 00425	KJ961586	–	Caçapava, SP
<i>Crossodactylus caramaschii</i>	CFBH 17647	CFBHT 06917	KJ961569	–	Itanhaém, SP
<i>Cycloramphus boraceiensis</i>	CFBH 15188	CFBHT 06336	KJ961570	–	Parque Estadual de Ilhabela, SP

To infer the phylogenetic relationships of *H. regius* relative to other species of the *H. lateristrigatus* species group, we aligned the new 16S sequences along those of other *Hylodes* sequences obtained from GenBank using MAFFT v7 (default parameters; KATO & STANDLEY 2013). We selected the best-fitting evolutionary model under the Akaike Information Criterion (AIC; AKAIKE 1974) as incorporated in jModelTest v2.1.5 (DARRIBA et al. 2012). The selected model for the 16S fragment was GTR+I+G. We then performed a Bayesian inference (BI) analysis in MrBayes v3.2.6 (RONQUIST & HUELSENBECK 2003) at the CIPRES Science Gateway (MILLER et al. 2010). We ran two independent analyses with four chains each, adopting the Markov Chain Monte Carlo approach (MCMC). We ran analyses for 10 million generations with sampling every 1,000 generations, discarding the first 25% of samples. We checked the convergence of parameters using Tracer v1.6 (RAMBAUT et al. 2014) and support of nodes was assessed using the posterior probability values in the consensus tree. We edited the tree using FigTree v1.4 (RAMBAUT 2014).

#### Morphology and behaviour of adults and tadpoles

We identified adult males by their calling activity in the field and by the presence of extended vocal sacs. In spite of their representing distinct colour morphs, we confirmed the three new adult males and the tadpole as all being individuals of *Hylodes regius* via genetic comparisons. We measured 12 standard metric traits for adult males, following HEYER et al. (1990) and DUELLMAN (2001): snout–vent length (SVL), head length (HL), head width (HW), eye diameter (ED), tympanum diameter (TD), eye–nostril distance (END), interorbital distance (IOD), internostril distance (IND), thigh length (THL), tibia length (TBL), tarsus length (TAL), and foot length (FL). Following the general format of tadpole description in the genus *Hylodes* (e.g., DE SÁ et al. 2015), we measured eight standard metric traits for the tadpole, following McDIARMID & ALTIG (1999) and ALTIG (2007): total length (TL), body length (BL), maximum body height (BH), maximum body width (BW), internostril distance (IND; between centres of the nostrils),

interorbital distance (IOD; between centres of the pupil), eye–nostril distance (END; between centre of the pupil and centre of the nostril), and eye diameter (ED). Tadpole staging follows GOSNER (1960), and tadpole tooth row formula follows ALTIG (1970, 2007). We measured the adult males and the tadpole with the aid of an ocular micrometer of a Zeiss stereomicroscope, except for SVL, HL, HW, THL, TBL, TAL, FL, TL, and BL, which we measured with callipers and rounded to the nearest 0.1 mm. Measurements are given as mean  $\pm$  standard deviation and range in parentheses. For the descriptions of male and tadpole behavioural expressions, we used focal animal and all-occurrence sampling methods (ALTMANN 1974, LEHNER 1998).

#### Advertisement and territorial calls

We recorded male calls with a Marantz digital recorder (PMD-660) with a Sennheiser external unidirectional microphone (ME-66), and measured air and water temperatures at the time of each recording. These recordings are deposited in the Fonoteca Neotropical Jacques Vielliard sound collection (FNJV) at the Instituto de Biociências of Universidade Estadual de Campinas (UNICAMP). We analysed 32 advertisement calls and 16 territorial calls recorded from the three collected males.

We analysed calls at a sampling frequency rate of 48 kHz and with 16-bit resolution in the mono pattern with Raven Pro v1.5 (Cornell Lab of Ornithology, Ithaca, NY, USA). We analysed seven call parameters, following DUELLMAN & TRUEB (1994) and KÖHLER et al. (2017): call duration (s), intercall duration (s), number of notes per call, note repetition rate (notes per s), note duration (s), internote duration (s), and dominant frequency (peak frequency of the call; in

kHz). Notes here are the smaller subunits interspaced in each call (KÖHLER et al. 2017).

## Results

### Rediscovering *Hylodes regius*

After continuous and regular visits to the type locality area of *Hylodes regius* and its surroundings, we found in 2012 a population of this species at fast-flowing streams from the mist forests on the highlands of the PARNA Itatiaia, southeastern Brazil, which was revisited in 2013 and 2016. We noted eight calling males and we collected three of them: CFBH 30970, collected on 8 February 2012 by FÁBIO P. DE SÁ, MARIANA L. LYRA and CÉLIO F. B. HADDAD, and CFBH 34910 and 34911, both collected on 1 February 2013 by FÁBIO P. DE SÁ and NADYA C. PUPIN. The three collected calling males were found to be associated with medium-sized streams (around 2 m in width) at a narrow altitudinal range in the montane mist forest area of the PARNA Itatiaia, municipality of Itamonte, state of Minas Gerais, southeastern Brazil (Fig. 1; 22.3654° S, 44.7450° W, 1,951 m a.s.l.; 22.3658° S, 44.7454° W, 1,957 m a.s.l.; and 22.3389° S, 44.7367° W, 1,957 m a.s.l., respectively). We also discovered one tadpole (CFBH 40054) at the same altitude as the adults, collected on 16 February 2016 by MARIANA L. LYRA and CÉLIO F. B. HADDAD (22.3305° S, 44.7357° W, 1,913 m a.s.l.).

### Phylogenetic relationships

The mitochondrial phylogenetic inference recovered all included species as independent evolutionary lineages (Fig. 2). Also, tree topology confirmed that all *H. regius*



Figure 1. Habitat of three endemic *Hylodes* spp. with restricted distribution ranges: *H. glaber*, *H. ornatus*, and *H. regius*. A) The highlands hosting the montane mist forest in the Parque Nacional do Itatiaia, southeastern Brazil; and B) a fast-flowing stream exemplifying the habitat where the tadpole and breeding adults of *H. regius* were found in our study (between 1,913 and 1,957 m a.s.l.).

individuals sampled (the darker and lighter individuals) belonged to the same taxonomic entity. The *Hylodes lateristrigatus* species group was represented in our study by *H. amnicola*, *H. caete*, *H. fredii*, *H. japi*, *H. ornatus*, *H. perere*, *H. phyllodes*, *H. pipilans*, *H. regius*, and *H. sazimai* (50% of the known species of this group). This taxonomic group was recovered as a monophyletic cluster with strong support (Fig. 2). Nested within this group, a clade composed by *H. amnicola*, *H. japi*, *H. ornatus*, *H. perere*, *H. regius*, and *H. sazimai*, was recovered also with strong support (Fig. 2).

#### Morphology of adult males and colour morphs

The external morphometric measurements of the three collected males of *Hylodes regius* are provided in Table 2. In life, they had light oblique lateral stripes (an attribute shared by the species of the *Hylodes lateristrigatus* species group) formed by bright golden yellow lines or serially aligned dots that may start off at the tip of the snout. The supralabial area was also patterned with bright golden yellow blotches. The ventral faces of the upper and lower arms, tibiae and thighs, as well as the concealed dor-

Table 2. Measurements (mm) of the sampled *Hylodes regius* males (n = 3). For abbreviations, see materials and methods.

Trait	CFBH 30970	CFBH 34910	CFBH 34911	Mean ± standard deviation
SVL	35.5	33.6	35	34.74 ± 0.98
HL	12.7	12.6	12.5	12.61 ± 0.12
HW	10.9	11.1	10.9	10.98 ± 0.13
ED	4.6	4.5	4.3	4.44 ± 0.15
TD	2.5	2.7	2.8	2.66 ± 0.19
END	2.6	2.5	2.9	2.65 ± 0.2
IOD	6.7	6.4	6.7	6.6 ± 0.19
IND	4.1	4.2	4.3	4.2 ± 0.1
THL	19	17.8	18.7	18.48 ± 0.61
TBL	20.8	20.7	19.7	20.38 ± 0.59
TAL	11	11.5	10.8	11.09 ± 0.33
FL	19.7	19.7	17.9	19.16 ± 1.14

sal faces of tarsi and feet were coloured a vivid purple-red. Two distinctive colour morphs were represented: a greenish brown or greenish black dorsal colouration, both with scattered golden yellow blotches and white dots. These

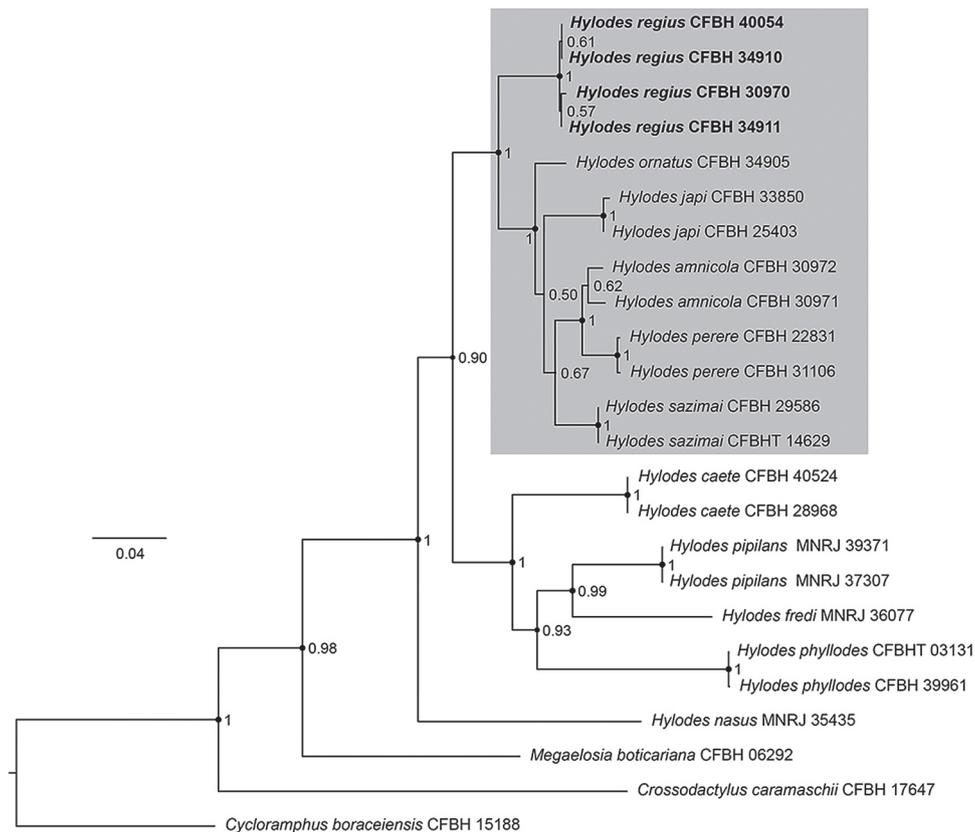


Figure 2. Phylogenetic tree obtained from a Bayesian inference analysis of the mitochondrial gene 16S for species of the genus *Hylodes* and outgroups. This inference focused on the positioning of *H. regius* (in boldface type) among its congeners, mainly including available sequences from species of the *H. lateristrigatus* species group (HEYER 1982). The shaded area highlights the *H. ornatus* molecular clade, in which *H. regius* is nested. Values adjacent to each node are posterior probabilities.

bright dots and blotches were also scattered on the dorsal faces of the fore and hind limbs. The darker males (CFBH 30970 and CFBH 34911) had smaller dorsal blotches and dots, whereas the blotches were clearly larger in the lighter male (CFBH 34910). The belly was reddish black, and the gular region was black in the darker males, but all ventral faces were peppered with well-defined white spots in all individuals. These two colour morphs of *H. regius* males in life are depicted in Fig. 3.

#### Advertisement and territorial calls

We included in our analyses the advertisement and territorial calls of *Hylodes regius* recorded at an air temperature of 16°C and a water temperature of 15°C (FNJV 45459–45467).



Figure 3. *Hylodes regius*, breeding males in life and representing the two distinctive colour morphs observed in this species. A) CFBH 30970, with the darker colour pattern (SVL = 35.5 mm), and B) CFBH 34910, with the lighter pattern (SVL = 33.6 mm). Photographs taken in the Parque Nacional do Itatiaia, municipality of Itamonte, state of Minas Gerais, southeastern Brazil, bordering the state of Rio de Janeiro.

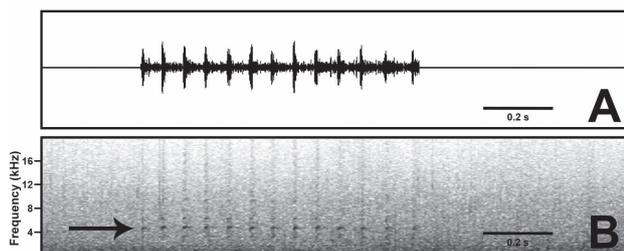


Figure 4. Advertisement call of *Hylodes regius*, FNJV 45460: A) waveform and B) spectrogram of one advertisement call composed of 13 notes. The dominant frequency (indicated by the arrow) lies in the third harmonic; the fast-flowing stream produced the background noise, closer to the bottom of the figure, and masks the first harmonic (the fundamental frequency). Call recorded on 8 February 2012; air temperature 16°C and water temperature 15°C.

Advertisement calls (Fig. 4) lasted  $0.98 \pm 0.26$  s ( $0.65$ – $1.49$  s;  $n = 32$  calls), at intervals of  $7.49 \pm 2.28$  s ( $2.55$ – $12.21$  s;  $n = 31$  intervals), and had  $18.31 \pm 6.79$  notes per call ( $11$ – $32$  notes per call;  $n = 32$  calls) that were emitted at a rate of  $18.21 \pm 2.21$  notes/s ( $15.21$ – $21.48$  notes/s;  $n = 32$  calls). Notes lasted  $0.023 \pm 0.005$  s ( $0.011$ – $0.049$  s;  $n = 355$  notes) and were emitted at intervals of  $0.033 \pm 0.008$  s ( $0.013$ – $0.059$  s;  $n = 354$  intervals). Each note consisted of a rising, frequency-modulated whistle with a harmonic structure. The dominant frequency was situated in the third harmonic, at  $4.66 \pm 0.16$  kHz ( $4.41$ – $4.91$  kHz;  $n = 32$  calls); often the first note was lower, with a dominant frequency at  $4.50 \pm 0.21$  kHz ( $3.88$ – $4.91$  kHz;  $n = 32$  calls).

Territorial calls (Fig. 5) were composed by two note types, a peep-like (type A) and a squeal-like (type B), which were variably alternated and combined as follows:

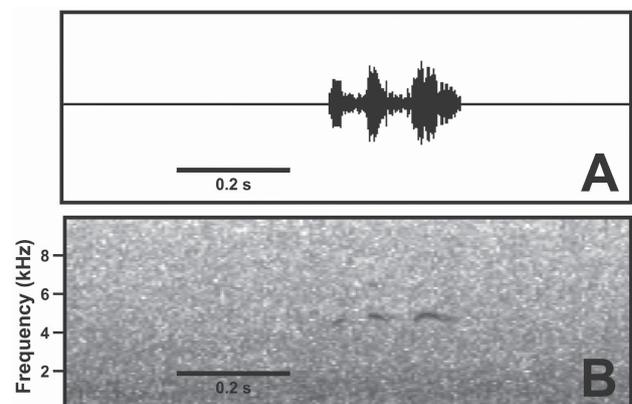


Figure 5. Complex territorial call of *Hylodes regius*, composed of two beep-like notes (type A; the first and second notes) combined with one squeal-like note (type B; the third note), FNJV 45467. Harmonic structures are apparent, but the background noise masks most of them. A) waveform and (B) spectrogram of one territorial call. Call recorded on 1 February 2013; air temperature 16°C and water temperature 15°C.

A (n = 4), AA (n = 2), B (n = 5), AB (n = 4), and AAB (n = 1). Territorial calls were alternated also with advertisement calls, sometimes with a territorial call emitted right before an advertisement call (0.059–0.078 s; n = 2).

There were simple A territorial calls, composed of one (n = 4) or two A notes (n = 2), and simple B territorial calls, composed always of one B note (n = 5). Simple A calls lasted  $0.07 \pm 0.05$  s (0.04–0.15 s; n = 6 calls) and had  $1.33 \pm 0.52$  notes per call (1–2 notes per call; n = 6 calls). Their notes lasted  $0.041 \pm 0.006$  s (0.034–0.052 s; n = 8 notes) and were emitted at intervals of  $0.045 \pm 0.009$  s (0.038–0.052 s; n = 2 intervals). Their dominant frequencies were at  $4.60 \pm 0.23$  kHz (4.31–4.91 kHz; n = 6 calls). Simple B calls lasted  $0.08 \pm 0.02$  s (0.06–0.10 s; n = 5 calls). Their dominant frequencies were also at  $4.33 \pm 0.04$  kHz (4.31–4.41 kHz; n = 5 calls).

There were also more complex territorial calls, which were composed of one A note combined with one B note (n = 4), or by two A notes combined with one B note (n = 1). Complex territorial calls lasted  $0.15 \pm 0.05$  s (0.12–0.23 s; n = 5 calls) and had  $2.20 \pm 0.45$  notes per call (2–3 notes per call; n = 5 calls). Their A notes lasted  $0.030 \pm 0.010$  s (0.021–0.048 s; n = 6 beep notes) and their B notes lasted  $0.068 \pm 0.015$  s (0.044–0.084 s; n = 5 squeal notes), with both note types being emitted at intervals of  $0.038 \pm 0.009$  s (0.030–0.053 s; n = 6 intervals). In complex territorial calls, the dominant frequencies of A notes were at  $4.28 \pm 0.32$  kHz (3.96–4.74 kHz; n = 6 calls) whereas the dominant frequencies of B notes were situated at  $4.79 \pm 0.05$  kHz (4.74–4.82 kHz; n = 5 calls). Overall, an A and a B call or two consecutive A calls, B calls, or complex territorial calls were emitted at intervals of  $4.38 \pm 0.28$  s (3.93–4.78 s; n = 6 intervals).

#### Tadpole description

The tadpole of *Hylodes regius* was found to be benthic, exotrophic, and hard to detect during our surveys. The single discovered and collected tadpole (CFBH 40054) was at Stage 35; we collected information from this sample for the following description. A small piece of muscle from its tail was removed (not interfering with the tadpole measurements) for molecular analysis. TL 52.1 mm, BL 18.8 mm, BH 6.7 mm, BW 9.9 mm, IND 3.8 mm, IOD 5.4 mm, END 3.1 mm, and ED 1.9 mm. Body oval in dorsal and ventral views, and oblong in lateral view, widest medio-anteriorly (Figs 6A–C); snout rounded in dorsal and lateral views; eyes small, dorsolateral; nostrils dorsolateral, small, rounded, nearly midway between the eyes and the tip of the snout; spiracle sinistral, situated more anteriorly on the body, opening posterodorsal; vent tube short, opening dextral, attached to ventral fin; caudal musculature robust, gradually tapering to a pointed tip; dorsal fin slightly larger than the ventral fin. Lateral line system composed of 12 lateral lines, 6 on each side of the body and tail. A ventral depression anterior to the coiled intestine was perceptible. Oral disc directed ventrally and bordered by two rows of

small papillae interrupted on the anterior labium; tooth row formula 2(2)/3(1) (Fig. 6D). The jaw sheaths are not described here because they were dekeratinized. In preservative, dorsum brown to light brown; flank gradually turning translucent toward the venter, ventral faces translucent; edge of spiracle matching body colouration; translucent fins with irregular, brown blotches distributed along the caudal fins; iris black; nostril surrounded by whitish shading; edge of nostril sparsely coloured with black. In life (Figs 6A–C), the colouration was overall dominated by distinct shades of brown, dorsally exhibiting black dots and spots interspersed with yellow blotches; spiracle with a distinctly lighter colouration; in lateral view, the upper region of the caudal musculature sported darker brown blotches that were loosely aligned longitudinally.

#### Natural history and geographic distribution

All *Hylodes regius* males recorded in our study were calling during the day. All males and the tadpole were collected at the beginning of February (between 1<sup>st</sup> and 16<sup>th</sup>) and in the shaded areas of fast-flowing streams. The first male (CFBH 30970) was discovered calling from between emerged rocks, the second (CFBH 34910) on the ground on the bank of a stream, and the third male (CFBH 34911) was calling perched on the trunk of a shrub, approximately 60 cm right above the stream. When disturbed, all males promptly stopped calling and leaped into the water or hid

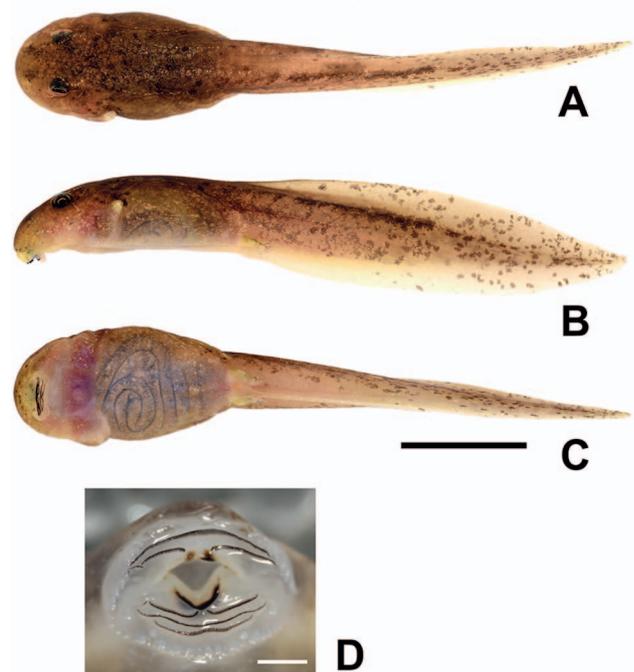


Figure 6. Tadpole of *Hylodes regius*, CFBH 40054 (Gosner Stage 35): A) dorsal, B) lateral, and C) ventral views (scale bar = 10 mm) in life. Photograph: M. VENCES. D) Detail of the oral disc (scale bar = 1 mm). Note the dekeratinized mouthparts.

themselves in crevices among or under emerged rocks, returning after few minutes to the same calling sites and resuming their calling activity. The tadpole was found during the day on the sandy floor of a stream. In Figure 7, we detail the distribution of *H. regius*, indicating where calling males and the tadpole were observed and collected, indicating the currently known geographic distribution limits of this species.

### Discussion

The present study adds information on the taxonomy, phylogenetic position, and status of *H. regius*, which is exemplary also for the family Hylodidae. Adult morphology, colour pattern variation, calls, and larval morphology are of critical importance to species identification, taxonomy

and to understanding evolutionary processes (HADDAD et al. 2003). Our more detailed description of the morphology of the males is exactly as originally described by GOUVÊA (1979). The ventral depression perceptible anterior to the coiled intestine in the tadpole is a putative synapomorphy of the genus *Hylodes* (see HADDAD & POMBAL 1995). As is typical of *Hylodes* spp., *H. regius* males call during the day and exhibit signs of site-fidelity and territoriality. Using molecular data, we proved that the two distinct colour morphs found in individuals of this *H. regius* population (darker and lighter individuals; GOUVÊA 1979; present study) are indicative of high intrapopulational polymorphism. Also, our genetic data demonstrate that, within the *Hylodes lateristrigatus* species group, *H. regius* is nested within the *H. ornatus* clade, suggesting its being phylogenetically closer to *H. amnicola*, *H. japi*, *H. ornatus*, *H. perere*, and *H. sazimai* than to the other *Hylodes* spp. in-

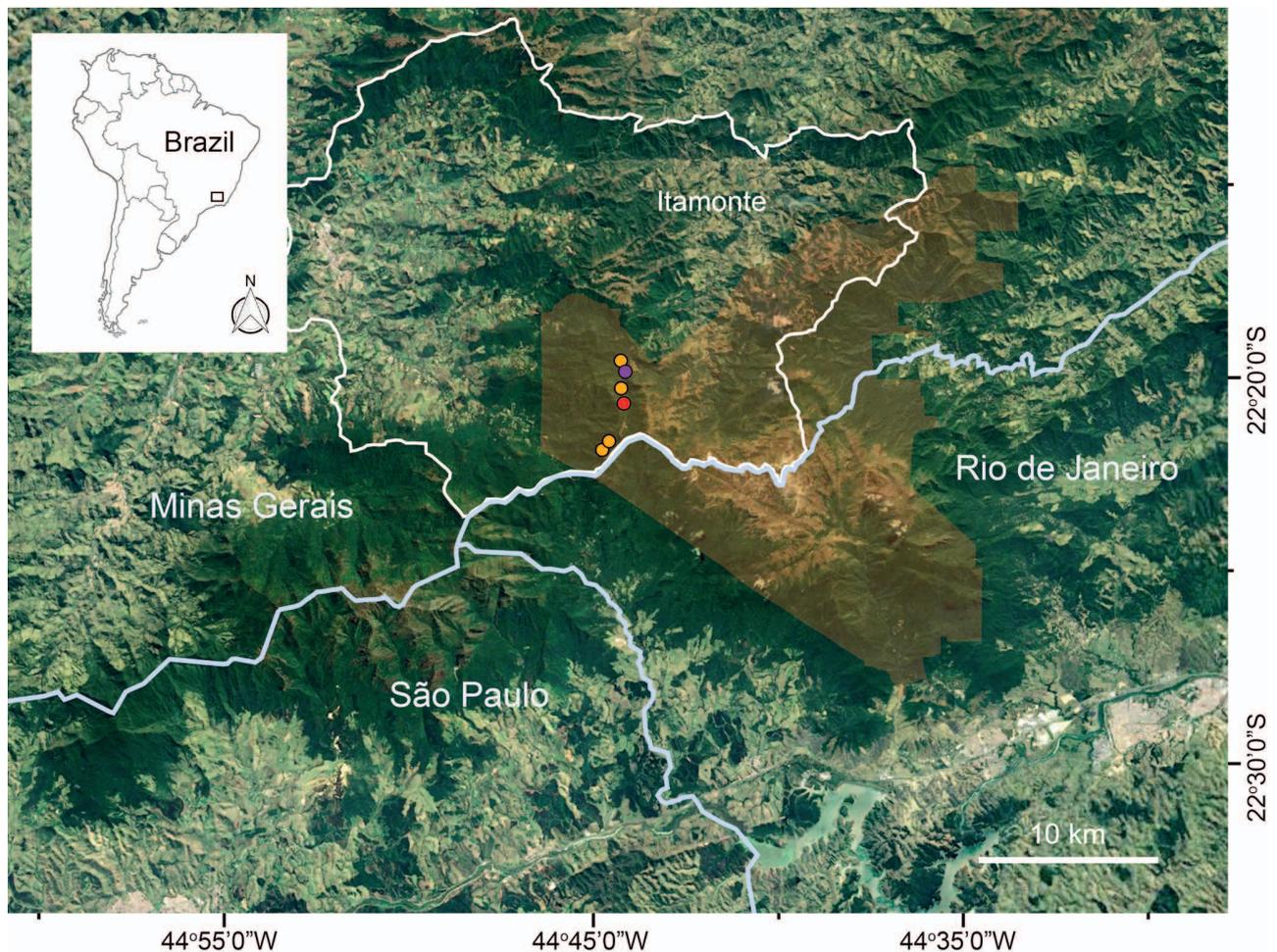


Figure 7. Geographic distribution of *Hylodes regius* on the highlands of the Parque Nacional do Itatiaia, southeastern Brazil: The small square in the insert map of South America indicates the area where we collected *Hylodes regius* individuals. Blue lines indicate state boundaries, white lines outline the municipality of Itamonte, and the area of the PARNA Itatiaia is shaded in orange. The type locality of *H. regius* (holotype and five paratopotypes) is indicated by a purple circle, and a red circle points out the locality where another paratopotype was collected (GOUVÊA 1979). Orange circles indicate where calling males and the single tadpole were found in the present study. All circles together designate the currently known geographic range of this species, between 1,850 and 2,100 m a.s.l. Satellite image from Google Earth.

cluded in our analysis. Clades within the *H. lateristrigatus* species group might be associated with topographic patterns of the Atlantic forest domain, but a more complete dataset of the group is necessary to confirm this.

Although our rediscovery of *Hylodes regius* here is exciting, it is still difficult to fully assess the actual dimension of threats this species currently faces. The difficulty in finding this species in nature for 35 years may be a result of natural demographic oscillations, natural geographic dynamics, or an actual decline. We know that *Hylodes* spp. tadpoles are often susceptible to *Batrachochytrium dendrobatidis* (*Bd*), as has been reported for *H. asper* (GRÜNDLER et al. 2012, RUGGERI et al. 2015), *H. cardosoi* (BECKER et al. 2017), *H. japi* (SCHLOEGEL et al. 2012, ROSENBLUM et al. 2013, VIEIRA et al. 2013, DE SÁ et al. 2015), *H. magalhaesi* (TOLEDO et al. 2006), and *H. phyllodes* (BECKER et al. 2014, RUGGERI et al. 2015, FISHER et al. 2018). Despite a lack of evidence in the literature that *Bd* has infected *H. regius* in the past, the dekeratinized jaw sheaths of the one tadpole found might be a sign of a *Bd* infection (CARVALHO et al. 2017, FISHER et al. 2018). In Brazil, anuran declines have been reported since the 1980s and mainly afflict populations native to the mountains in the Atlantic forest realm (which includes the PARNA Itatiaia; HEYER et al. 1988, WEYGOLDT 1989, GUIX et al. 1998, POMBAL & HADDAD 1999, ETEROVICK et al. 2005). Whereas evidence of the prevalence and persistence of *Bd* in the PARNA Itatiaia is scant (CARVALHO et al. 2017), it has been proved that most historical declines of Brazilian anurans in the late 1970s to mid-1980s were the result of infections with *Bd* (CARVALHO et al. 2017), matching the general global pattern (SCHEELE et al. 2019). Therefore, oscillations in *Hylodes* spp. populations in the PARNA Itatiaia might be linked to chytridiomycosis. New research projects are needed to clarify the host-pathogen dynamics in the PARNA Itatiaia and help to understand the current and past threats *Bd* poses on *Hylodes* spp. populations. Also, the currently known geographic distribution of *H. regius* is still limited to the type locality and suggests it to be a microendemite. However, there are still uncertainties as to the ecological requirements and the total distribution range of this species. Long-term projects and new surveys focusing in particular on the conservation of endemic *Hylodes* spp. with restricted distributions in the PARNA Itatiaia (*H. regius*, *H. glaber*, and *H. ornatus*) should delineate more accurately their actual, current distributions. Particularly, data on resource use and demographics over time will show more precisely how vulnerable those species are today.

Rediscovering a missing species might give us a sensation of optimism regarding its survival, but the discovery alone may not denote an optimistic reality. A global review of rediscovered species conducted by SCHEFFERS et al. (2011) demonstrated that these are likely to have a higher probability of being threatened. Species with small populations and small distribution ranges are even more vulnerable and difficult to protect, typically requiring intensive conservation efforts (SCHEFFERS et al. 2011). At least three endemic frog species that are restricted to the PARNA Itatiaia have been missing since the late 1970s and early 1980s:

*Holoaden bradei* (family Craugastoridae; HADDAD et al. 2016, MARTINELLI & TOLEDO 2016), *Paratelmatobius lutzii* (family Leptodactylidae; HADDAD et al. 2016), and *Hylodes glaber* (HADDAD et al. 2016; C. F. B. HADDAD pers. obs.). These missing species together with the temporary disappearances of *H. ornatus* and *H. regius* underscore the need for intensive conservation measure in the park. At present, promoting the protection of the environment in Brazil is a challenge due to the national policy of the current federal government, which deliberately disregards science to the detriment of the environment and biodiversity (THOMÉ & HADDAD 2019) in favour of agribusiness, logging, and mineral exploitation (RAFTOPOULOS & MORLEY 2020). The unique Brazilian biodiversity is now at serious risk due to the dismantling policy of the inspection agencies and the relaxation of laws to permit the predatory exploitation of protected areas (CARVALHO et al. 2019, THOMÉ & HADDAD 2019, RAFTOPOULOS & MORLEY 2020). It is imperative to keep, expand, and improve our protected natural areas (such as the PARNA Itatiaia), applying conservation strategies to protect the Brazilian biodiversity.

#### Acknowledgements

We thank N. C. PUPIN and M. VENCES for helping with our fieldwork; M. VENCES took the pictures of the tadpole in life. This research was supported by grants from the São Paulo Research Foundation (FAPESP; #2010/14117-9 to FPS, #2010/50124-0 and #2017/26162-8 to MLL, and #2008/50928-1, #2013/50741-7, and #2014/50342-8 to CFBH) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; #306623/2018-8 to CFBH). Field research was approved by the committee on animal care ethics at the Universidade Estadual Paulista, Rio Claro, Brazil (#020/2011). Field research and collection permits were issued by the relevant Brazilian government environmental agencies (ICMBio #14846-3 and #22511-3). Genetic heritage assessment: SISGEN: ADFB611.

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