Evolutionary history of secondary sexual characters in the most species-rich genus of anurans, the direct-developing frogs of the genus *Pristimantis* (Anura: Strabomantidae)

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Ancestral character-state reconstruction is a powerful method in phylogenetics that can be applied to elucidate the evolutionary history of secondary sexual characters. Here, we surveyed the variation and reconstructed the ancestral states of secondary sexual characters (i.e. sexual dichromatism, vocal slits and nuptial pads) for the most species-rich genus of anurans (*Pristimantis*) using maximum parsimony, maximum-likelihood and Bayesian methods. This study demonstrates that at least five independent transformation series account for the occurrence of sexual dichromatism in *Pristimantis*: dorsum, throat, venter, groin and posterior surface of thighs. The ancestral reconstructions suggest that the most recent common ancestor of *Pristimantis* lacks sexual dichromatism on these five body areas. Likewise, the occurrence of vocal slits and the absence of nuptial pads were inferred as ancestral conditions. Morphological synapomorphies were identified for *Yunganastes* and two infrageneric units within *Pristimantis* (the *Pristimantis devillei* and *Pristimantis unistrigatus* species groups). Our results demonstrate that the evolutionary history of the secondary sexual characters in *Pristimantis* followed a rather complex pattern of multiple independent gains and losses for which this genus is a promising model to investigate the evolution of secondary sexual characters in the context of the complex interactions between natural and sexual selection.

ADDITIONAL KEYWORDS: amphibian – character evolution – evolution – morphology – phylogenetics – selection.

INTRODUCTION

Natural selection is one of the central mechanisms of evolutionary change, as individuals that survive to maturity will be able to mate and leave offspring (Darwin, 1859; Mayr, 1972; Rivera, 2009; Losos, 2016). In addition to natural selection, the transformation of species through time can also be explained through sexual selection, which depends upon efforts to monopolize the gene pool, resulting from variance in mating success (Darwin, 1871; Lande, 1981; Ghiselin, 2006, 2016).

Both natural selection and sexual selection may cause phenotypic differences between males and females, which is recognized as sexual dimorphism (McPherson & Chenoweth, 2012). There are three realms of sexual phenotypic differences, namely primary sexual, secondary sexual and tertiary or non-reproductive characters (Ghiselin, 2010; Motani *et al.*, 2018). Features that are directly associated with sexual reproduction constitute the primary sexual characters, features related to competition for mates between individuals of the same sex account for the secondary sexual characters, and finally, features that are not directly

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related to reproduction comprise the tertiary sexual characters (e.g. in some flies, males and females have different feeding sources, thus, some structures of the mouth differ between the sexes) (Darwin, 1871; Ghiselin, 2010). The distinction between primary and secondary characters, according to Darwin (1871: 256), is whether the characters have evolved through natural selection or are the product of sexual selection, respectively. However, it has become increasingly clear that primary sexual characters can also be sexually selected (Eberhard, 1985, 1996, 2010), which means that it is often difficult to determine the relative importance of natural and sexual selection given these accompany one another in the evolution of sexual characters (Ghiselin, 2010).

In animals, gonads, gametes and genital structures required for ova and sperm to unite are considered primary sexual characters (Leonard & Aguilar-Córdoba, 2010). Among the sexual characters considered secondary, sexual dichromatism (i.e. intersexual variation in colour, pattern or both) has been extensively studied in animals (Badyaev & Hill, 2003; Stuart-Fox & Ord, 2004; Friis & Milá, 2020; Gazda *et al.*, 2020; van der Bijl, 2020; Miller *et al.*, 2021). There are two classes of sexual dichromatism within frogs, namely dynamic and ontogenetic (Bell & Zamudio, 2012). Dynamic dichromatism refers to temporary colour change in males during the breeding season, whereas ontogenetic dichromatism refers to permanent colour change in either sex, usually once sexual maturity is reached (Bell & Zamudio, 2012; Bell *et al.*, 2017).

Some reviews on anuran coloration found that contrary to birds, fishes and butterflies, sexual dichromatism in anurans is rare, with less than 5% of the known species being sexually dichromatic (Bell & Zamudio, 2012; Rojas, 2016; Lambert *et al.*, 2017). Nevertheless, in recent years several studies have demonstrated that the number of sexually dichromatic anurans may be underestimated (Bell *et al.*, 2017; Engelbrecht-Wiggans & Tumulty, 2019; Portik *et al.*, 2019; Greener *et al.*, 2020).

In addition to sexual dichromatism, other sexual characters traditionally reported as secondary in anurans include the occurrence of nuptial pads, vocal slits and sac, among others (Noble, 1931; Liu, 1950; Shine, 1979; Buchanan, 1994; Emerson, 1996; Blackburn, 2009; Magalhães *et al.*, 2018; Bossuyt *et al.*, 2019; Portik *et al.*, 2019). Vocal slits are a pair of openings into the vocal sac in the floor of the mouth present in adult males of most species (Liu, 1935; Duellman & Lehr, 2009). These structures allow the air from the lungs to flow between the vocal sac and the buccal cavity during acoustic and/or visual communication (Colafrancesco & Gridi-Papp, 2016; Elias-Costa *et al.*, 2017). The nuptial pads are a thickened area of the epidermis or dermis, or both, with skin glands located on the medial margin of the thumb

of most male anurans (Epstein & Blackburn, 1997; Duellman & Lehr, 2009; Luna *et al.*, 2018). In some species, the nuptial pads are permanent, whereas, in others, they last during some seasons (Solomonova *et al.*, 2011; Luna *et al.*, 2012). It has been proposed that the function of nuptial pads may be for male-male combat, for release of protein pheromones, to facilitate the grip of a female or to avoid being dislodged from a female by another male during amplexus (Kurabuchi, 1993; Thomas *et al.*, 1993; Duellman & Trueb, 1994; Kyriakopoulou-Sklavounou *et al.*, 2012; Luna *et al.*, 2012, 2018; Willaert *et al.*, 2013; Bossuyt *et al.*, 2019).

In anurans, sexual dichromatism, vocal slits and nuptial pads are characters commonly used for taxonomic purposes (Savage & Heyer, 1969; Poynton, 1986; Lynch & Ruiz-Carranza, 1996; Lynch & Duellman, 1997; Cisneros-Heredia & McDiarmid, 2007; Lehr & Trueb, 2007; Vaughan & Mendelson, 2007; Duellman & Lehr, 2009; Grant & Myers, 2013; Fouquet *et al.*, 2015; Zhao *et al.*, 2015; Luna *et al.*, 2018) and less commonly in phylogenetic studies (e.g. Scott, 2005; Wiens *et al.*, 2005; Grant *et al.*, 2006; Araujo-Vieira *et al.*, 2019; Pereyra *et al.*, 2021). Nevertheless, the inference of evolutionary history based on ancestral character-state reconstruction (ACSR) for these characters is relatively scarce. For example, only two studies have investigated the occurrence of sexual dichromatism (Bell *et al.*, 2017; Portik *et al.*, 2019) and nuptial pads (Emerson, 1994; Pereira *et al.*, 2015) in a phylogenetic context, whereas four works have been performed on the evolution of vocal slits (Emerson, 1994; Elias-Costa & Faivovich, 2019; Targino *et al.*, 2019; Moura *et al.*, 2021). This milieu hampers the possibility to exploit analytical possibilities derived from ACSR such as morphological evolution assessment, correlated character evolution analyses and the possible interplay between natural selection and sexual selection as evolutionary drivers for secondary sexual characters (Joy *et al.*, 2016).

With more than 590 described species, direct-developing frogs of the genus *Pristimantis* Jiménez de la Espada, 1870 make up the largest genus of anurans (Waddell *et al.*, 2018; Frost, 2022). Frogs of the genus *Pristimantis* are markedly dimorphic in size and exhibit interspecific variation in the presence of sexual dichromatism, vocal slits and nuptial pads (Lynch & Duellman, 1997). Despite the number of studies dedicated to unravelling the complex phylogenetic relationships within the genus *Pristimantis* (e.g. Hedges *et al.*, 2008; Padial *et al.*, 2014; Waddell *et al.*, 2018; Jetz & Pyron, 2018), the diversity and evolution of the secondary sexual characters in *Pristimantis* have not been studied in a large-scale phylogenetic context. Thus, in this study we aimed to: (i) study the diversity of sexual dichromatism, proposing phylogenetic characters according to the precepts of phylogenetic

inference (Grant & Kluge, 2004) and (ii) perform ACSR analyses to reconstruct major evolutionary patterns of sexual dichromatism, and occurrence of vocal slits and nuptial pads. Reconstructing the evolutionary patterns of sexual dichromatism, and occurrence of vocal slits and nuptial pads in *Pristimantis* has broad implications for understanding the diversification of this group of frogs, and offers opportunities to study the action of selective forces acting on individuals. On this basis, here we provide initial insights into the evolution of secondary sexual characters in the context of complex interactions between natural and sexual selection.

MATERIAL AND METHODS

TAXON SAMPLING AND CHARACTER SCORING

We studied the occurrence of sexual dichromatism, nuptial pads and vocal slits for 171 species of *Pristimantis* (representing nearly 30% of its specific diversity) and 42 species of closely related genera. The selection of *Lynchius* Hedges *et al.*, 2008, *Niceforonia* Goin & Cochran, 1963, *Oreobates* Jiménez de la Espada, 1872, *Phrynopus* Peters, 1873 and *Yunganastes* Padial *et al.*, 2007 as outgroups was based on the phylogenetic results of Jetz & Pyron (2018). We also included *Ceuthomantis* Heinicke *et al.*, 2009 as an outgroup based on previous evidence that supported a putative close relationships with *Pristimantis* and *Yunganastes* (see discussion in Padial *et al.*, 2014).

Sexual dichromatism was quantified by visually scoring intersexual differences in colour based on photographs of living and preserved specimens as well as by direct observations of preserved specimens. Museum information for all specimens is provided in the Supporting Information (Appendix S1). We also surveyed the literature to obtain data as a baseline to complement our data set (Supporting Information, Appendix S2). For the coding of vocal slits and nuptial pads occurrence, we examined males after sex determination (by direct gonad observation) or obtained this information from explicit mentions of their condition in the literature. When disagreements were evident in the information compiled between direct observations and the information extracted from the literature for the coding of vocal slits and nuptial pads, we opted for our observations. Transformation series (characters and character-states) were delimited following Grant & Kluge (2004). Species with both states (presence/absence) were coded as polymorphic. See Appendix S3 of the Supporting Information for character coding.

ANCESTRAL CHARACTER-STATE RECONSTRUCTIONS

ACSRs were reconstructed considering parsimony (MP) as the optimality criterion using Mesquite v.3.61

(Maddison & Maddison, 2019) on the topology of Anura obtained by Jetz & Pyron (2018). Alternative evolutionary scenarios were examined, for which we employed maximum likelihood (ML) and Bayesian inference (BI), specifically stochastic character mapping (Huelsenbeck *et al.*, 2003), using the package phytools (Revell, 2012) in R (R Core Team, 2013). Two transition rate models were assessed under ML: (i) equal transition rates between states (ER) and (ii) all rates different between states (ARD). We selected the best-fit model for each character individually using the Akaike information criterion corrected for small sample size (AICc). The selected model for each character was used to perform the analyses under both ML and BI. Under BI, we explored three scenarios with 10 000 simulations each: (i) no restrictions; (ii) restricting the ancestor to possess the character (i.e. sexual dichromatism, vocal slits and nuptial pads); and (iii) a Dollo's model (no regains possible) (Cunningham, 1999). Species with a polymorphic condition (0&1) or missing data (?) in any character were excluded from ML and BI analyses due to analytical restrictions of the methods. The R script used for ML and BI analyses is provided in Appendix S4 of the Supporting Information.

Ambiguous nodes in the most parsimonious reconstructions were taken as transformation. However, the type of transformation was not registered, i.e. they were not classified as gain or loss but solely counted in the row 'Total transitions' from Table 1. Given that only transformations from one character-state to another are relevant in phylogenetic inference (see Grant & Kluge, 2004: 25), we did not infer any transformation between the tip and the immediate ancestor in polymorphic terminal nodes. In ML and BI analyses, we counted a state change whenever the difference of proportional likelihood of a character state between two nodes was greater than 50% (Pereyra *et al.*, 2016).

We favoured parsimony because it uses the number of transformations as optimality criterion, minimizing the hypothetical transformation events required to explain the evidence (Kluge & Grant, 2006). Nonetheless, we comparatively considered the results obtained under ML and BI.

RESULTS

DEFINED CHARACTERS STATES

We defined seven transformation series (characters) that describe relevant variation in sexual dichromatism (Fig. 1), nuptial pads and vocal slits.

Sexual dichromatism

In the literature on frogs of the genus *Pristimantis*, the occurrence of sexual dichromatism is treated as

Table 1. Comparison of estimated transformations across parsimony, maximum likelihood and stochastic character mapping analyses for sexual dichromatism across five body regions (dorsum, throat, venter, groin and posterior surface of thighs), vocal slits and nuptial pads in *Pristimantis*

Character	Model	AICc	No. of losses	No. of gains	Total transitions	Support for ancestor lacking the character (Yes/No, %)
Dorsum	Parsimony	N/A	0	5	5	Yes, N/A
	ARD ML	53 556	0	5	5	Yes, 99.983%
	ER ML	66 902	0	5	5	Yes, 99.983%
	ARD SIMMAP	N/A	0	5	5	Yes, 99.98%
	ARD + RA‡ SIMMAP	N/A	1	5	6	N/A
	Dollo's model SIMMAP	N/A	21	0	21	N/A
Throat	Parsimony	N/A	0	17	23*	Yes, N/A
	ARD ML	156 888	2	21	23	Yes, 99.9828%
	ER ML	210 932	2	21	23	Yes, 99.9828%
	ARD SIMMAP	N/A	2	21	23	Yes, 100%
	ARD + RA SIMMAP	N/A	3	21	24	N/A
	Dollo's model SIMMAP	N/A	49	0	49	N/A
Venter	Parsimony	N/A	0	10	10	Yes, N/A
	ARD ML	97 443	0	10	10	Yes, 99.983%
	ER ML	138 952	0	10	10	Yes, 99.983%
	ARD SIMMAP	N/A	0	10	10	Yes, 99.94%
	ARD + RA SIMMAP	N/A	1	10	11	N/A
	Dollo's model SIMMAP	N/A	39	0	39	N/A
Groin	Parsimony	N/A	0	7	7	Yes, N/A
	ARD ML	70 057	0	7	7	Yes, 99.983%
	ER ML	99 410	0	7	7	Yes, 99.983%
	ARD SIMMAP	N/A	0	7	7	Yes, 99.98%
	ARD + RA SIMMAP	N/A	1	7	8	N/A
	Dollo's model SIMMAP	N/A	32	0	32	N/A
Posterior surface of thighs	Parsimony	N/A	0	6	6	Yes, N/A
	ARD ML	57 207	0	6	6	Yes, 99.983%
	ER ML	79 129	0	6	6	Yes, 99.983%
	ARD SIMMAP	N/A	0	6	6	Yes, 99.97%
	ARD + RA SIMMAP	N/A	1	6	7	N/A
	Dollo's model SIMMAP	N/A	31	0	31	N/A
Vocal slits	Parsimony	N/A	19	4	29*	No, N/A
	ARD ML	218 755	25	4	29	No, 2.5458%
	ER ML	229 317	25	4	29	No, 2.5458%
	ARD SIMMAP	N/A	25	4	29	No, 2.64%
	ARD + RA SIMMAP	N/A	25	4	29	N/A
	Dollo's model SIMMAP	N/A	33	0	33	N/A
Nuptial pads	Parsimony	N/A	5	20	38*	Yes, N/A
	ARD ML	250 667	12	26	38	Yes, 67.7329%
	ER ML	248 677	12	26	38	Yes, 67.7329%
	ER SIMMAP	N/A	12	26	38	Yes, 67.94%
	ER + RA SIMMAP	N/A	18	21	39	N/A
	Dollo's model SIMMAP	N/A	45	0	45	N/A

SIMMAP: stochastic character mapping. RA: ancestor restricted to possess the character. N/A: not applicable. *: transformations which were not classified as gain or loss but solely counted in the row 'Total transitions'.

one character (e.g. Lynch, 1996a; Lynch & Duellman, 1997; Duellman & Lehr, 2009). That is, a single transformation series is assumed to account for the presence of sexual dichromatism over the whole body. Nevertheless, we report that the occurrence of sexual dichromatism varies independently between different
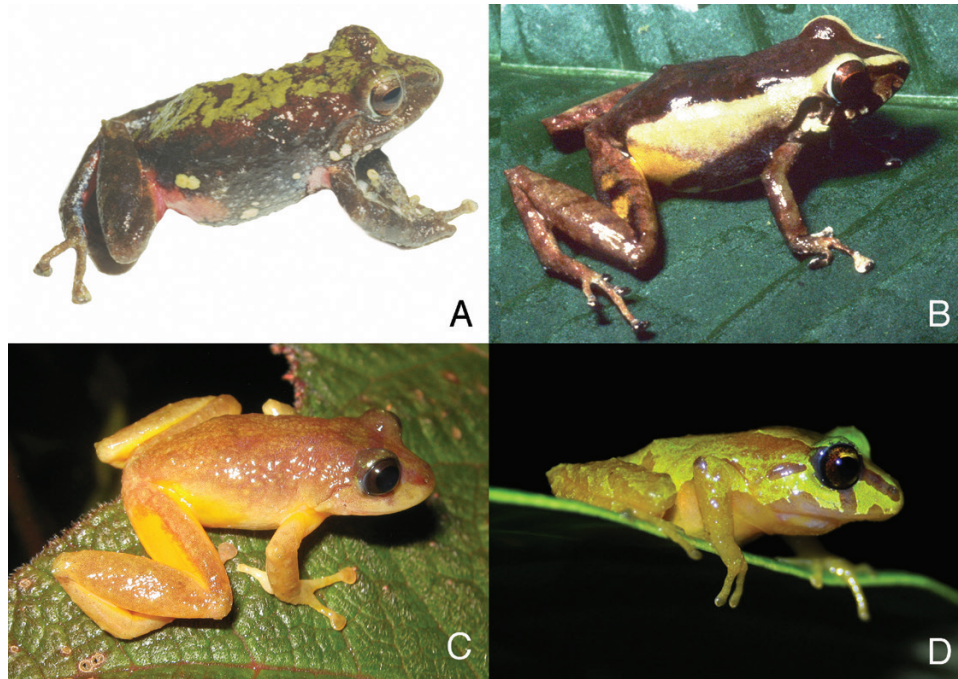
Figure 1. Examples of sexual dichromatism in *Pristimantis*. A-B, female and male of *Pristimantis erythropleura*. C-D, female and male of *Pristimantis restrepoi*.

body regions and the different patterns of co-occurrence of dichromatism in these regions [conjunction test; Patterson (1982); see also de Pinna *et al.* (1991)] support its recognition as an independent character (see Appendix S3 of Supporting Information). Some species are sexually dichromatic on multiple body regions, whereas others are on one body region alone. For example, *Pristimantis bambu* Arteaga-Navarro & Guayasamin, 2011 has sexual dichromatism on the dorsum, throat and venter (Arteaga-Navarro & Guayasamin, 2011), *Pristimantis inusitatus* (Lynch & Duellman, 1980) exhibits sexual dichromatism on the dorsum, throat, venter and groin (Guayasamin & Funk, 2009) and *Pristimantis erythropleura* (Boulenger, 1896) has sexual dichromatism on the venter, groin and posterior surface of the thighs (Fig. 1A-B; Lynch, 1996a). On the other hand, *Pristimantis acerus* (Lynch & Duellman, 1980) and *Pristimantis gryllus* Barrio-Amorós *et al.*, 2012 have sexual dichromatism on the throat (Lynch & Duellman, 1980; Barrio-Amorós *et al.*, 2012), *Pristimantis acatallelus* (Lynch & Ruiz-Carranza, 1983) and *Pristimantis bromeliaceus* (Lynch, 1979) exhibit sexual dichromatism only on the venter (Lynch & Ruiz-Carranza, 1983; Duellman & Lehr, 2009) and *Pristimantis restrepoi* (Lynch, 1996) exhibits sexual dichromatism on the dorsum, groin and posterior surface of the thighs (Fig. 1C-D; Lynch, 1996b).

To account for the independently varying transformation series, we propose the following five characters and character-state for the occurrence of sexual dichromatism on all body regions:

Character 1. Sexual dichromatism on the dorsum: (0) absent; (1) present.
Character 2. Sexual dichromatism on the throat: (0) absent; (1) present.
Character 3. Sexual dichromatism on the venter: (0) absent; (1) present.
Character 4. Sexual dichromatism on the groin: (0) absent; (1) present.
Character 5. Sexual dichromatism on the posterior surface of the thighs: (0) absent; (1) present.

Nuptial pads

The nuptial pads are modified epidermal and dermal tissues typically located on the first digit of the hand of several species of anuran amphibians (Noble, 1931; Luna *et al.*, 2018). In some anurans, the nuptial pads may also be present on the median or dorsal surfaces of the second and third fingers (Duellman & Trueb, 1994; Luna *et al.*, 2018). Nuptial pads are differentiated from the adjacent skin by the increase in thickness of the dermis and the presence of sexually dimorphic skin glands, which in most cases are specialized mucous glands (Luna *et al.*, 2018). With respect to morphological variation in nuptial pads, it has been reported that these vary in terms of their distribution, size, texture and coloration (Luna *et al.*, 2012). In *Pristimantis*, the nuptial pads can

be absent or present, and when present, they are white, nonspinous and located on the dorsolateral and medial surfaces of the base of the thumb in breeding males (Lynch & Duellman, 1997). On this basis, we propose the following character for the occurrence of nuptial pads:

Character 6. Nuptial pads: (0) absent; (1) present.

Vocal slits

Males of most species have one small aperture on one or both sides of the mouth floor called vocal slits, which are located between the midlateral base of the tongue and the angle of the jaw and, when present, are commonly used to determinate adulthood of males (Liu, 1935; Lynch & Duellman, 1997; Duellman & Lehr, 2009; Elias-Costa *et al.*, 2017). Vocal slits vary extensively in shape and length (Elias-Costa *et al.*, 2017). In adult males of *Pristimantis*, vocal slits may be present or absent (Lynch & Duellman, 1997). Considering this, we propose the following character for the occurrence of vocal slits:

Character 7. Vocal slits: (0) absent; (1) present.

ANCESTRAL CHARACTER-STATE RECONSTRUCTION

Sexual dichromatism evolution: MP scenarios

The ancestral character-state analysis allowed us to identify that the absence of sexual dichromatism across five different body regions (i.e. dorsum, throat, venter, groin and posterior surface of thighs) is plesiomorphic in *Pristimantis* and closely related genera (*Lynchius*, *Niceforonia*, *Oreobates*, *Phrynopus*, *Yunganastes*) (Figs 2–4; see Appendices S5–S9 of the Supporting Information). With respect to the sequences of gains and losses of sexual dichromatism across body regions in *Pristimantis*, ACSRs revealed that sexual dichromatism has arisen multiple times independently with no subsequent losses (Table 1). Among body regions, the throat and dorsum exhibited the greatest and least number of independent origins, 17 and five, respectively (Table 1). Also, it is important to point out that six independent transformations were observed in the throat; however, the optimization did not distinguish between gains and losses (see Appendix S6 of the Supporting Information). Outside *Pristimantis*, we found that sexual dichromatism is plesiomorphically absent across each one of the five body regions, but has been gained independently at least three times in the throat [in *L. parkeri* (Lynch, 1975), *Phrynopus pesantesi* Lehr *et al.*, 2005, *Y. bisignatus* (Werner, 1899); see Fig. 2; see Appendix S6 of the Supporting Information] and twice in the venter (in *L. parkeri*, *Phrynopus pesantesi*; see Fig.

2; see Appendix S7 of the Supporting Information). In addition to these gains, sexual dichromatism in the groin has arisen once in *Phrynopus pesantesi* (see Fig. 2; see Appendix S8 of the Supporting Information).

Sexual dichromatism evolution: ML and stochastic character reconstructions

All rates different between states (ARD model) was chosen as the best-fit model for each character (Table 1). Our ML and stochastic character reconstructions analyses under various evolutionary scenarios exhibited results similar to the parsimony reconstructions of sexual dichromatism across the five different body regions with strong support for an ancestor of *Pristimantis* lacking sexual dichromatism (see Appendices S10–S14 of the Supporting Information). Likewise, these analyses estimated similar patterns of sexual dichromatism gains and losses to the parsimony reconstructions, except for four gains and two losses in the throat (Table 1). When we assumed the ancestor of *Pristimantis* to be sexually dichromatic across the five body regions and a model of different transition rates (ARD), we found support for an additional loss in all the five body regions (Table 1). When restricting regains from occurring (Dollo's model), we found that throat and dorsum exhibit the greatest and least number of losses across *Pristimantis*, 49 and 21, respectively (Table 1).

Vocal slits and nuptial pads evolution: MP scenarios

The presence of vocal slits is plesiomorphic in *Pristimantis* (see Appendix S15 of the Supporting Information) but has been lost independently at least 19 times (Table 1; Figs 3–4). The occurrence of vocal slits is also plesiomorphic in *Niceforonia* and *Yunganastes* (see Appendix S15 of the Supporting Information). On the other hand, ancestral reconstruction suggests that the ancestor of the genus *Phrynopus* lacks vocal slits (see Appendix S15 of the Supporting Information). Finally, vocal slits are absent in *Oreobates lundbergi* (Lehr, 2005) but present in all other species of the genus, resulting in an ambiguous optimization for *Oreobates* (Fig. 2; see Appendix S15 of the Supporting Information). With respect to the sequences of gains and losses of vocal slits in *Pristimantis*, ACSRs revealed a total of 19 losses and four regains (Table 1). In addition, six independent transformations are also observed in the evolution of vocal slits across *Pristimantis*; however, the optimization does not distinguish between gains and losses (Table 1).

Nuptial pads are plesiomorphically absent in *Pristimantis* (see Appendix S16 of the Supporting Information) but have been gained independently at least 20 times (Table 1; Figs 3–4). The absence of
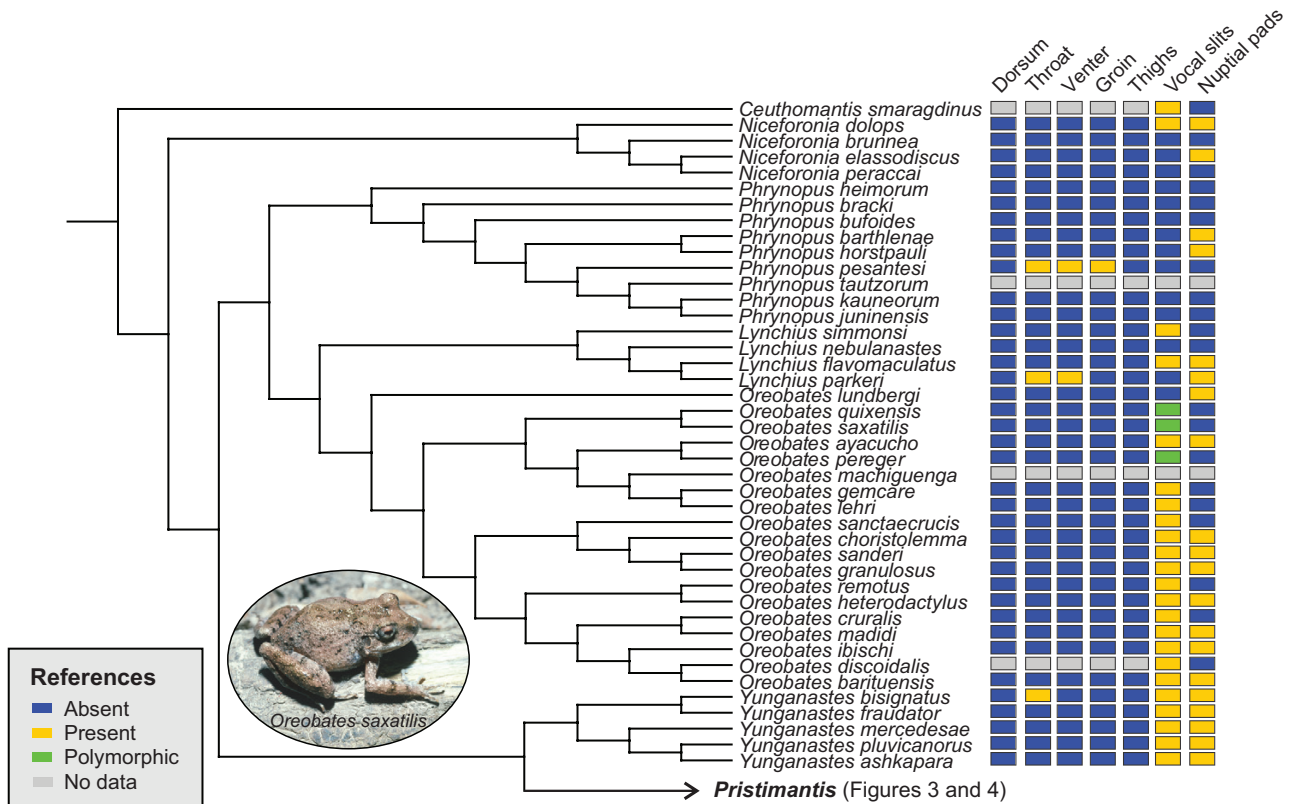
Figure 2. Distribution of Characters 1–7 in clades outside *Pristimantis*. The phylogenetic hypothesis for *Pristimantis* and the outgroups are that of Jetz & Pyron (2018). The complete parsimony ACSRs for all characters are shown in the Supporting Information (Appendices S5–S9, S15–S16).

nuptial pads is also plesiomorphic in *Niceforonia*, *Phrynopus* and *Lynchius* (see Appendix S16 of the Supporting Information). Ancestral reconstruction suggests that the ancestor of the genus *Yunganastes* presents nuptial pads (see Appendix S16 of the Supporting Information). In the case of the genus *Oreobates*, the presence and/or absence of nuptial pads is highly variable and optimizes ambiguously (Fig. 2; see Appendix S16 of the Supporting Information). With respect to the sequences of gains and losses of nuptial pads in *Pristimantis*, ACSR reveals a total of 20 regains and five losses (Table 1). In addition, thirteen independent transformations are also observed in the evolution of nuptial pads across *Pristimantis*; however, the optimization does not distinguish between gains and losses (Table 1).

Vocal slits and nuptial pads evolution: ML and stochastic character reconstructions

All rates different (ARD model) and equal rates (ER model) between states were chosen as the best-fit models for vocal slits and nuptial pads, respectively (Table 1). Our ML and stochastic character reconstructions analyses under various evolutionary scenarios showed

similar results to the parsimony reconstructions for both vocal slits and nuptial pads with strong support for an ancestor of *Pristimantis* having vocal slits but lacking nuptial pads (see Appendices S17–S18 of the Supporting Information). Furthermore, these analyses inferred similar patterns of gains and losses to the parsimony reconstructions, except for additional six losses in vocal slits and seven losses and six regains in nuptial pads (Table 1). When we assumed the ancestor of *Pristimantis* having vocal slits and a model of different transition rates (ARD), we found the same pattern of losses and gains, whereas when we assumed the ancestor of *Pristimantis* having nuptial pads and a model of equal transition rates (ER), we found support for six additional losses but only 21 gains (Table 1). When restricting regains from occurring (Dollo's model), we found 33 and 45 losses for vocal slits and nuptial pads, respectively (Table 1).

DISCUSSION

SEXUAL DICHROMATISM EVOLUTION

Our results demonstrate that several *Pristimantis* species are sexually dimorphic in coloration, increasing
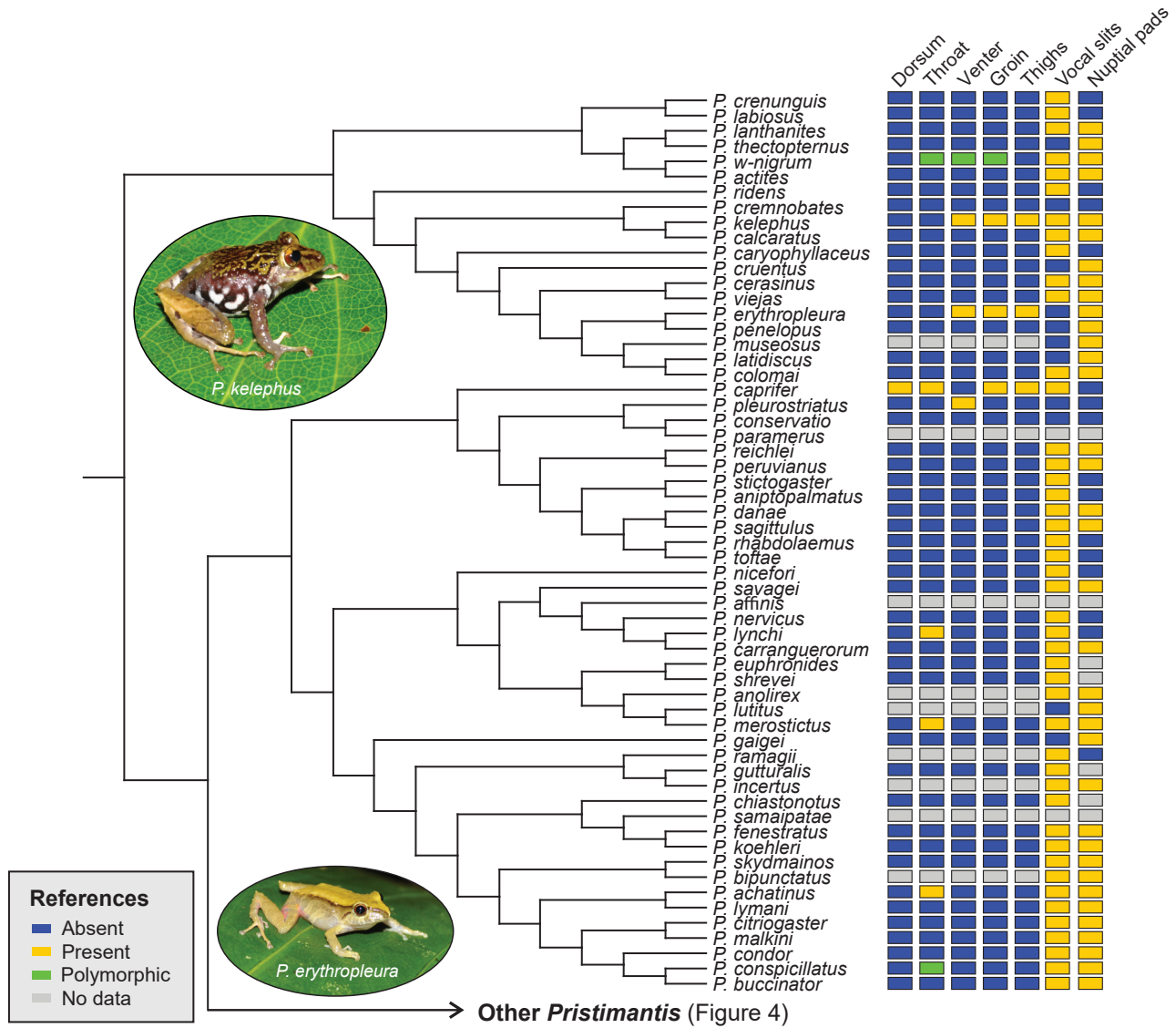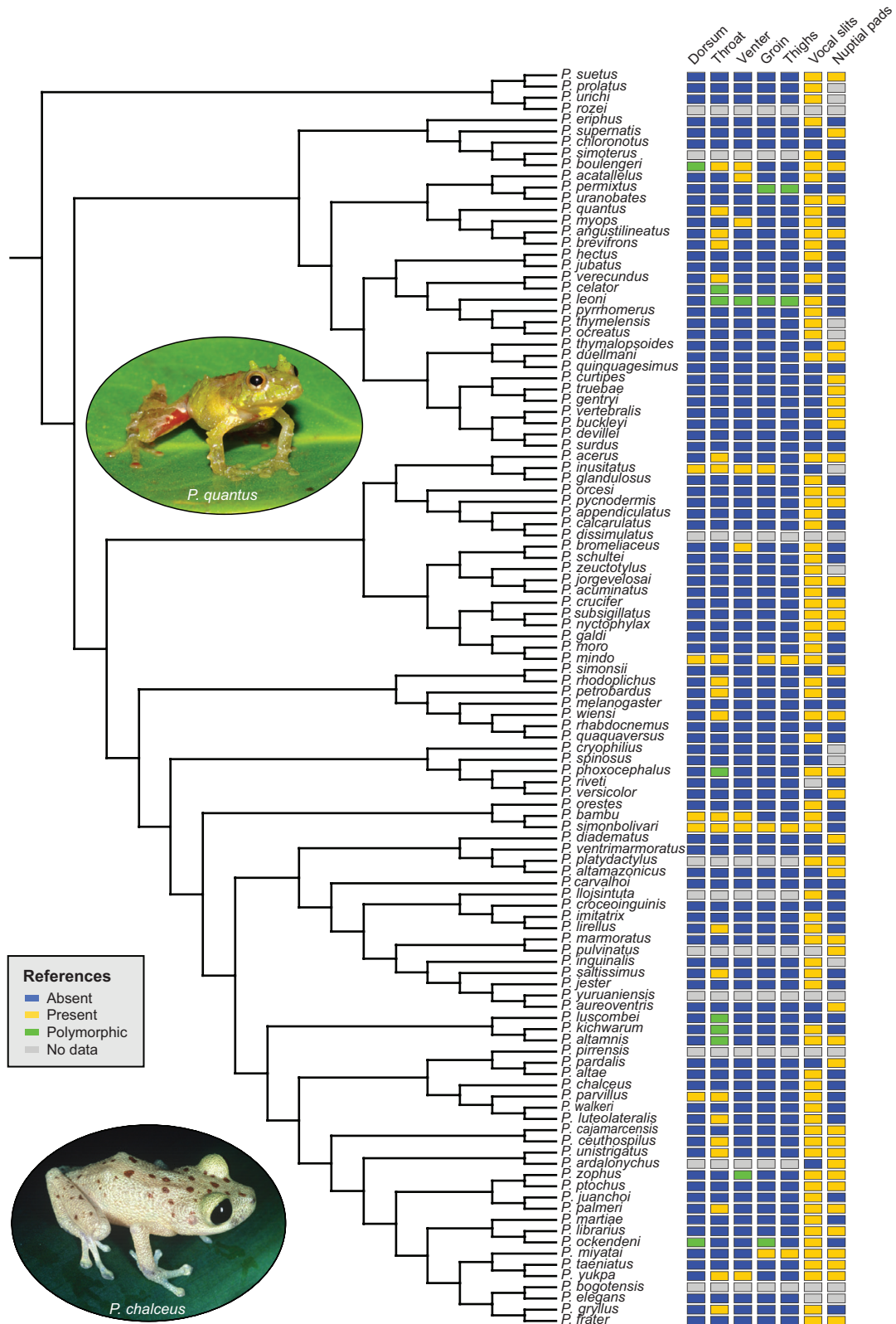
Figure 3. Partial distribution of Characters 1–7 in *Pristimantis*. The phylogenetic hypothesis for *Pristimantis* is that of Jetz & Pyron (2018). The complete parsimony ACSRs for all characters are shown in the Supporting Information (Appendices S5–S9, S15–S16).

the number of species recognized as sexually dichromatic from 6 (Bell & Zamudio, 2012; Bell *et al.*, 2017) to 36. By considering outgroup species, our study updates the reported number of sexually dichromatic anurans from 350 (Bell & Zamudio, 2012; Bell *et al.*, 2017; Engelbrecht-Wiggans & Tumulty, 2019; Portik *et al.*, 2019; Greener *et al.*, 2020) to 383 species.

This work contributes to the understanding of sexual dichromatism in *Pristimantis*, demonstrating that at least five independent transformation series are involved in it. The body regions in which the dichromatism is present are traditionally linked to intraspecific communication (i.e. dorsum, throat, venter, groin and posterior surface of thighs; Hödl &

Amezquita, 2001; Maan & Cummings, 2008; de Sá *et al.*, 2016; Engelbrecht-Wiggans & Tumulty, 2019). Our ACSRs reveal major evolutionary patterns of sexual dichromatism in *Pristimantis*, including multiple independent gains with no losses and differences in the number of independent origins between the body regions (Table 1). Among the few studies available in the literature on the evolution of sexual dichromatism in anurans, Portik *et al.* (2019) found one single origin of sexual dichromatism with subsequent losses in frogs of the family Hyperoliidae. However, it is important to note that Portik *et al.* (2019) considered the occurrence of sexual dichromatism as a single character, despite its variation across body regions is not coextensive and

Figure 4. Partial distribution of Characters 1–7 in further species of *Pristimantis*. The phylogenetic hypothesis for *Pristimantis* is that of Jetz & Pyron (2018). The complete parsimony ACSRs for all characters are shown in the Supporting Information (Appendices S5–S9, S15–S16).

there appears to be several independent characters involved. In contrast, we analysed the body regions independently, finding that the throat has the highest number of independent origins (17). It is worth mentioning that the throat is commonly involved in visual signalling behaviour in anurans (Rosenthal *et al*., 2004; Hirschmann & Hödl, 2006; Starnberger *et al*., 2014a; de Sá *et al*., 2016), therefore further investigation will be required to determine if colour diversity on the throat is driven by sexual selection.

In tetrapods, there is strong evidence showing that sexual dichromatism varies independently between the body regions (Stuart-Fox & Ord, 2004; Marcondes & Brumfield, 2019; Delhey, 2020). For example, some studies found that some body areas are more subject to exhibit sexual dichromatism than others, such as the dorsum and the venter in lizards (Pérez i de Lanuza *et al*., 2013) and the dorsum, head and chest in birds (Negro *et al*., 2018). Similar results have been reported for non-tetrapod vertebrates. Hodge *et al*. (2020) reported the occurrence of conspicuous colours on exposed body regions in males of wrasse and parrotfish (Labridae). In contrast, Bossu & Near (2015) reported the ability of darters (Percidae) to conceal sexually dichromatic body parts such as dorsal fins until females or rival males are present. Despite the accumulation of evidence showing that sexual dichromatism evolves independently between the regions of the body; sexual dichromatism in phylogenetic studies is generally treated as one character (e.g. Pérez i de Lanuza *et al*., 2013; Simpson *et al*., 2015; Møller *et al*., 2016; Negro *et al*., 2018; Portik *et al*., 2019). Such reasoning undoubtedly conflated characters and character-states. The position we take here is that the occurrence of sexual dichromatism is transformationally independent between the regions of the body, and, as such, they are defensibly coded separately for analysis.

Among the studies that treated the occurrence of sexual dichromatism as transformationally independent between the regions of the body, it has been demonstrated that natural selection constrains the evolution of sexual dichromatism of body regions exposed to visual predators whereas dichromatism of 'concealed' body regions appears to be driven to a greater degree by sexual selection (Stuart-Fox & Ord, 2004; Bossu & Near, 2015; Hodge *et al*., 2020). In comparing our results with these studies, we found that in *Pristimantis* frogs, the greatest number of independent origins of sexual dichromatism appears in concealed body regions (throat and venter, see Table 1). In contrast, the least number of independent origins occurs in the most exposed body region (dorsum). That is, our results seem to agree with previous findings regarding the contribution of natural and sexual selection in the evolution of sexual dichromatism. In

particular, natural and sexual selection might help explain the remarkable differences in the number of independent origins of sexual dichromatism between exposed and concealed body regions in *Pristimantis*. Further investigation into the evolution of sexual dichromatism in the context of complex interactions between natural and sexual selection in frogs of the genus *Pristimantis* is warranted.

Another topic that deserves further investigation in the evolution of sexual dichromatism in *Pristimantis* frogs is the association between sexual dichromatism and diversification. The occurrence of sexual dichromatism is often associated with diversification rates in some taxonomic groups, including fishes (e.g. Wagner *et al*., 2012), birds (e.g. Seddon *et al*., 2008), lizards (e.g. Stuart-Fox & Owens, 2003) and anurans (Portik *et al*., 2019). In frogs, Portik *et al*. (2019) reported that sexual dichromatism was indeed related to the diversification rates within the Afrobatrachia clade, where sexually dichromatic lineages nearly double the average diversification rate of monochromatic lineages. Considering that *Pristimantis* is the most diverse genus of anuran with over 590 species (Frost, 2022), as well as the complex pattern of multiple independent gains with no losses involved in the evolution of sexual dichromatism, future research will define whether any character state related to sexual dichromatism could be linked to the diversification in this genus.

Our results also demonstrated that the absence of sexual dichromatism across body regions is plesiomorphic in *Pristimantis* and closely related genera (*Lynchius*, *Niceforonia*, *Oreobates*, *Phrynopus*, *Yunganastes*) (see Appendices S5–S9 of the Supporting Information). Similar findings regarding monochromatism as the ancestral state were reported in Hylidae (Bell *et al*., 2017) and Afrobatrachia (Portik *et al*., 2019). In addition, Bell *et al*. (2017) suggested that in hylids, dynamic sexual dichromatism may be an evolutionary intermediate from the plesiomorphic state of monochromatism to ontogenetic sexual dichromatism. We are unaware of any *Pristimantis* species in which dynamic sexual dichromatism occurs; however, it should be pointed out that further studies may reveal that we have conflated transformations involving dynamic and ontogenetic dichromatism.

In Anura, previous studies have linked colour characteristics and chromatic diversity to sexual selection (Summers *et al*., 1997; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008, 2009; Hettyey *et al*., 2009; Rojas, 2016; Dreher *et al*., 2017). For example, empirical evidence shows that sexual dichromatism may act as a type of visual signal evolved to promote swift mate recognition and mate finding in dense aggregations (Sztatecsny *et al*., 2012; Rehberg-Besler *et al*., 2015). Accordingly, Bell *et al*. (2017) found a correlation between the evolution of dynamic sexual

dichromatism and the evolution of large breeding aggregations in the family Hylidae. In *Pristimantis*, the reproductive behaviour of many species remains unknown (Waldez *et al.*, 2011), and there are few reproductive behaviour data available for sexual dichromatic species. Despite this lack of information, the available data suggest that there is no association between reproductive modes and sexual dichromatism in *Pristimantis* given that explosive breeding occurs in monochromatic as well as in dichromatic species (e.g. *Pristimantis* aff. *fenestratus* and *Pristimantis merostictus* (Lynch, 1984); Waldez *et al.*, 2011; Ramírez-Pinilla & Granados-Pérez, 2020; see Appendix S2 of the Supporting Information). Further studies of *Pristimantis* mating and reproductive behaviour are needed to test the role of sexual dichromatism on mate choice in anurans.

Nuptial pads and vocal slits evolution

Our results suggest that in *Pristimantis*, nuptial pads and vocal slits possess more complex evolutionary histories than any of the characters of sexual dichromatism (see Table 1). By comparing our results with previous works regarding nuptial pads (i.e. Emerson, 1994; Pereira *et al.*, 2015), it is suggested that in *Pristimantis* occurs a more complex scenario for the evolution of this character. In total, 38 evolutionary transformation events (20 regains, five losses and 13 transformations in which optimization does not distinguish between gains and losses; see Table 1) were inferred to occur along *Pristimantis*, four times those reported in Leptodactylinae by Pereira *et al.* (2015).

The occurrence of nuptial pads has been cited in most alpha taxonomic studies of anurans (e.g. Lynch & Duellman, 1997; Duellman & Lehr, 2009). Although there is some variation among species in the extension, coloration, size, occurrence and structure of epidermal projections, pore morphology, nuptial gland structure and histochemistry of the nuptial pads (Luna *et al.*, 2018), explicit delimitations of character-states within the nuptial pads are generally lacking. In addition, we are also aware of some *Pristimantis* species that possess nuptial pads on two parts of the hand: (i) the dorsomedial surface on the base of the thumb and (ii) the outer edge of the thenar tubercle [*Pristimantis librarius* (Flores & Vigle, 1994), *Pristimantis pulvinatus* (Rivero, 1968), *Pristimantis avius* (Myers & Donnelly, 1997) and *Pristimantis reichlei* Padial & De la Riva, 2009; Flores & Vigle, 1994; Myers & Donnelly, 1997; Padial & De la Riva, 2009]. Future studies will undoubtedly advance considerably beyond the current study by scoring more of the phenotypic diversity of the nuptial pads in Anura.

Vocal slits and vocal sacs are coextensive and are clearly functional and phylogenetically dependent. The occurrence of vocal slits in a phylogenetic context has been assessed mostly through the occurrence of vocal sacs (Emerson, 1994; Elias-Costa & Faivovich, 2019; Targino *et al.*, 2019; Moura *et al.*, 2021). By comparing our results with these works, we found that the presence of vocal sacs in males is also plesiomorphic in Microhylidae, but has been lost independently at least in two internal clades, *Hoplophryne* Barbour & Loveridge, 1928 (Hoplophryninae) and *Ctenophryne* Mocquard, 1904 (Gastrophryninae) (Targino *et al.*, 2019), as well as in Rhacophoridae, Mantellidae, Dicroglossidae and Ranidae (Elias-Costa & Faivovich, 2019), and in the Lophyohylini (also plesiomorphic for the Hylidae), with three independent losses (Moura *et al.*, 2021). In addition to addressing the evolution of vocal sacs, Moura *et al.* (2021) identified three character-states accounting for the variation in the shape of vocal slits in Hylidae (small orifice, elongated slits gaping hole). Considering that there is growing evidence demonstrating that vocal slits present variation in relation to the number, shape, length and position (Savage & Heyer, 1969; Elias-Costa *et al.*, 2017; Moura *et al.*, 2021), future research may likely determine that our vocal slits coding was a simplification.

Previous studies have suggested that vocal sacs can be involved in multimodal signalling in intra- and intersexual interactions, enhancing the attractiveness to females and aggression during territorial male-male interactions (Rosenthal *et al.*, 2004; Hirschmann & Hödl, 2006; Taylor *et al.*, 2008; Starnberger *et al.*, 2014a; de Sá *et al.*, 2016). Consequently, the evolution of vocal slits seems to be more influenced by divergent sexual selection than natural selection (Rosenthal *et al.*, 2004; Ryan & Guerra, 2014; Starnberger *et al.*, 2014b). Nonetheless, multiple experiments suggest that the dynamically moving vocal sac in calling males increases detection by predators (Halfwerk *et al.*, 2014, 2017; Rhebergen *et al.*, 2015; Gomes *et al.*, 2016, 2017), suggesting that natural selection may be acting on the evolution of vocal slits. Thus, further work is needed to understand how the complex interaction between natural and sexual selection shapes the evolution of vocal slits/sacs in *Pristimantis*.

An overview of secondary sexual characters in anurans

Patterns of sexual dichromatism, and occurrence of vocal slits and nuptial pads in Anura have been mainly studied from the point of view of sexual selection (Liu, 1950; Shine, 1979; Buchanan, 1994; Emerson, 1996; Blackburn, 2009; Magalhães *et al.*, 2018; Bossuyt *et al.*, 2019; Portik *et al.*, 2019). Nevertheless, recent studies have demonstrated that evolution of secondary sexual characters such as vocal slits seem to be more

influenced by natural selection (Halfwerk *et al.*, 2014, 2017; Rhebergen *et al.*, 2015; Gomes *et al.*, 2016, 2017). Our results also suggest that natural selection might constrain the evolution of sexual dichromatism of body regions exposed to visual predators in *Pristimantis*.

In the case of nuptial pads, the selective forces governing their evolution are still poorly understood. According to Noble (1931: p. 108), the best-known secondary sexual characters are the nuptial pads which appear on the prepollex region of many frogs and toads during the breeding season. Nevertheless, Noble defined secondary traits operationally and makes no mention of either natural or sexual selection. Considering that nuptial pads may play an important role for male-male combat, releasing protein pheromones, facilitating the grip of a female or to avoid being dislodged from a female by another male during the amplexus (Kurabuchi, 1993; Thomas *et al.*, 1993; Duellman & Trueb, 1994; Kyriakopoulou-Sklavounou *et al.*, 2012; Luna *et al.*, 2012, 2018; Willaert *et al.*, 2013; Bossuyt *et al.*, 2019), it is not easy to infer to what extent each of the two selective forces might be involved. For example, when nuptial pads play a role associated with obtaining mates or access to mates (e.g. male-male combat or to avoid being dislodged from a female), the nuptial pads would be directly affected by sexual selection; however, when nuptial pads may make it easier and less costly to engender offspring and thereby increase reproductive output, such characters would be primary sexual characters, produced by natural selection (Ghiselin, 2010). To date, there is no evidence that nuptial pads of any *Pristimantis* play a role associated with obtaining mates or access to mates, which suggests that the evolution of nuptial pads in *Pristimantis* could be explained by natural selection.

By compiling the existing information, it is suggested that some sex characters that might be thought of as secondary in anurans are really primary. In addition, it is also evident that the extent to which we consider a sex character primary or secondary may be a challenging task given the serious difficulties in identifying the selective agents that bring about evolutionary diversity. This is clearly an area that would benefit from additional work.

New phenotypic synapomorphies

Our results demonstrated that the presence of nuptial pads is a new morphological synapomorphy for the genus *Yunganastes* (see Appendix S16 of the Supporting Information). Currently, *Yunganastes* is diagnosed based on molecular data and the path of the mandibular ramus of the trigeminal nerve with respect to the m. adductor mandibulae (Padial *et al.*, 2007, 2014). Our results also demonstrated that the

absence of vocal slits and the occurrence of nuptial pads are two putative new synapomorphies for the *P. devillei* species group (see Appendices S15–S16 of the Supporting Information), a clade only diagnosed by the presence of a cranial crest (Hedges *et al.*, 2008; Padial *et al.*, 2014). Finally, the presence of nuptial pads was recovered as a synapomorphy for a clade composed of 18 species currently not included in any recognized species group within the genus *Pristimantis sensu* Padial *et al.* (2014): *P. cajamarcensis* (Barbour & Noble, 1920), *P. ceuthospilus* (Duellman & Wild, 1993), *P. unistrigatus* (Günther, 1859), *P. ardalonychus* (Duellman & Pramuk, 1999), *P. zophus* (Lynch & Ardila-Robayo, 1999), *P. ptochus* (Lynch, 1998), *P. juanchoi* (Lynch, 1996), *P. palmeri* (Boulenger, 1912), *P. martiae* (Lynch, 1974), *P. librarius*, *P. ockendeni* (Boulenger, 1912), *P. miyatai* (Lynch, 1984), *P. taeniatus* (Boulenger, 1912), *P. yukpa* Barrio-Amorós *et al.*, 2008, *P. bogotensis* (Peters, 1863), *P. elegans* (Peters, 1863), *P. gryllus* and *P. frater* (Werner, 1899) (see Appendix S16 of the Supporting Information). On this basis, we propose to recognize this clade as the *Pristimantis unistrigatus* group. The *Pristimantis unistrigatus* group was defined by Lynch & Duellman (1997) based on overall similarity in external morphology rather than synapomorphies. The group was demonstrated non-monophyletic by Hedges *et al*. (2008); however, they retained it as defined by Lynch & Duellman (1997). Herein, we propose to recognize a *P. unistrigatus* group that includes 18 species, diagnosed on the presence of nuptial pads. In addition to these unambiguously optimized synapomorphies, our results also showed that character-states related to vocal slits and nuptial pads optimize ambiguously for some clades (e.g. *Lynchius*, *Oreobates* and the *Pristimantis conspicillatus* species group). In order to clarify these ambiguities, future studies that increase taxon sampling and improve the delimitation and scoring of the transformation series will define whether any character-state related to vocal slits and nuptial pads could be considered as a synapomorphy of some of these clades.

To date, no study has combined DNA sequences with phenotypic evidence in the phylogenetic analyses of *Pristimantis*. Consequently, most recognized clades within *Pristimantis* lack phenotypic synapomorphies. This situation has led to a lack of confidence in phenotypic evidence and even to speculate the well-recognized failure of morphology to describe the diversity within *Pristimantis* (Waddell *et al.*, 2018). Contrary to this assumption, recent empirical evidence demonstrates that phenotypic data are a valuable source of evidence in testing phylogenetic hypotheses in these frogs (Ospina-Sarria & Grant, 2021). Hence, it has been suggested that instead of simply optimizing taxonomic characters on trees, it is imperative to delimit the characters objectively as transformation

series and test their phylogenetic significance. Our results support the assertion that phenotypic data are a valuable source of evidence for testing phylogenetic hypotheses in frogs of the genus *Pristimantis*.

CONCLUSION

Here we provide the first comprehensive assessment of the diversity and evolution of the secondary sexual characters in frogs of the species-rich genus *Pristimantis*. We demonstrate that sexual dichromatism in *Pristimantis* evolves independently across body regions (dorsum, throat, venter, groin, posterior surface of thighs). We also show differences in the sequence of gains with no losses of sexual dichromatism between concealed and exposed body regions. By delimiting and scoring transformation series related to sexual dichromatism, we hope to contribute to the understanding of the evolution of sexual dichromatism in *Pristimantis*. In line with previous studies (Stuart-Fox & Ord, 2004; Maan & Cummings, 2009; Bossu & Near, 2015; Hodge *et al*., 2020) and considering that sexual dichromatism acts differentially on sexes and is expressed in regions linked to communication, future studies dealing with chromatic communication across body regions may contribute to revealing the selective pressures acting on sexual dichromatism in *Pristimantis*.

Ancestral reconstructions of nuptial pads and vocal slits reveal more complex evolutionary histories in comparison to characters related to sexual dichromatism in *Pristimantis*. Also, available evidence clearly indicates that the presence of vocal slits and the absence of nuptial pads are plesiomorphic for *Pristimantis*. To date, the evolution of nuptial pads and vocal slits has been poorly documented in Anura. We demonstrate that nuptial pads and vocal slits are phylogenetically informative at different taxonomic levels, allowing us to identify four synapomorphies. Overall, our findings provide new opportunities to test functional hypotheses related to the occurrence of nuptial pads and vocal slits in Anura.

To summarize, we have shown that the evolution of sexual dichromatism, vocal slits and nuptial pads in *Pristimantis* follows a rather complex pattern of multiple independent transformation events, making them a suitable system for studying the interaction of natural and sexual selection in the evolution of secondary sexual characters in Anura.

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DATA AVAILABILITY

The data underlying the work are available in the Supporting Information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Specimens examined.
Appendix S2. Sources for characters scored by species.

Appendix S3. Distribution of secondary sexual characters and their states (numbers) among the studied species.

Appendix S4. R script used for ML and BI analyses.

Appendix S5. Parsimony ACSR of Character 1: Sexual dichromatism on the dorsum in *Pristimantis*.

Appendix S6. Parsimony ACSR of Character 2: Sexual dichromatism on the throat in *Pristimantis*.

Appendix S7. Parsimony ACSR of Character 3: Sexual dichromatism on the venter in *Pristimantis*.

Appendix S8. Parsimony ACSR of Character 4: Sexual dichromatism on the groin in *Pristimantis*.

Appendix S9. Parsimony ACSR of Character 5: Sexual dichromatism on the posterior surface of the thighs in *Pristimantis*.

Appendix S10. ACSRs of Character 1 using ML and BI: Sexual dichromatism on the dorsum. A, considering all rates different with maximum likelihood (ARD). B, considering equal transition rates with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 1 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.

Appendix S11. ACSRs of Character 2 using ML and BI: Sexual dichromatism on the throat. A, considering all rates different with maximum likelihood (ARD). B, considering equal transition rates with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 2 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.

Appendix S12. ACSRs of Character 3 using ML and BI: Sexual dichromatism on the venter. A, considering all rates different with maximum likelihood (ARD). B, considering equal transition rates with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 3 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.

Appendix S13. ACSRs of Character 4 using ML and BI: Sexual dichromatism on the groin. A, considering all rates different with maximum likelihood (ARD). B, considering equal transition rates with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 4 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.

Appendix S14. ACSRs of Character 5 using ML and BI: Sexual dichromatism on the posterior surface of thighs. A, considering all rates different with maximum likelihood (ARD). B, considering equal transition rates with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 5 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.

Appendix S15. Parsimony ACSR of Character 6: Vocal slits in *Pristimantis*. Presence of vocal slits is plesiomorphic in *Pristimantis*, with 19 losses, four regains and six transformations in which optimization does not distinguish between gains and losses.

Appendix S16. Parsimony ACSR of Character 7: Nuptial pads in *Pristimantis*. Absence of nuptial pads is plesiomorphic in *Pristimantis*, with 19 independent origins of nuptial pads, followed by five losses and 13 transformations in which optimization does not distinguish between gains and losses.

Appendix S17. ACSRs of Character 6 using ML and BI: Vocal slits. A, considering all rates different with maximum likelihood (ARD). B, considering equal transition rates with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 6 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.

Appendix S18. ACSRs of Character 7 using ML and BI: Nuptial pads. A, considering equal transition rates with maximum likelihood (ARD). B, considering all rates different with maximum likelihood (ER). C, considering all rates different with stochastic character mapping. D, restricting the ancestor to possess Character 7 + all rates different with maximum likelihood. E, considering Dollo's model (no regains possible) with stochastic character mapping.