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Phenotypic plasticity raises questions for taxonomically important traits: a remarkable new Andean rainfrog (*Pristimantis*) with the ability to change skin texture

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We describe a new frog, *Pristimantis mutabilis* sp. nov., from the Andes of Ecuador. Individuals of the new species are remarkable for their ability to change skin texture from tuberculate to almost smooth in a few minutes, being the first documented amphibian species to show such dramatic phenotypic plasticity. The new taxon is assigned to the *P. myersi* group. It differs from other members of its group by body size (adult males 17.2–17.4 mm; adult females 20.9–23.2 mm), arboreal habitat, and red flash coloration in females. We document three call types for the new species, which differ through their number of notes and amplitude peaks. The three types are pulsed calls that share a dominant frequency of 3186.9–3445.3 Hz. Surprisingly, we also document similar skin texture plasticity in species (*P. sobetes*) from a different species group, suggesting that this ability might be more common than previously thought. The discovery of these variable species poses challenges to amphibian taxonomists and field biologists, who have traditionally used skin texture and presence/absence of tubercles as important discrete traits in diagnosing and identifying species. Reciprocal monophyly and genetic distances also support the validity of the new species, as it has distances of 15.1–16.3% (12S) and 16.4–18.6% (16S) from the most similar species, *Pristimantis verecundus*. Additionally, each of the two known populations of *Pristimantis mutabilis* are reciprocally monophyletic and exhibit a high genetic distance between them (5.0–6.5%). This pattern is best explained by the presence of a dry valley (Guayllabamba River) that seems to be acting as a dispersal barrier.

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ADDITIONAL KEYWORDS: Amphibia – Andes – intraspecific variation – new species – *Pristimantis myersi* group – *Pristimantis sobetes* – skin texture – taxonomy.

INTRODUCTION

Although intraspecific variation in traits is widespread in nature, there is still a marked tendency to describe species based on a single specimen. This practice is a legacy of Plato's archetype concept, which assumes that intraspecific variation is limited or nonexistent. As a clear example of how our understanding of natural variation is limited, Lim, Balke & Meier (2012) reported that, in a 10-year period (2000–2010), a large proportion of newly described species (17.7% of invertebrates; 19% of vertebrates) are known only from a single specimen, a practice that is likely

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to result in an overestimation of diversity and taxonomic confusion.

In amphibians, reports of intraspecific variation tend to be limited to coloration or conspicuous sexual dimorphism. For example, many species of frogs have intraspecific variation in coloration that act as a warning signal for toxicity in poisonous species (e.g. Santos, Coloma & Cannatella, 2003), sexual selection (e.g. Siddiqi *et al.,* 2004), or crypsis that aids in concealment from predators (Wells, 2007). Additionally, permanent sexually dimorphic traits include humeral spines of glassfrogs (see Guayasamin *et al.,* 2009), thumb-daggers in Otton frogs (Tokita & Iwai, 2010), and vocals sacs of most anuran males (Duellman & Trueb, 1994). However, some morphological traits are temporary, such that males may acquire barbs, glands or tubercles during the breeding season that facilitate territory defence or mating (Tsuji & Matsui, 2002; Tsuji, 2004; Cadle, 2008). In these cases, taxonomists have used these traits for diagnosing species, as they can be easily observed on museum specimens after preservation.

Phenotypic plasticity, defined as the ability of an individuals to alter its phenotype in response to environmental changes (see West-Eberhard, 2003), is either rare or has been largely overlooked in amphibians (but see Buskirk & Relyea, 1998; Relyea, 2001; Auld, Agrawal & Relyea, 2010). Outside of the well studied cases of predator-induced phenotypic plasticity in amphibians (reviewed by Bernard, 2004), one of the few common examples is individual rapid colour change in amphibians and fish. This change is produced by the synchronous movement of pigment organelles within pigmented cells (chromatophores) in the skin, as well as in changes in angles of light-reflecting crystals in iridophores and leucophores (e.g. fish: Fujii & Oshima, 1994; Fujii, 2000; frogs: Aspengren *et al.,* 2009). Rapid colour change may allow the individual to match its coloration to the background substrate (e.g. surface of a leaf, or leaf litter) to become concealed and avoid predators. Additionally, individuals may change their coloration for communication and sexual display (Nilsson Sköld, Aspengren & Wallin, 2013 and references therein). Despite the commonness of phenotypic plasticity in colouration, plasticity in other traits frequently used in taxonomic studies is extremely rare or has not been observed.

Herein, we describe a striking case of phenotypic plasticity in a new frog species (genus *Pristimantis*) from the western Andes of Ecuador. The new species has the ability to dramatically alter its skin texture within minutes, changing between two discrete character states. Historically, amphibian taxonomists have consistently used skin texture and presence/absence of tubercles as discrete traits to identify and describe species (e.g., Jiménez de la Espada, 1872; Boulenger, 1882; 1918; Noble, 1924; Lynch & Duellman, 1997; Savage, 2002). Surprisingly, we also find a second species (*Pristimantis sobetes* (Lynch, 1980)) from a different species group that shares this extreme phenotypic plasticity. These discoveries unveil a new challenge for amphibian taxonomists, as plasticity in taxonomically important traits thought to be stable (e.g., presence or absence of tubercles) to our knowledge has not been reported previously.

METHODS

Terminology and morphological data

Generic and family names follow the taxonomy proposed by Hedges, Duellman & Heinicke (2008), as modified by Pyron & Wiens (2011; also see Frost, 2014). Specimens were sacrificed with 20% benzocaine, fixed and stored in 75% ethanol (no formalin was used). Diagnoses and descriptions follow those described by Lynch & Duellman (1997). We examined comparative alcohol-preserved specimens from the amphibian collections at the Museo de Zoología of the Universidad Tecnológica Indoamérica (MZUTI) and Instituto de Ciencas Naturales of the Universidad Nacional de Colombia (ICN) (Appendix 1). Morphological measurements were taken with Mitutoyo® digital caliper to the nearest 0.1 mm, as described by Guayasamin & Bonaccorso (2004), except when noted. Measurements were as follow: (1) snout–vent length (SVL); (2) tibia length; (3) foot length; (4) head length; (5) head width; (6) interorbital distance; (7) upper eyelid width; (8) internarial distance; (10) eye diameter; (11) tympanum diameter; (12) radioulna length; (13) hand length; (14) Finger I length; (15) Finger II length = distance from outer margin of palmar tubercle to tip of Finger II; (16) disc of Finger III. Sexual maturity was determined by the presence of vocal slits in males and by the presence of eggs or convoluted oviducts in females.

Skin texture variation

Phenotypic variation of skin texture was recorded in a series of photographs taken across a timeframe of 330 s. The frog was sighted at 2200 h on July 28, 2009 on Santa Rosa River Trail of Reserva Las Gralarias upon a *Melastoma* sp. leaf approximately 1 m above the ground. Photographs were taken at approximately 9 h post-capture using a Nikon D200, Sigma 28–135 mm D lens and Sigma EM-140 DG ring flash. Upon capture, the frog had a strong tuberculate appearance and was stored in a polyethylene holding container. The frog was then placed back into the container and moss was added to provide moisture and shelter. Several minutes later, the frog was removed from the container and placed immediately onto a smooth white surface and photographs were taken over the 330 s period. Variation of skin texture was observed and

photographed in two additional individuals (i.e. adult male MZUTI 2190 and sub-adult male MZUTI 2191) in order to confirm that the phenomenon of plasticity is recurrent in the species.

GENETICS

We targeted the mitochondrial ribosomal genes 12S and 16S and the methods for DNA extraction, amplification, and sequencing are described in Guayasamin *et al*. (2008). We obtained genetic data for 23 specimens (Appendix 1) from the newly described taxon, *Pristimantis verecundus* (Lynch & Burrowes, 1990), *P. sobetes*, and *Pristimantis* sp., as they are morphological similar to the new species.

We supplemented these new data with published sequences of *Pristimantis* specimens from GenBank. Recent molecular studies have shown that most species groups need revision (e.g. Pinto-Sánchez *et al*., 2012; Padial, Grant & Frost, 2014) and, as a result, the selection of genetic data can be challenging. To address this aspect, we first created a preliminary maximum likelihood tree (see methods below) using all individuals of *Pristimantis* for which 12S and 16S rRNA data were available on GenBank. We included multiple specimens per species, as previous studies have noted the presence of cryptic and misidentified specimens, especially in *Pristimantis* (Padial *et al*., 2014, see Table 1). Next, we selected the specimens most closely related to our new samples, choosing those specimens that formed the largest clade that included all members of the *P. surdus* and *P. myersi* species groups (the clade was highly supported). The selection included 22 species (with 34 total individuals) from the *P. surdus*, *P. myersi*, and *P. unistrigatus* species groups. Finally, we included distantly related *Pristimantis actites* as the outgroup (Hedges *et al*., 2008). Specimens used in these analyses and their GenBank accession numbers are included in Appendix 2.

The sequences were initially aligned using Geneious (Biomatters, 2014) using MAFFT v7 (Katoh & Standley, 2013). We then manually aligned the RNA sequence data to the stem and loop secondary structures. These structures can differ in their evolutionary model and rate of substitution and were partitioned separately (following Wiens *et al*., 2005). We conducted all analyses for 12S and 16S separately and then concatenated the genes.

For the maximum likelihood estimation, we used the RAxML 7.2.0 program (Stamatakis, 2006). We used the GTR + Γ model of nucleotide substitution, which accounts for invariant sites by using 25 rate categories for Γ. The dataset was partitioned by stem and loop structures for each gene (12S and 16S). Next, we used the '-f a' function to search simultaneously for the optimal likelihood tree and conduct a bootstrap analysis. We performed 100 tree searches and assessed node support using 1000 bootstrap replicates.

For Bayesian analyses, we used the MrBAYES 3.2.1 program (Huelsenbeck & Ronquist, 2001). First, we used JMODELTEST 2.0 (Posada, 2008) for each partition to select the model of sequence evolution that best fitted the data, using Akaike's information criterion (AIC; Akaike 1974). The selected model for 12S and 16S stem partitions was the SYM + I + G model (Symmetrical Model with equal base frequencies and a proportion of invariant sites and a gamma distribution for rates across sites). The best-fit model for 12S and 16S loop partitions was the GTR + I + G model (General

Table 1. Morphometrics (in mm) of the holotype (MZUTI 2190) and additional specimens of *Pristimantis mutabilis* sp. nov.

	MZUTI 2190 Male	MZUTI 912 Male	MZUTI 413 Female	MZUTI 910 Female	MZUTI 911 Female	MZUTI 913 Female
SVL	17.2	17.4	21.1	23.2	20.8	20.9
Tibia	8.2	8.3	10.3	10.8	10.9	10.6
Foot	8.3	8.7	11.4	11.8	10.5	10.7
Head length	6.4	6.9	7.8	8.4	8.1	7.7
Head width	6.2	6.3	7.7	7.8	7.8	7.5
Snout to eye distance	2.8	2.8	3.2	3.7	3.2	3.2
Interorbital distance	1.7	1.9	1.8	2.0	2.1	2.2
Upper eyelid width	1.6	1.7	2.0	1.9	2.0	1.8
Eye diameter	2.4	2.4	2.6	3.0	2.8	2.8
Tympanum	0.9	0.7	1.0	0.8	0.8	0.8
Radioulna length	4.1	4.3	5.0	5.6	5.4	5.5
Hand	4.8	5.2	6.4	6.8	6.1	6.1
Finger I	2.4	2.9	3.7	3.9	3.8	3.6
Finger II	3.4	3.4	4.1	4.3	4.0	4.0

Time-Reversible with a proportion of invariable sites and a gamma-shaped distribution for rates across sites). Next, we conducted an analysis for 20 million generations (sampling every 1000) with four Markov chains and default heating values. We used a uniform Dirichlet prior for the rate matrix and did not incorporate prior information on the topology. We ran the analysis twice to assess consistent convergence and stationarity, where we examined the standard deviation of split frequencies and plotted the –lnL per generation. We discarded the trees generated before stationarity as 'burn–in', which was the first 20% of trees.

Finally, to address genetic similarity, we calculated uncorrected pairwise genetic distances using Geneious (Biomatters, 2014). We also tested for reciprocal monophyly of the new species and its most morphologically similar species, *Pristimantis verecundus*. Reciprocal monophyly provides evidence that a lineage is separately evolving (and thus no gene flow) when a species clusters with its conspecifics on a phylogenetic tree. Significant statistical support is given through high bootstrap support (< 75%) in maximum likelihood analyses and high posterior probability support (< 0.95) in Bayesian analyses.

VOCALIZATIONS

Calls were recorded with an Olympus LS-10 Linear PCM Field Recorder and a Sennheiser K6–ME 66 unidirectional microphone with a sampling rate of 44.1 kHz s^{-1} with 16 bits/sample. Calls were analysed using the software RAVEN PRO 3.4 (Charif, Clark & Fristrup, 2004). The Fast Fourier Transformation size was set to 512 samples and the frequency grid resolution was 86.1 Hz. Digital recordings are deposited at MZUTI, and are available upon request.

Call parameter definitions follow Hutter *et al*. (2013) and references therein. Relevant call parameters used for this study are: call amplitude type (tonal or pulsed), number of calls per series, series duration (ms), series interval (ms), call duration (ms), interval between calls (s), pulses rate (/ms), call envelope (time of peak amplitude/call duration), dominant frequency, frequency modulation, lower and higher fundamental frequencies, and 1st harmonic and 2nd harmonic frequencies. We add an additional parameter to describe the frequency modulation of the calls throughout a series, which was measured by taking the difference between the dominant frequency (Hz) of the first and last call. A series of calls is defined as two or more calls emitted rapidly with consistent time intervals that are much shorter than intervals between series. A call is defined as the sound produced in a single exhalation of air. Pulsed calls are defined as having one or more clear amplitude peaks. Call variables were measured as described in Hutter & Guayasamin (2012). Measures are reported as the range followed by the mean ± two standard deviations from the mean.

RESULTS

Pristimantis mutabilis GUAYASAMIN, KRYNAK, KRYNAK, CULEBRAS, & HUTTER, SP. NOV.

Common English name
Mutable Rainfrog.

Common Spanish name
Cutín Mutable.

Holotype (Fig. 1)
MZUTI 2190, an adult male obtained by Juan M. Guayasamin on February 1, 2013, at Reserva Las Gralarias (00.00843° S, 78.7305° W; 2063 m.a.s.l.), Pichincha province, Ecuador.

Paratopotypes
MZUTI 2191, sub-adult male with same data as holotype. MZUTI 413, adult female collected by Carl R. Hutter on March 24, 2012, within Reserva Las Gralarias ('Puma Trail': 0.00954° S, 78.7346° W; 2030 m.a.s.l.).

Referred specimens
MZUTI 909 (juvenile), 910–911, 913 (adult females), and 912 (adult male) were collected by Amanda Delgado,

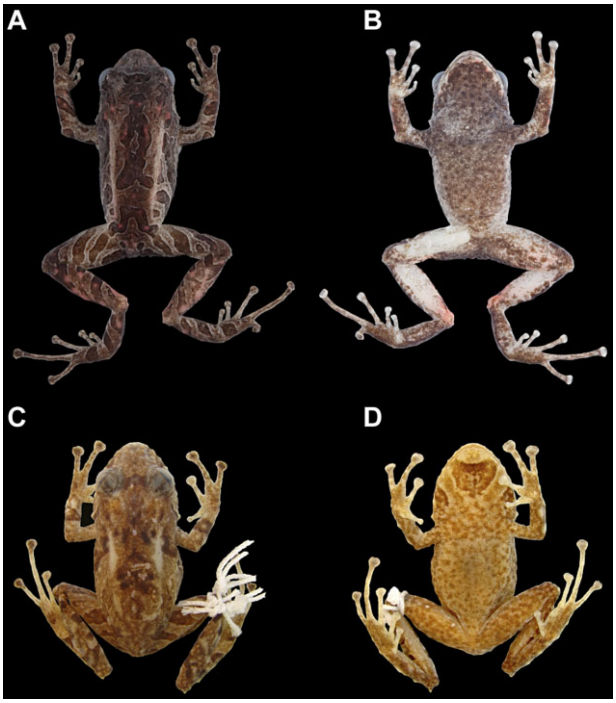

Figure 1. (A, B, *Pristimantis mutabilis* sp. nov., holotype, MZUTI 2190, in preservative. C, D, *Pristimantis verecundus*, holotype, IND-AN 1834, in preservative.

Jaime García, Fernando J. M. Rojas-Runjaic, Guissepe Gagliardi-Urrutia, Paloma Lima, and Juan M. Guayasamin, at Reserva Los Cedros (Sendero Oso: 0.3197 N, 78.7858 W, 1880 m; Sendero Cascada Nueva: 0.3249 N, 78.7809 W, 1850 m), Provincia Imbabura, Ecuador.

Generic and group placement

Pristimantis mutabilis is placed in the genus *Pristimantis*, as diagnosed by Hedges *et al*. (2008), because of the absence of cranial crests, and presence of dentigerous process of the vomers and T-shaped terminal phalanges. Within *Pristimantis,* the new species shares all traits that define the *P. myersi* group (*sensu* Hedges *et al*., 2008); these traits are: small body size (female SVL < 28 mm), robust body, short snout and relatively narrow head, Finger I shorter than Finger II, Toe V slightly longer than Toe III, tympanic membrane differentiated, cranial crests absent, vomerine teeth present and, in adult males, vocal slits present. Furthermore, generic and group placement is strongly supported by genetic analyses (see below).

Diagnosis

We provide a diagnosis based on preserved specimens; however, we emphasize that this species presents a marked skin texture variation that cannot be observed on museum specimens and is described in the *Variation of skin texture* section. *Pristimantis mutabilis* was diagnosed by having:

1. skin texture of dorsum shagreen, with low interspersed tubercles; dorsolateral folds present, low; occipital fold usually present, but low; venter areolate;
2. tympanic membrane present; tympanic annulus barely visible, with upper rim obscured by supratympanic fold;
3. snout short, rounded in dorsal and lateral views;
4. upper eyelid with one conical or sub-conical tubercle and several low tubercles, which are almost unnoticeable in some specimens; cranial crests absent;
5. dentigerous process of vomers small, oblique in outline, positioned posterior to level of choanae, each process bearing 1–4 teeth;
6. males with small sub-gular vocal sac; vocal slits present; nuptial pads absent;
7. first finger shorter than the second; discs on fingers elliptical, laterally expanded (Fig. 1);
8. fingers bearing narrow lateral fringes; palmar tubercle bifurcated distally; few supernumerary tubercles present, round, fleshy (Fig. 1);
9. ulnar tubercles present, but difficult to distinguish in some specimens; inner tarsal fold absent;
10. heel with conical tubercle; tarsal tubercles present;
11. toes bearing narrow fringes; webbing absent; Toe V longer than Toe III; toe discs rounded and slightly expanded (Fig. 1);
12. inner metatarsal tubercle elliptical, about 1.5–2 times the size of outer, rounded metatarsal tubercle; supernumerary plantar tubercles small, round, low, and fleshy (Fig. 1);
13. coloration in ethanol, dorsum pale brown to grey with darker chevrons outlined by thin, white lines; cream to pink dorsolateral stripes; flanks with diagonal dark stripes, outlined by a thin, white line; venter pale brown with small, darker brown spots. Coloration in life, dorsum light brown to pale greyish green, with dark brown chevrons outlined by cream or white, and green blotches; dorsolateral folds orange; venter greyish brown to brown with darker, diffuse spots, and few small white spots; iris cream to golden with thin black reticulation and reddish brown horizontal streak; in females, groin and hidden surfaces of legs red (Fig. 3);
14. relatively small, SVL in females 20.9–23.2 mm (mean = 21.5 ± 1.14, $N = 4$), in males 17.2–17.4 mm ($N = 2$).

Similar species

In the Pacific slopes of the Andes, the only species that has a similar size, morphology, and colour pattern is *P. verecundus* (Lynch & Burrowes, 1990). *Pristimantis verecundus* is easily distinguished by having short dorsolateral folds that only reach the level of sacrum (dorsolateral folds extend posteriorly beyond the level of sacrum in *P. mutabilis*). Females also have a unique chevron pattern on the throat (Fig. 1), which is absent in *P. mutabilis*. Additionally, adult males of *P. verecundus* are slightly larger [18.0–21.9 mm ($N = 4$); Lynch & Burrowes 1990] than males of *P. mutabilis* (17.2–17.3 mm; $N = 2$), although sample sizes are low. Genetically, populations of *P. verecundus* and *P. mutabilis* are reciprocally monophyletic with strong support and have a substantial uncorrected genetic distance of 16.2–16.8% in the combined 12S and 16S dataset (Fig. 4; see *Genetics* below).

Pristimantis sobetes (Lynch, 1980) co-occurs with *P. mutabilis* and also shares some morphological traits that could lead to misidentification (i.e. dorsolateral folds, dorsal colour pattern, skin texture plasticity). *Pristimantis sobetes* differs mainly by its non-overlapping larger size (adult male SVL = 20–23 mm; adult female SVL = 30–41 mm; Arteaga, Bustamante & Guayasamin, 2013), bright copper-red iris lacking a horizontal stripe (cream to gold iris with reddish horizontal stripe in *P. mutabilis*), and by lacking red flash coloration on the groin and hidden surfaces of thighs (present in *P. mutabilis* females). Furthermore, *P. sobetes* is genetically distant from *P. mutabilis* and is placed in the

P. surdus species group based on morphology (Hedges *et al.*, 2008); such placement is confirmed by genetics (Fig. 4).

Description of the holotype

We provide a description based on the preserved holotype; however, we emphasize that skin texture variation is conspicuous (see *Variation of skin texture* section). Adult male (MZUTI 2190; Fig. 1). Head slightly longer than wide, narrower than body; upper eyelid bearing one conical tubercle and several low tubercles; head width 36.0% of SVL; head length 37.2% of SVL; snout of moderate length (snout to eye distance 16% of SVL), rounded in dorsal and lateral views; tongue longer than wide, with posterior margin round (not notched); eye diameter larger than eye–nostril distance; nostrils not protuberant, directed anterolaterally; canthus rostralis weakly concave in profile; loreal region slightly concave; upper eyelid width 94% of interorbital distance; cranial crests absent; tympanic annulus distinct, except for upper border, which is obscured by supratympanic fold; tympanic membrane distinct; two postrictal tubercles situated postero-ventrally to tympanic annulus; choanae elliptical, not concealed by palatal shelf of maxillary; vomerine odontophores postero-medial to choanae, low, oblique in outline, separated medially by distance less than width of odontophore, each bearing two teeth; skin on dorsum finely shagreen with interspersed low tubercles; low dorsolateral folds present; skin of throat and venter with numerous low, round warts homogenously distributed; no discoidal or thoracic folds; cloacal sheath absent; two low ulnar tubercles evident; outer palmar tubercle large, bifurcated distally (Fig. 2); subarticular tubercles prominent, round; supernumerary palmar tubercles present, but few and low; fingers bearing narrow lateral fringes; Finger I conspicuously shorter than Finger II (Finger I length 70.6% of Finger II length); disc of Finger I slightly expanded; all other discs conspicuously expanded, elliptical in shape (Fig. 3); ventral pads defined by circumferential grooves.

Tibia length 47.7% of SVL; foot length 48.3% of SVL; heel tubercle conical; tarsal tubercles small, barely evident; inner metatarsal tubercle oval, about twice the size of the outer, rounded tubercle; sub-articular tubercles round; plantar supernumerary tubercles indistinct; toes bearing narrow lateral fringes; webbing absent; all other toe discs expanded, rounded to elliptical in shape; toes with ventral pads well defined by circumferential grooves; relative length of toes: I < II < III < V < IV; Toe V longer than Toe III.

Measurements of type series and referred specimens: Meristic data are shown in Table 1.

Coloration in preservative

Dorsum pale brown to grey with darker chevrons outlined by thin, white lines; cream to pink dorsolateral stripes; arms, legs, and flanks with diagonal dark

Figure 2. Skin texture variation in one individual frog (*Pristimantis mutabilis*) from Reserva Las Gralarias (Pichincha, Ecuador). Note that skin texture shifts from highly tubercular to almost smooth; also, note the relative size of tubercles on the eyelid, lower lip, and limbs. The frog was found on a leaf during the night (*left photograph*) and photographed in the laboratory (*photograph with white background*) the following morning.

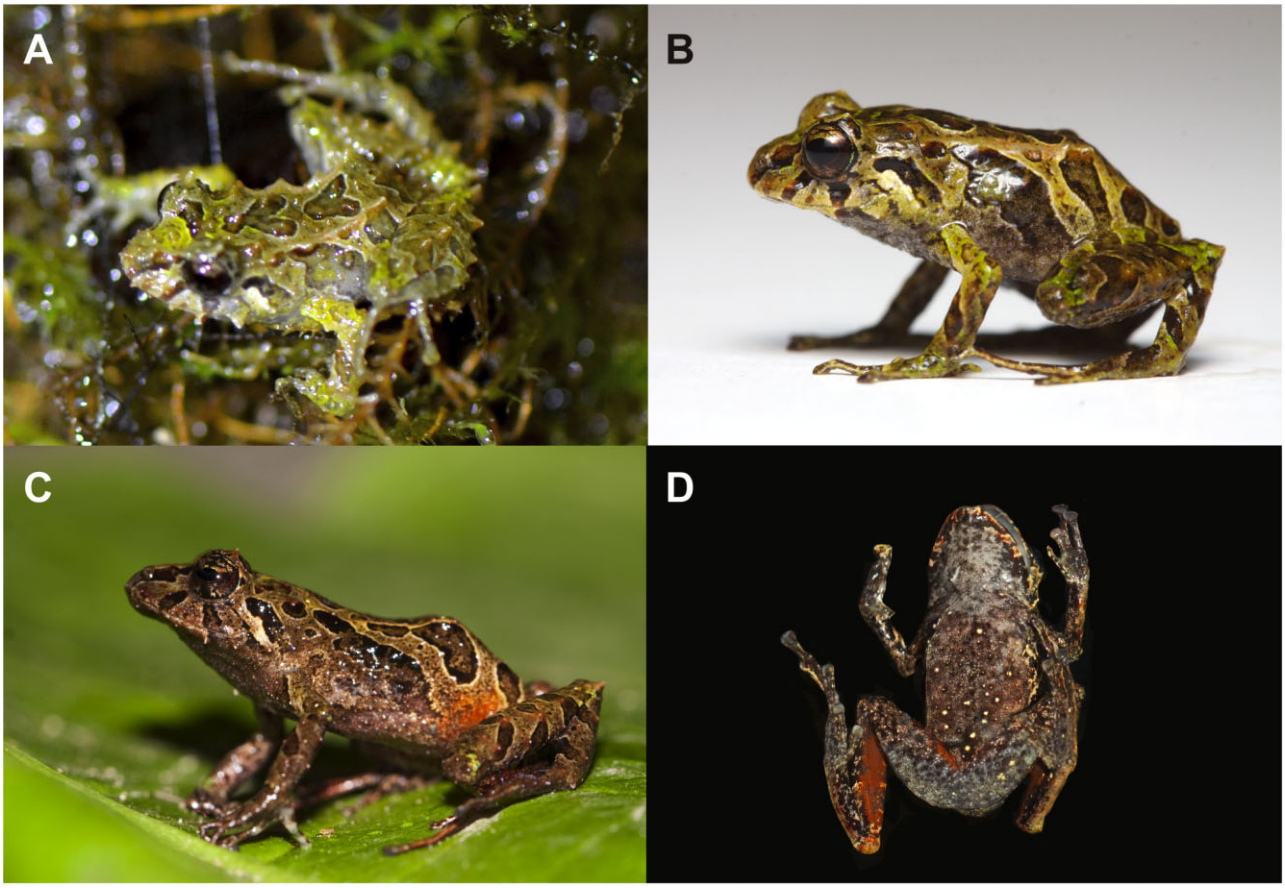

Figure 3. *Pristimantis mutabilis* sp. nov. in life. A, B, Sub-adult male, MZUTI 2191, photographed in its natural habitat during the night *(top left)* and under laboratory conditions during the day *(top right)*. C, Adult female in dorsolateral view, MZUTI 910. D, Adult female in ventral view, MZUTI 911.

stripes, outlined by thin, white line; venter pale brown with small, darker brown spots, and minute white spots (Fig. 1); iris silver.

Coloration in life

Dorsum light brown to pale greyish green, with bright green marks and grey to dark brown chevrons, outlined by thin cream or white line; dorsolateral folds orange; venter pale grey to brown with darker, diffuse spots, and few minute white spots; iris cream to golden with thin black reticulation and reddish horizontal streak; in females, groin and hidden surfaces of legs red (Figs 2, 3).

Variation of skin texture (Figs 2, 3)

All individuals of *Pristimantis mutabilis* presented a markedly tubercular skin texture when found on vegetation or hidden in moss during the night. Large tubercles were evident on the dorsum, upper and lower lips, upper eyelid, arms and legs. After frogs were captured, they all showed a sudden and drastic change in skin texture; all tubercles became reduced in size, and the dorsal skin became smooth or nearly smooth (i.e., few tubercles are visible, mainly on the upper eyelid and heel). When frogs were returned to mossy, wet environments, they recovered a tuberculate skin texture. We speculate that explanatory variables involved in frog skin texture change are stress, humidity, and background. Our observations do not support light availability as a source of texture variation as we observed skin texture change at day and night. The time rate of skin texture variation might depend on the variables mentioned above; we only have one quantitative measure, which is summarized in Figure 2.

Genetics

The genetic results supported the morphological analyses by placing the new species in the *Pristimantis myersi* species group (Fig. 4), although the group itself is only strongly supported in Bayesian analyses and contains species from the *P. unistrigatus* group. Reciprocal monophyly of the new species is supported in maximum likelihood and Bayesian analyses (for 12S, 16S, and concatenated datasets) with high bootstrap

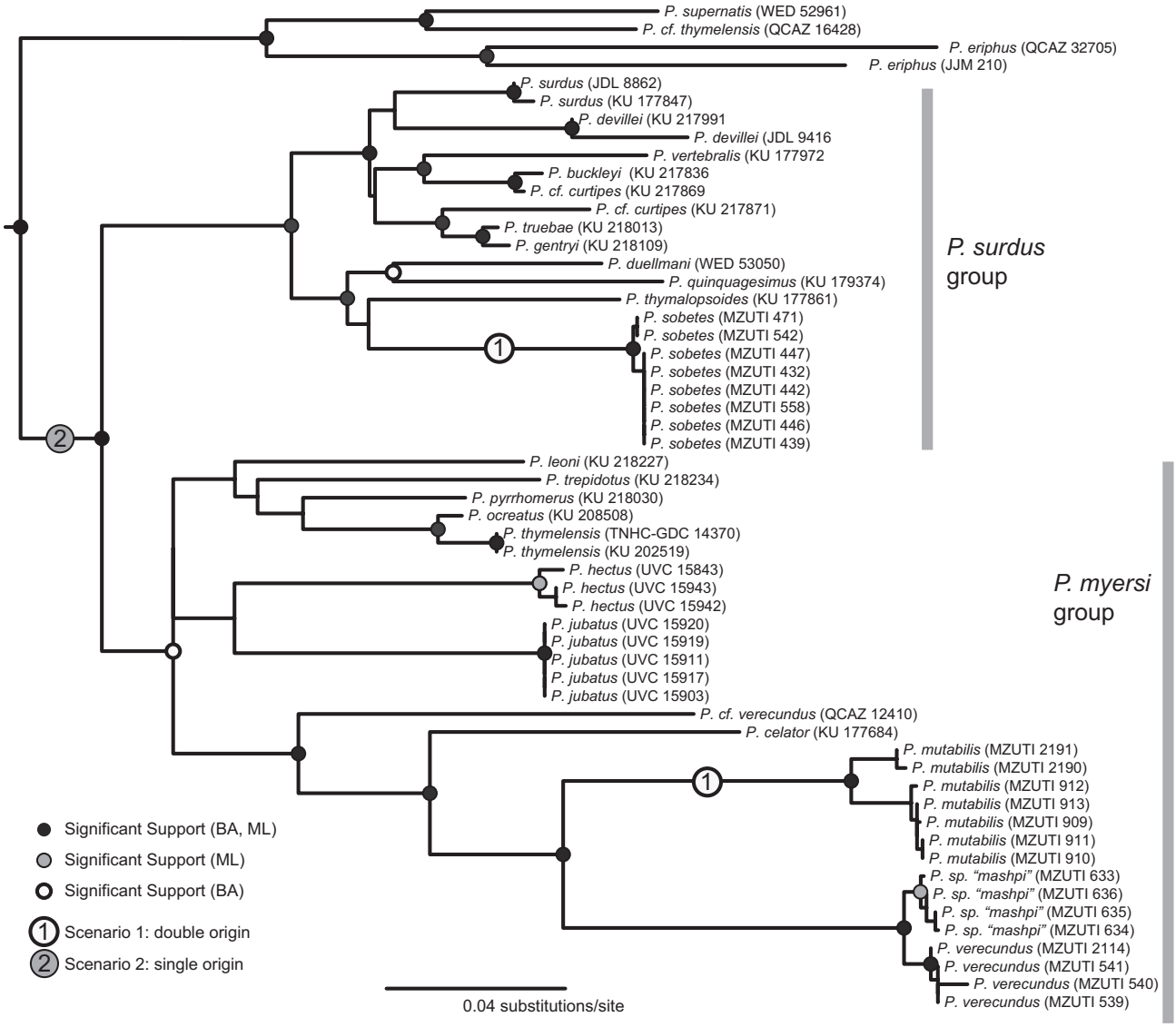

Figure 4. Maximum likelihood (ML) and Bayesian (BA) genetic topology for the combined 12S and 16S dataset. Separate gene trees not shown because the results were similar. The circles at the nodes show ML and BA significant support values (ML: bootstrap > 75%; BA: posterior probability > 0.95). The lack of a circle indicates that the node was not significantly supported by either analysis. The (1) and (2) represent two possible evolutionary scenarios given current data. Scenario 1 hypothesizes that skin texture plasticity originated independently twice while Scenario 2 hypothesizes that skin plasticity originated once in the common ancestor to the *P. myersi* and *P. surdus* groups.

and posterior probabilities (Fig. 4). The populations from Reserva Las Gralarias and Reserva Los Cedros differ by large genetic distances of 5.0–6.5% while individuals from the same area show differentiation of 0.01–0.04%. This result suggests the existence of a cryptic species, however data were not available to assess this possibility. We find substantial genetic differentiation between the new species and *P. verecundus,* which are morphological similar and possibly evolutionary sister species. We find genetic distances of 15.1–16.3% and 16.4–18.6% for the new species compared with *P. verecundus* for 12S and 16S, respectively. For the concatenated dataset, we find genetic distances of 16.2–16.8% from *P. verecundus*.

Vocalization (Fig. 5, Table 2)

MZUTI 2190; adult male recorded from Reserva Las Gralarias on February 1, 2013; night with light rain, temperature of 14.2 °C. *Pristimantis mutabilis* emitted calls at an approximate rate of five calls per minute ($N = 1$). Three distinct types of calls were emitted by this species: (1) a single-note call with a single strong

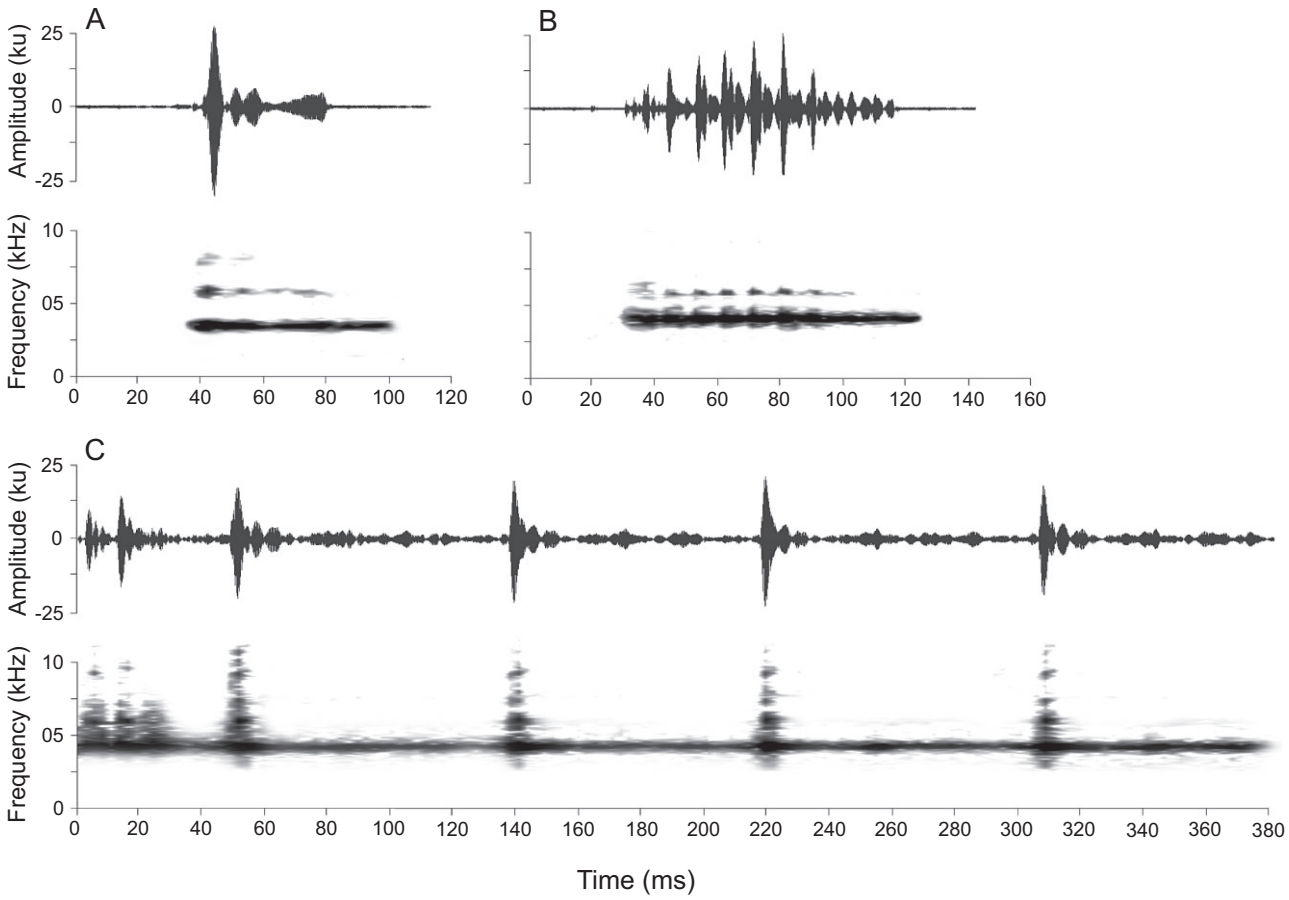

Figure 5. Oscillogram and spectrogram for the three call types A–C, of *Pristimantis mutabilis* on the same time scale. Note that call type C is a series of calls.

amplitude peak (Fig. 5A); (2) a single-note call with four or more strong amplitude peaks (Fig. 5B); and (3) a series of 5–6 calls with very short time intervals and frequency modulation increasing for each call throughout the series (Fig. 5C). The biological function of each call type is not known.

The first call type is composed of a single pulsed note that sounds like a short ring to the ear ($N = 14$; Fig. 5A). The call has one strongly amplitude modulated pulse, and 2–4 weakly amplitude modulated pulses. The pulse rate of a call is 0.041–0.111 pulses ms^{-1} (0.094 ± 0.019). The call duration is 45.7–49.0 ms (45.7 ± 1.3). Calls of this type are not frequency modulated and the dominant frequency of a call is 3273–3359 Hz (3285 ± 31). The lower limit of the fundamental frequency is 3255–3346 Hz (3275 ± 39) and the upper limit is 3617–3708 Hz (3688 ± 39).

The second call type is composed of a single pulsed note that sounds like a rough screech to the ear ($N = 7$; Fig. 5B). The call has two or more strongly amplitude modulated pulses, and several weakly amplitude modulated pulses. The pulse rate of a call is 0.138–0.274 pulses ms^{-1} (0.211 ± 0.041). The call duration is 68.0–99.0 ms (82.6 ± 11.0). Calls of this type are not frequency modulated and the dominant frequency of a call is 3358–3445 Hz (3421 ± 42). The lower limit of the fundamental frequency is 3256–3345 Hz (3269 ± 34) and the upper limit is 3708–3799 Hz (3773 ± 44). Notable non-overlapping differences of this call type from the first includes more than one strongly amplitude modulated pulse, a higher pulse rate, a longer duration, and a higher fundamental frequency.

The third call type is arranged in a series of 5–6 (5.6 ± 0.548) pulsed calls that sound like a long, coarse trill to the ear ($N = 5$; Fig. 5C). The series duration is 301.0–456.0 ms (375.0 ± 56.0) with a series interval of 4.2–7.3 s (5.3 ± 1.2). Each call has one strongly amplitude modulated pulse, and several weakly amplitude modulated pulses. The pulse rate is 0.055–0.089 (0.066 ± 0.010) and is most similar to call type A. The dominant frequency of a call is 3187–3445 Hz (3342 ± 94). Calls of this type have some frequency modulation increasing 86–173 Hz (141.7 ± 48.3)

Table 2. Comparisons of call types recorded for *Pristimantis mutabilis*. Calls were recorded from a single male calling from a container the same night it was collected. Call envelope is the ratio of the time of peak amplitude to call duration. Call interval for Type C is only for calls within a series. Frequency modulation across a series was measured by using the difference in dominant frequency between the first and last call in a series. Data are the mean ± two standard deviations, and the range (in parentheses)

	Call type		
Parameter	Type A	Type B	Type C
N – calls (series)	14 (0)	7 (0)	27 (5)
Note amplitude structure	Pulsed	Pulsed	Pulsed
Number of notes/call	1	1	1
Calls/series	–	–	5.6 ± 0.548 (5–6)
Series duration (ms)	–	–	374.6 ± 55.6 (301.0–456.0)
Series interval (ms)	–	–	5.3 ± 1.2 (4.2–7.3)
Call duration (ms)	45.7 ± 1.3 (44.0–49.0)	82.6 ± 11.0 (68.0–99.0)	54.1 ± 0.026 (50.0–59.1)
Call interval (s)	16.7 ± 6.3 (12.0–33.7)	7.6 ± 3.9 (3.8–14.9)	0.041 ± 0.009 (0.022–0.053)
Pulse rate (/ms)	0.094 ± 0.019 (0.041–0.111)	0.211 ± 0.041 (0.138–0.274)	0.066 ± 0.010 (0.055–0.089)
Call envelope	0.159 ± 0.016 (0.136–0.196)	0.829 ± 0.059 (0.750–0.899)	0.665 ± 0.162 (0.528–0.931)
Dominant frequency (Hz)	3285 ± 31 (3273–3359)	3421 ± 42 (3358–3445)	3342 ± 94 (3186–3445)
Frequency modulation within a call (Hz)	0	0	0
Frequency modulation across a series (Hz)	–	–	141.7 ± 48.3 (86–173)
Lower fundamental frequency (Hz)	3275 ± 39 (3255–3346)	3269 ± 34 (3256–3345)	3256 ± 64 (3165–3346)
Higher fundamental frequency (Hz)	3689 ± 39 (3617–3708)	3773 ± 44 (3708–3799)	3744 ± 50 (3708–3799)
First harmonic (Hz)	6626 ± 23 (6546–6632)	6718 ± 70 (6632–6805)	6632 ± 61 (6546–6718)
Second harmonic (Hz)	9278 ± 252 (9043–9733)	–	9027 ± 187 (8699–9130)

throughout the series. The lower limit of the fundamental frequency is 3165–3346 Hz (3256 ± 64) and the upper limit is 3708–3799 Hz (3744 ± 50). Notable non-overlapping differences of this call type from the first and second type are that calls are arranged in a series and frequency modulated.

Etymology

The specific epithet *mutabilis* is the Latin word for changeable and refers to the ability of this species to modify its skin texture.

Distribution (Fig. 6)

Pristimantis mutabilis is known from the following Andean localities in northwestern Ecuador: Reserva Las Gralarias (0.0167° S, 78.7333° W, 2063 m, Pichincha province) and Reserva Los Cedros (Sendero Oso: 0.3197° N, 78.7858° W, 1880 m; Sendero Cascada Nueva: 0.3249° N, 78.7809° W, 1850 m, Imbabura province).

Natural history

Pristimantis mutabilis has been found in both primary and secondary Andean forests. At Reserva Las Gralarias, the two males (MZUTI 2190, 2191) were found concealed in moss on a tree 230 cm above the ground; calling males were heard during January and February 2013. Additionally, other individuals (MZUTI 413; 909–913) were found on the surfaces of leaves about a metre above the ground (Fig. 3A). All individuals were observed to be displaying the tuberculate state while perched on leaves.

At the two localities where *Pristimantis mutabilis* has been recorded, the species seems to be abundant, based on the vocalizations that are commonly heard during the night. However, given the arboreal habits of the species, observations are rare. As an example, at Reserva Las Gralarias, we have only observed three individuals during a three-year study.

Conservation status

Given the available information on *Pristimantis mutabilis,* and following IUCN (International Union for Conservation of Nature) (2001) criteria, we suggest placing this species in the Data Deficient category.

DISCUSSION

We describe the new species *Pristimantis mutabilis* and document a striking phenotypic plastic ability to rapidly

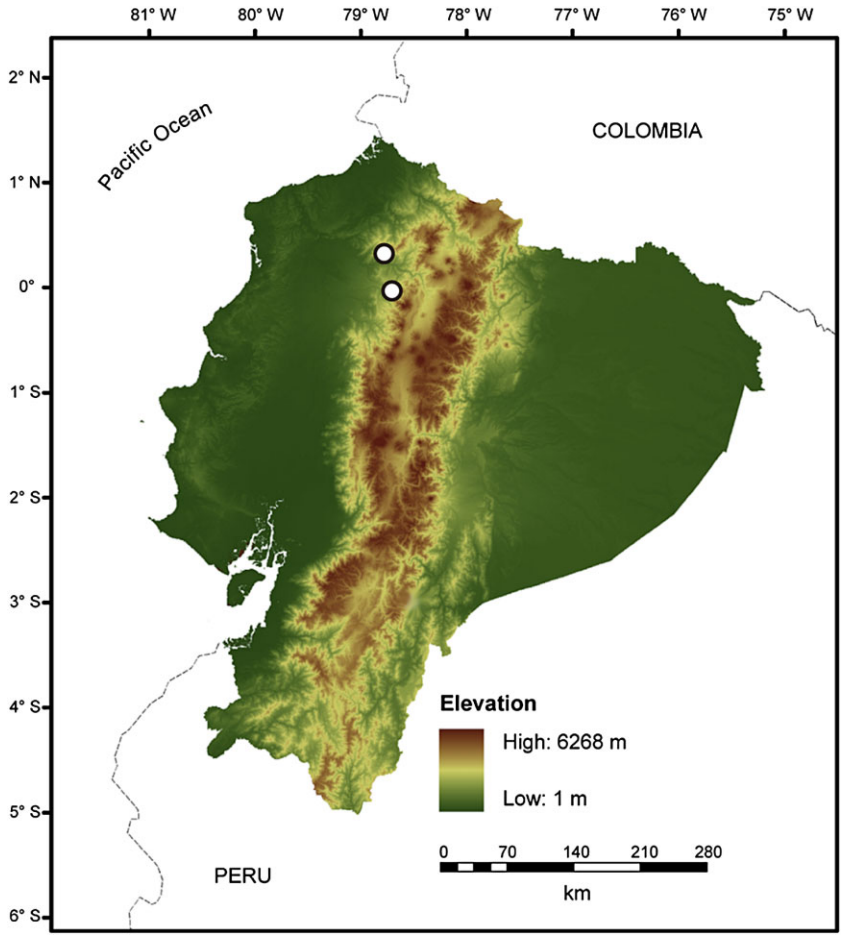

Figure 6. Map showing the distribution of *Pristimantis mutabilis* (white circles) in Ecuador.

change skin texture between two character states, smooth and tuberculate. We also document a second, sympatric Andean species (*Pristimantis sobetes*) with the same type of phenotypic plasticity (Fig. 7). This is surprising because of the lack of similar observations in the literature for any frog species. Together, our observations suggest that this phenomenon might be present in other amphibians, and could be relatively widespread in the genus *Pristimantis*.

Previous reports on skin texture differences in frogs are associated with sexual dimorphism. In groups such as glassfrogs and harlequin toads, males may have tubercles that are absent in females; however, these tubercles are present during the entire breeding season (Guayasamin *et al.,* 2009; Coloma *et al.,* 2010). Also, some frogs temporarily gain barbs, glands, or tubercles during the breeding season, which are used for sexual selection or territory defence (e.g. Tsuji & Matsui, 2002; Cadle, 2008). These frogs differ substantially from *P. mutabilis* and *P. sobetes* by the time scale in which the change occurs (minutes versus seasonal) and probably its function (camouflage versus breeding). Furthermore, we documented the same skin texture plasticity in females, thus a relationship to sexual dimorphism or sexual selection mechanism is unlikely.

We suggest that skin plasticity is associated with environmental camouflage rather than sexual selection or dimorphism. *Pristimantis mutabilis* and *P. sobetes* are geographically distributed in montane cloud forest habitats that are abundant in epiphytes, vegetation, and moss. In these habitats, skin texture that has the appearance of moss or detritus likely conceals the individual from visual predators, such as birds and arachnids (Fig. 3). While the physiological mechanisms of how texture changes in such a short time are unknown, we speculate that it could involve allocation of more or less water to existing small structures (e.g. warts and tubercles) on the skin. Further laboratory studies are needed to understand the mechanisms that underlie these species' rapid plasticity.

We can only speculate on how widespread this ability to modify skin texture is in anurans, as to our knowledge our study is the first to describe this ability. We reviewed noteworthy studies on

Figure 7. Phenotypic plasticity in skin texture in *Pristimantis sobetes*, MZUTI 412, from Reserva Las Gralarias, Ecuador. Frog photographed under natural *(top)* and laboratory *(bottom)* conditions.

amphibian taxonomy and have not found any references to such plasticity (e.g., Duellman, 1978; 2005; Lynch & Duellman, 1997; Savage, 2002; Faivovich *et al.,* 2005; Grant *et al.,* 2006; Hedges *et al.,* 2008; Duellman & Lehr, 2009; Coloma *et al.,* 2010; 2012; Arteaga *et al.,* 2013). In contrast, all these studies explicitly mention skin texture and the presence/absence of tubercles as discrete traits to distinguish species.

Given that *P. mutabilis* and *P. sobetes* are not closely related taxa and share similar phenotypic plasticity in skin texture, several scenarios of trait evolution are possible. One possibility is that skin plasticity evolved twice independently in *P. mutabilis* and *P. sobetes* (Scenario 1, Fig. 4). Skin plasticity in this scenario could have originated in older ancestral lineages of these species if future observations find the trait in closely related species. An alternate possibility would involve a single origin in the most recent common ancestor of the *P. myersi* and *P. surdus* groups (Scenario 2, Fig. 4). This scenario would result in all species in this clade sharing the trait (with at least 22 species with undocumented skin texture plasticity). It is also possible that the trait was retained by *P. mutabilis* and *P. sobetes* (or their ancestral lineages) and lost in the ancestral lineages of extant species that are later found to not have this trait. The likely scenario remains an open question as Scenario 1 would be highly coincidental and Scenario 2 implies that the trait has been overlooked for numerous frog species. Furthermore, the evolutionary history of the trait could be a complex sequence of multiple losses or gains and all of the species in this clade will have to be investigated individually to fully understand its evolutionary history.

We urge amphibian taxonomists to exercise caution as our findings raise some fundamental challenges. A major challenge for amphibian taxonomists working with few preserved specimens and without records (e.g. photographs, descriptions) is that the appearance and variation of species in natural conditions are unknown. This situation could also result in other assumed discrete taxonomic traits having similar plasticity. Additionally, it remains unclear how preservation techniques, time at preservation, and long-term preservation alter this plastic skin texture observed from museum specimens. It is possible, for example, that intraspecific variation we observed in preserved specimens of *P. mutabilis* could be caused by the skin texture at the moment of preservation, which we did not evaluate during this study.

Potential taxonomic over-splitting due to intraspecific variation in taxonomically relevant traits is expected to be more problematic in species that have been described using one or very few specimens. This is problematic because intraspecific variation cannot be assessed and reported. Other challenging cases are morphologically similar species in which skin texture is the key morphological diagnostic trait originally used to diagnose the species (e.g. *Pristimantis actites*/*P. w-nigrum*; *P. curtipes*/*P. truebae*/*P. vertebralis*; see Lynch & Duellman, 1997). It is possible that a species could have been erroneously described based on an unknown plastic trait, but to determine this, each species would have to be individually evaluated.

A few simple procedures would help to address these problems. In the field, taxonomists should evaluate the stability and variation of important traits (tubercles, skin texture, coloration) used for diagnosing and comparing species, both among and within individuals. We recommend that taxonomists photograph live animals in different environmental conditions (e.g. before and after handling; during day and night). This procedure would not only decrease the probability of diversity overestimation (describing species that have already being described), but would facilitate species identification, especially for non-taxonomic researchers. Being able to accurately identify species is a critical aspect for all studies that use species as the

taxonomic unit, especially ecology and conservation biology (Margules & Pressey, 2000).

Finally, genetic differentiation patterns within the new species presents some interesting questions for future research. *Pristimantis mutabilis* contains two reciprocally monophyletic populations with a relatively high genetic distance (5.0–6.5%), even though they are geographically close (distance by air = 37.7 km). Given the high divergence between the two populations, it is plausible that they represent distinctive lineages; however, a larger sample size for all data (i.e., genetic, morphologic, acoustic) is required to test this hypothesis. The most likely explanation for such deep genetic divergence between otherwise morphologically similar populations is the presence of the dry valley of the Guayllabamba River, which seems to be acting as a biogeographic barrier, limiting gene flow between populations. Studies with other taxa distributed north and south of the Guayllabamba valley are necessary to further test its relevance in diversification processes.

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APPENDIX 1

Specimens examined and corresponding GenBank accession numbers

Pristimantis bicantus. – Ecuador: *Provincia Napo:* Oyacachi–El Chaco trail (02.25728°S, 77.9466°W; 2340 m), MZUTI 729–734, 751–760.

Pristimantis festae. – Ecuador: *Provincia Napo:* Papallacta, Páramo de la Virgen (00.32316°S, 78.2007°W; 4221 m), MZUTI 2620–2623.

Pristimantis gladiator. – Ecuador: *Provincia Napo:* near Guango river (0.37639°S, 78.07471°W; 2708 m), MZUTI 1124–1133

Pristimantis hectus. – Ecuador: *Provincia Pichincha:* Reserva Las Gralarias (0.02557°S, 78.70391°W; 2136 m), MZUTI 2025–2033.

Pristimantis leoni. – Ecuador: *Provincia Pichincha:* near Laguna de Mojanda (0.1675°S, 78.2939°W; 3358 m), MZUTI 1803–1815.

Pristimantis lucidosignatus. – Ecuador: *Provincia Cotopaxi:* Reserva Otonga (0.41549°S, 79.0048°W; 2115 m), MZUTI 2092–2095.

Pristimantis mutabilis. – GenBank numbers: KM675434–440, KM675457–463.

Pristimantis pteridophyllus. – Ecuador: *Provincia Imbabura:* Reserva Siempre Verde (00.37537°N, 78.42276°W; 2532 m), MZUTI 3165, 3168–69.

Pristimantis pyrrhomerus. – Ecuador: *Provincia Cotopaxi:* Sigchos, Unache–Santa Rosa road (00.6836°S, 78.900°W; 2803 m), MZUTI 1925–1930.

Pristimantis sirnigeli. – Ecuador: *Provincia Imbabura:* Reserva Siempre Verde (00.370°N, 78.416°W; 2808–2025 m), MZUTI 3153, 3159.

Pristimantis sobetes. – Ecuador: *Provincia Pichincha:* Reserva Las Gralarias (0.025°S, 78.704°W; 2100 m), MZUTI 432–450, 558; Mindo, Yellow House (0.043°S, 78.750°W; 1657 m), MZUTI 542. GenBank numbers: KM675428–433, KM675449–456.

Pristimantis sp. – Ecuador: *Provincia Pichincha:* San Francisco de Pachijal and Mashpi biological corridor (0.112°S, 77.398°W; 1241 m), MZUTI 633–636. GenBank numbers: KM675441–444, KM675464–467.

Pristimantis verecundus. – Ecuador: *Provincia Pichincha:* Cascadas de Mindo (0.07919°S, 78.76336°W; 1404 m), MZUTI 540–541; Mindo, Séptimo Paraíso (0.0285°S, 78.766°W; 1521 m), MZUTI 539, 2114. Colombia: *Departamento Nariño:* Reserva La Planada, 1780 m, IND-AN 1834 (holotype). GenBank numbers: KM675424–427, KM675445–448.

APPENDIX 2

GenBank accession numbers of specimens used for phylogenetic analyses

Pristimantis actites. – Ecuador: *Cotopaxi, Pilaló*, KU 217830. 12S GenBank accession: EF493696; 16S GenBank accession: EF493696.

Pristimantis buckleyi. – Ecuador: *Carchi, 9.0 km E El Angel*, KU 217836. 12S GenBank accession: –; 16S GenBank accession: EF493350.

Pristimantis celator. – Ecuador: *Carchi, Maldonado*, KU 177684. 12S GenBank accession: EF493685; 16S GenBank accession: EF493685

Pristimantis curtipes. – Ecuador: *Carchi: 26.6 km W Tulcán on road to Maldonado, near Volcán Chiles*, KU 217869. 12S GenBank accession: AY819343; 16S GenBank accession: DQ679379

Pristimantis curtipes. – Ecuador*: Pichincha, Bosque Pasocha*, KU 217871. 12S GenBank accession: EF493513; 16S GenBank accession: EF493513

Pristimantis devillei. – Ecuador: *Napo, 6.1 km E Papallacta*, KU 217991. 12S GenBank accession: EF493688; 16S GenBank accession: EF493688

Pristimantis duellmani. – Ecuador: *Carchi; ~5 km W La Gruel*, WED 53050; KU 202404. 12S GenBank accession: AY326003; 16S GenBank accession: AY326003

Pristimantis eriphus. – *Locality unavailable*, JJM 210. 12S GenBank accession: –; 16S GenBank accession: DQ195458

Pristimantis eriphus. – Ecuador: *Napo, Yanayacu,* QCAZ 32705. 12S GenBank accession: EU186671; 16S GenBank accession: EU186671

Pristimantis gentryi. – Ecuador: *Cotopaxi, 27.6 km E Pilaló*, KU 218109. 12S GenBank accession: –; 16S GenBank accession: EF493511

Pristimantis hectus. – Colombia, UVC 15942. 12S GenBank accession: –; 16S GenBank accession: JN104680

Pristimantis hectus. – Colombia, (2.6642° N 76.9025° W), UVC 15943. 12S GenBank accession: –; 16S GenBank accession: JN371037

Pristimantis hectus. – Colombia, (2.6642° N 76.9025° W), UVC 15843. 12S GenBank accession: –; 16S GenBank accession: JN371038

Pristimantis jubatus. – Colombia, (2.6642° N 76.9025° W), UVC 15903. 12S GenBank accession: –; 16S GenBank accession: JN370986

Pristimantis jubatus. – Colombia, (2.6642° N 76.9025° W), UVC 15911. 12S GenBank accession: –; 16S GenBank accession: JN370982

Pristimantis jubatus. – Colombia, (2.6642° N 76.9025° W), UVC 15917. 12S GenBank accession: –; 16S GenBank accession: JN371000

Pristimantis jubatus. – Colombia, (2.6642° N 76.9025° W), UVC 15919. 12S GenBank accession: –; 16S GenBank accession: JN370989
Pristimantis jubatus. – Colombia, (2.6642° N 76.9025° W), UVC 15920. 12S GenBank accession: –; 16S GenBank accession: JN370990
Pristimantis leoni. – Ecuador*: Carchi, 51.3 km W Tulcán*, KU 218227. 12S GenBank accession: EF493684; 16S GenBank accession: EF493684
Pristimantis pyrrhomerus. – Ecuador: *Bolívar, Bosque Protector Cashca Totoras*, KU 218030. 12S GenBank accession: EF493683; 16S GenBank accession: EF493683
Pristimantis quinquagesimus. – Ecuador*: Pichincha, Quebrada Zapadores*, KU 179374. 12S GenBank accession: EF493690; 16S GenBank accession: EF493690
Pristimantis supernatis. – Ecuador: *Napo; 3.5 km E Santa Bárbara*, WED 52961; KU 202432. 12S GenBank accession: AY326005; 16S GenBank accession: AY326005
Pristimantis surdus. – Ecuador: *Imbabara, Le Delicia*, KU 177847. 12S GenBank accession:; 16S GenBank accession: EF493687
Pristimantis thymalopsoides. – Ecuador: *Cotopaxi, Pilaló*, KU 177861. 12S GenBank accession: EF493514; 16S GenBank accession: EF493514
Pristimantis thymelensis. – Ecuador: *Napo, Páramo de Guamaní*, QCAZ 16428. 12S GenBank accession: EF493516; 16S GenBank accession: EF493516
Pristimantis thymelensis. – *Locality unavailable*, TNHC-GDC 14370. 12S GenBank accession: JX564889; 16S GenBank accession: JX564889
Pristimantis trepidotus. – Ecuador*: Imbabara, 13.8 km W Tabacundo*, KU 218234. 12S GenBank accession: EF493515; 16S GenBank accession: EF493515
Pristimantis truebae. – Ecuador*: Cotopaxi, 24.6 km E Pilaló*, KU 218013. 12S GenBank accession: EF493512; 16S GenBank accession: EF493512
Pristimantis aff verecundus. – Ecuador: *Cotopaxi, Reserva Otonga*, QCAZ 12410. 12S GenBank accession: EF493686; 16S GenBank accession: EF493686
Pristimantis vertebralis. – Ecuador: *Imbabara, La Delicia*, KU 177972. 12S GenBank accession: EF493689; 16S GenBank accession: EF493689.