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Testing concordance in species boundaries using acoustic, morphological, and molecular data in the field cricket genus *Itaropsis* (Orthoptera: Grylloidea, Gryllidae: Gryllinae)

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In most taxa, species boundaries are inferred based on differences in morphology or DNA sequences revealed by taxonomic or phylogenetic analyses. In crickets, acoustic mating signals or calling songs have species-specific structures and provide a third data set to infer species boundaries. We examined the concordance in species boundaries obtained using acoustic, morphological, and molecular data sets in the field cricket genus *Itaropsis*. This genus is currently described by only one valid species, *Itaropsis tenella*, with a broad distribution in western peninsular India and Sri Lanka. Calling songs of males sampled from four sites in peninsular India exhibited significant differences in a number of call features, suggesting the existence of multiple species. Cluster analysis of the acoustic data, molecular phylogenetic analyses, and phylogenetic analyses combining all data sets suggested the existence of three clades. Whatever the differences in calling signals, no full congruence was obtained between all the data sets, even though the resultant lineages were largely concordant with the acoustic clusters. The genus *Itaropsis* could thus be represented by three morphologically cryptic incipient species in peninsular India; their distributions are congruent with usual patterns of endemism in the Western Ghats, India. Song evolution is analysed through the divergence in syllable period, syllable and call duration, and dominant frequency.

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ADDITIONAL KEYWORDS: calling song – cluster analysis – cryptic species – distribution – India – phylogeny – song evolution.

INTRODUCTION

Although the species is a widely used and fundamental unit of analysis in biology, the identification of species and delineation of species boundaries continues to be a difficult problem (e.g. Coyne & Orr, 2004; but see de Queiroz, 2007). The problem of species delineation and identification is most severe in groups characterized by large morphological variation and polymorphisms, such as marine molluscs (Knowlton, 2000) and reptiles (Wiens & Penkrot, 2002), and, at the other end of the spectrum, in groups that exhibit little variation. The latter are exemplified by 'cryptic' species, typically closely related species that are very similar and difficult to distinguish morphologically (discussed in Bickford *et al*., 2007). These species may be revealed by an alternate level of analysis, using molecular data sets, specifically the DNA sequences of

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specific mitochondrial or nuclear genes (Packer & Taylor, 1997; Bickford *et al*., 2007; Goldstein & DeSalle, 2010; Padial *et al*., 2010). Cryptic species have important implications for biodiversity assessment and identifying them can sometimes have enormous consequences for disease and pest management (Bickford *et al*., 2007).

Cryptic species may most likely be found in taxonomic groups that use non-visual mating signals (Bickford *et al*., 2007) such as crickets. The peculiarities of the mating system of many cricket species, wherein adult males attract potential mates by producing species-specific acoustic signals or calling songs (Walker, 1957), offers an alternative acoustic data set that may be used to demarcate species boundaries. Even though there is not always a one-to-one correspondence between species and songs, with a few species having identical songs (e.g. allochronic species: Alexander & Bigelow, 1960), most female crickets are tuned to the spectral and/or temporal properties of the calling songs of their own species (e.g. Gerhardt & Huber, 2002), and stabilizing selection on these features may allow them to serve as acoustic signatures of species identity.

Morphological, acoustic, ecological, and molecular data sets may be subjected to different kinds of analyses, either separately or in combination. These include cluster analysis and multidimensional scaling, which construct groups on the basis of overall similarity between individuals (Manly, 1986), ecological niche modelling (Raxworthy *et al*., 2007), and phylogenetic methods, which reconstruct groups that reflect hypotheses of evolutionary relationships based on shared derived characters (Eldredge & Cracraft, 1980; Brooks & McLennan, 1991; Harvey & Pagel, 1991). The results of these analyses may be used to propose hypotheses of species boundaries and ultimately to support taxonomic decisions. Using a unified species concept as proposed by de Queiroz (2007), the most reliable species boundaries may be those that are concordant when produced by the analysis of the most relevant data sets, according to species biology and amount of variation, using the most relevant methods.

In this study, we have used the field cricket genus *Itaropsis* as a model system to test concordance in species boundaries using different data sets. The genus *Itaropsis* was formally created by Chopard (1925) to incorporate *Gryllus parviceps* Walker, 1871 from Bombay, India, and *Gryllodes microcephalus* Bolivar, 1893 from Western Africa. *Itaropsis* has striking morphological characteristics, such as a very narrow frontal rostrum, a small head, and a highly reduced, almost abortive, ovipositor in females. Chopard (1936) further transferred to this genus *Gryllus tenellus* Walker, 1869 from Sri Lanka, first placed in the genus *Anurogryllus* (Chopard, 1925), and synonymized this species with *parviceps*. Finally, *Gryllus fletcheri* Chopard, 1935 known from one female from Coimbatore was synonymized with *I. tenella* (Chopard, 1967).

Thus, the genus *Itaropsis* is, at the time of writing, represented in India by only one valid species, *Itaropsis tenella*, whose distribution extends from Sri Lanka in the South to Bombay in the north-west. This taxonomic hypothesis is supported by the homogeneous morphology of the genus over its whole range. It is invalidated, however, by the wide diversity of male calling songs recorded in the field. This pattern of diversity could indicate the presence of several morphologically cryptic species, as documented for other crickets and frogs (Gerhardt & Huber, 2002).

To test this hypothesis, we have examined individuals of the genus *Itaropsis* from four different localities extending from the north to south of peninsular India at the levels of morphology, acoustics, and DNA sequences, using both mitochondrial and nuclear genes. We examine and answer the following questions: Are concordant lineage boundaries obtained from the analysis of the different data sets? Is the acoustic data set (advertisement songs, involved in reproductive isolation) more relevant for cricket taxonomy than the morphology and molecular lines of evidence? Whatever the taxonomic results, what is the pattern of distribution and song differentiation within this genus?

Our results do not support the hypothesis of several cryptic allopatric species with divergent calling songs in India. Instead, the genus *Itaropsis* could be represented by three incipient species that are morphologically cryptic, but separated by acoustic and genetic divergence.

MATERIAL AND METHODS

Study sites

The choice of sampling sites was intended to cover a wide dispersion of latitude and altitude within the known range of distribution of the cricket genus *Itaropsis*. Song recordings of adult males and collection of recorded singing individuals were carried out at four sites. These included Bombay (19.13°N, 72.91°E, the type locality of *Itaropsis parviceps*), Bangalore (13°N, 77.5°E), Kadari (13.2°N, 75°E), and Valparai (10.32°N, 76.95°E) (Fig. 1). Of these, Bombay (in Maharashtra) was the northernmost site, located at the north-western end of the Indian peninsula on the coast (50 m asl); Bangalore and Kadari in Karnataka state are approximately 1000 km south of Bombay, with Kadari representing a low-altitude site (50 m asl) in the Western Ghats close to the coast

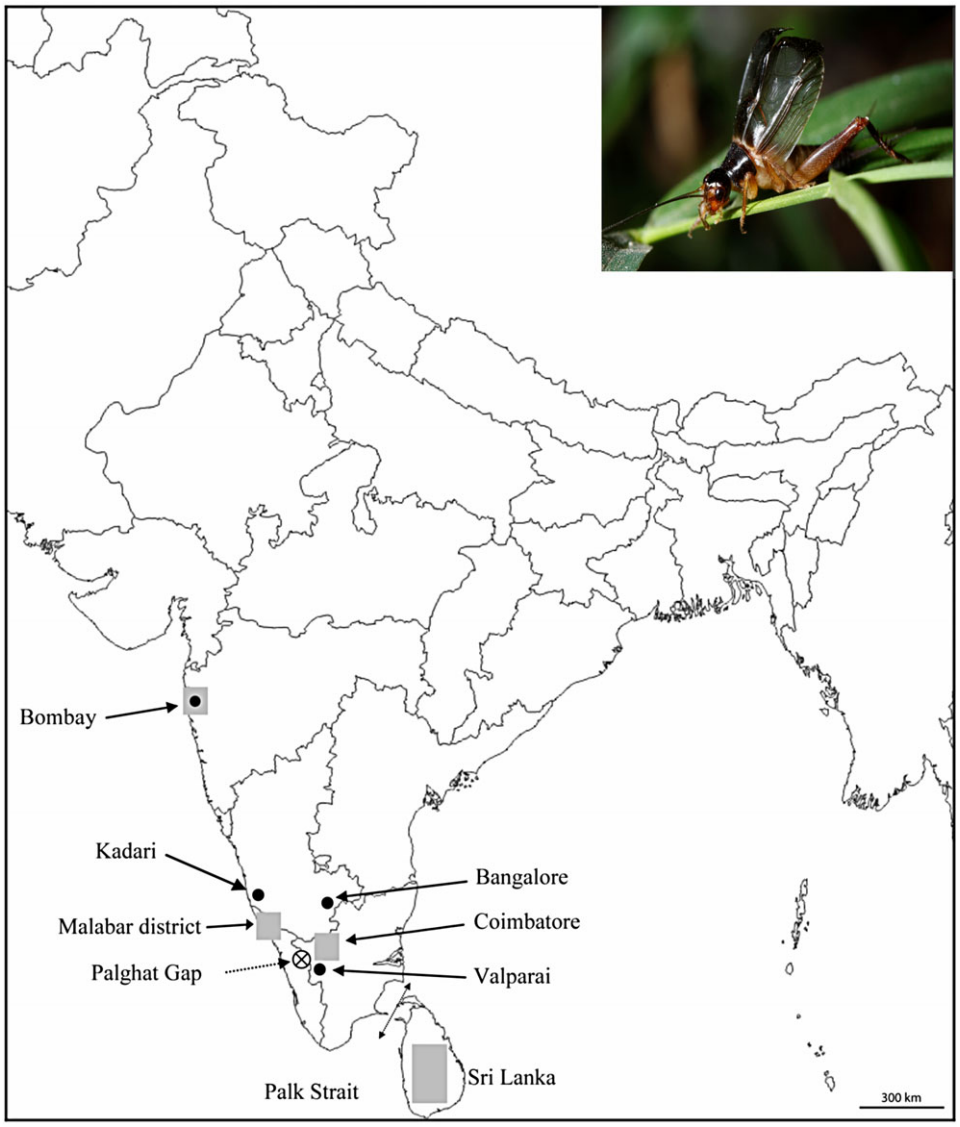

Figure 1. Map showing the four sampling sites for *Itaropsis* in peninsular India (black dots), together with the localities where the presence of *Itaropsis* has been acknowledged in the past (grey squares) and main biogeographical units (Palghat gap, Palk strait). Cricket picture courtesy of Ashok Kumar Mallik, CES, IISc, Bangalore, India.

and Bangalore (at approximately the same latitude in the centre of the peninsula) a relatively high-altitude site (940 m asl). The southernmost sampling site was Valparai, Tamil Nadu state, located in the Anaimalai Hills at an altitude of about 1000 m asl.

Studied material

Song analyses were performed on recordings of 5–8 males per locality (Table 1). Among these, four males were arbitrarily chosen for morphological and molecular analyses. Specimens and song files are deposited in the collection of the Centre for Ecological Sciences, Indian Institute of Science, Bangalore, and the Muséum national d'Histoire naturelle (MNHN), Paris.

Song recording and analysis

Individual calling *Itaropsis* males were located in the field by listening to their calling songs and tracking the source by ear. After visually verifying the position of each male, song recordings were carried out using a Sony WM-D6C Professional Walkman cassette recorder and a Sony ECM-MS957 microphone (flat frequency response from 50 Hz to 18 kHz) with the

Table 1. Material examined in this study, with date, time and area of collection, and temperature at which song was recorded

Code	Song type	Locality	Coordinates	Temperature (°C)	Time	Date
RFC 41*	Triller	Kadari	13.2°N, 75°E	28.3	19:30	20/11/07
RFC 42*	Chirper	Kadari	13.2°N, 75°E	28.3	19:30	20/11/07
RFC 53*	Chirper	Kalasanga	13.2°N, 75°E	25.1	19:30	27/11/07
RFC 69*	Triller	Kalasonga	13.2°N, 75°E	25	19:45	29/11/07
RFC 74	Triller	Mala	13.2°N, 75.1°E	26.8	19:50	02/12/07
RFC 94*	Triller	Bangalore, IISc	13°N, 77.5°E	22.7	01:15	01/03/08
RFC 95	Triller	Bangalore, IISc	13°N, 77.5°E	22.5	01:25	01/03/08
RFC 96*	Triller	Bangalore, IISc	13°N, 77.5°E	22.1	01:35	01/03/08
RFC 97	Triller	Bangalore, IISc	13°N, 77.5°E	21.8	01:48	01/03/08
RFC 99*	Triller	Bangalore, IISc	13°N, 77.5°E	21.4	01:56	01/03/08
RFC 100*	Triller	Bangalore, IISc	13°N, 77.5°E	21.5	02:15	01/03/08
RFC 101	Triller	Bangalore, IISc	13°N, 77.5°E	21.5	02:30	01/03/08
RFC 139	Triller	Ramana Plantation	13.2°N, 75°E	26.7	08.40	23/9/08
RFC 142*	Chirper	Ramana Plantation	13.2°N, 75°E	26.9	20:00	23/9/08
RFC 148	Chirper	Kadari Bridge	13.2°N, 75°E	26.5	20:15	24/9/08
RFC 150*	Chirper	Kadari bridge	13.2°N, 75°E	25.8	21:30	24/09/08
RFC 159*	Triller	Kadari village	13.2°N, 75°E	26	20:10	25/09/08
RFC 165	Triller	Perudaka	13.2°N, 75°E	26.1	20:30	26/09/08
RFC 166	Triller	Perudaka	13.2°N, 75°E	25.8	20:50	26/09/08
RFC 167*	Triller	Perudaka	13.2°N, 75°E	25.6	21:10	26/09/08
RFC 212*	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	26	23:15	26/02/09
RFC 213*	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	25.4	23:30	26/02/09
RFC 214*	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	25	23:45	26/02/09
RFC 215*	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	25	23:50	26/02/09
RFC 216	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	22.5	00:45	26/02/09
RFC 217	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	22.6	01:00	26/02/09
RFC 218	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	22.6	01:15	26/02/09
RFC 219	Triller	Bombay, IIT Bombay	19.13°N, 72.91°E	22.7	01:45	26/02/09
RFC 236*	Chirper	Valparai	10.32°N, 76.95°E	24	20:45	10/04/09
RFC 237*	Chirper	Valparai	10.32°N, 76.95°E	24	21:25	10/04/09
RFC 239*	Chirper	Valparai	10.32°N, 76.95°E	24	22:00	10/04/09
RFC 240	Chirper	Valparai	10.32°N, 76.95°E	23.9	22:25	10/04/09
RFC 241*	Chirper	Valparai	10.32°N, 76.95°E	23.9	22:45	10/04/09
RFC 244	Chirper	Valparai	10.32°N, 76.95°E	23.4	21:05	11/04/09
RFC 245	Chirper	Valparai	10.32°N, 76.95°E	23.4	21:50	11/04/09

All the individuals were used for acoustic analysis and the individuals marked with asterisks were used for both morphological and molecular studies (RFC 41 not present in molecular study).

microphone at a distance of 15 cm from the calling male. Ambient temperature was measured close to the calling male using a Kestrel 3000 Pocket Weather Station. The same male was captured and preserved in 70% ethanol for morphological and molecular studies.

Recorded calling songs were digitized using a Creative Sound Blaster A/D Card at a sampling rate of 44.1 kHz for spectral and temporal analysis. Spectral analysis was carried out using the signal processing software Spectra Plus Professional (1994, Version 3.0, Pioneer Hill Software, Poulsbo, WA, USA). Temporal pattern analysis was performed using a custom-built program (Chandra Sekhar, ECE, IISc) in Matlab (2001, Version 6.1.0.450, The Mathworks Inc., Natick, MA, USA).

The recorded song of each individual was divided into ten segments of 2 s each to examine intra-individual variation in the song. Spectral analysis was carried out on each segment using a fast Fourier transform (FFT), with a Hamming window and a window length of 2048 sampling points. The power spectra thus generated were used to measure the dominant frequency of the song. The dominant frequency (F_D) of each segment was measured as the frequency with maximum energy in the power

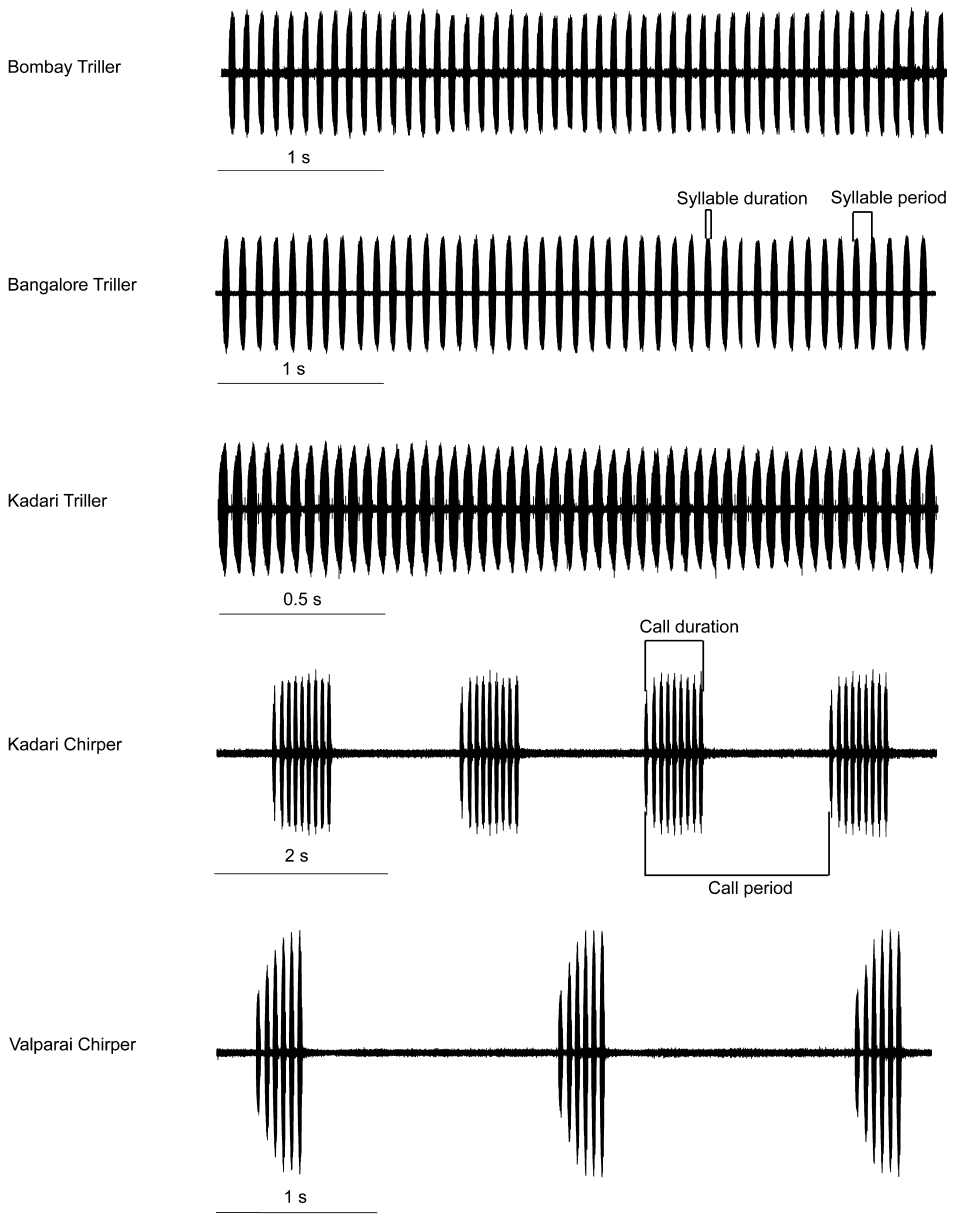

Figure 2. Oscillograms illustrating the five song types of *Itaropsis*.

spectrum. In field crickets, which emit almost tonal signals, F_D is very clearly defined as a narrow-bandwidth peak of highest amplitude. The mean dominant frequency for the ten segments per individual was taken as the value for that individual, and the mean of different individuals from one sampling site was used as the mean value for that site.

For temporal pattern analysis, we measured call duration, syllable period, syllable duration, and the number of syllables per chirp (Fig. 2). For trillers, song bouts 1–1.15 min in duration were analysed and used to measure call parameters; this represents from 500 (Bangalore trillers) to 1400 (Kadari trillers) individual syllables, which were all used to calculate syllable duration and syllable period. For chirpers, analysed song bouts lasted 1.20 min, the bouts representing 30 (Valparai) to 55 (Kadari) chirps; all recorded chirps were used to calculate the call duration and the number of syllables per chirp.

As crickets are poikilotherms, several of the song features may vary with temperature (e.g. Walker & Cade, 2003). To correct for differences due to differences in recording temperatures, each of the song features for individuals from a given locality were regressed against temperature. For this genus, we

did not find a significant correlation for any of the song features with change in temperature within the range over which the data were acquired, 22–27 °C, so the raw data were used for further analysis.

Statistical analysis

All statistical analyses of acoustic data were carried out using Statistica software (Statsoft Inc., Tulsa, OK, USA).

The data from the four sampling sites were analysed for each of four acoustic features, namely syllable period, syllable duration, call duration, and dominant frequency, to examine for significant differences between sampling sites. In a first step, groups were specified a priori based on the sampling sites and call structure, and two kinds of analyses were performed: each acoustic feature was described using basic statistical descriptors and compared; the data for each feature were then subjected to a one-way analysis of variance to examine overall differences between the four sites. If there were significant differences, pairwise comparisons between means were carried out for the four sites using Tukey's HSD test. Special attention was paid to syllable period and carrier frequency, as different species exploit one or the other, or both, as species indicators (Gerhardt & Huber, 2002).

In a second step, the data for individuals from all the sites for all four features were pooled and used to obtain measures of overall similarity (Euclidean distances) in pairwise fashion. This distance matrix was then subjected to a cluster analysis (single linkage) to examine groups emerging on the basis of overall song similarity between individuals.

The song features derived from the acoustic analysis were optimized onto the phylogeny as continuous values using the program Mesquite ver. 2.74 (Maddison & Maddison, 2010).

Morphological characters

In male crickets, the most significant characters for species identification are male genitalia, forewing venation, size, and coloration. We checked these characters for each terminal specimen. Male genitalia were dissected by cutting the membrane between the paraprocts and the subgenital plate, cleaned with cold KOH, and observed with a binocular Leica MZ12. Genitalic parts were named after Desutter (1987), as modified by Desutter-Grandcolas (2003). Male tegminal veins (see Chopard, 1969, fig. 74) were named according to homology statements proposed by Desutter-Grandcolas (2003) for Ensifera. The CuA branching stems and resulting cells were named after Robillard & Desutter-Grandcolas (2004): CuA stems numbered from the diagonal vein (CuA1) distad (CuA2 to CuAi); the cell alignments delimited by successive anal and cubital branches identified from the innermost alignment (A alignment, delimited by 3A and 2A veins) to the outermost alignment (delimited by CuAi-1 and CuAi veins). The first cells of C and D alignments (c1 and d1 cells, respectively) are always clearly delimited, the d1 cell corresponding to the mirror. The number of teeth of the stridulatory file has been checked using SEM pictures of all terminals, using a JEOL JSM-840 electron microscope (15 kV) at the Plateforme de Microscopie Electronique of the MNHN, Paris.

DNA extraction, PCR amplification and sequencing

DNA extractions from tissue samples were performed using a QIAamp DNA MicroKit (QIAGEN) following the manufacturer's instructions. Molecular work was carried out at the MNHN, Service de Systématique Moléculaire. The oligonucleotide primers used for polymerase chain reaction (PCR) and sequencing are listed in Table 2. Amplifications were made in a 25-μL volume reaction with 0.4 μL of each 10 pM primers, 19.2 μL H_2O, 2.5 μL buffer, 1.25 μL DMSO, 1 μL MIX, 0.15 μL *Taq* polymerase and 1 μL DNA. The PCR consisted of an initial denaturing step at 94 °C for 4 min, 35–40 amplification cycles (denaturation at 94 °C for 30 s, annealing between 48 and 55 °C for 40 s, and extension at 72 °C for 40 s), and a final step at 72 °C for 7 min. PCR products were checked on agarose gels and sequenced in both directions with the same primers at Genoscope (Evry, France). Sequences were cleaned and coding sequences were translated using the invertebrate mitochondrial genetic code to check for the absence of stop codons using Sequencher (Gene Codes Corp., v. 4.8 Build 3767, 2007). All genes were screened for potential contamination using the Blastx algorithm on GenBank.

Molecular markers have been chosen among the most variable markers based on previous studies on cricket phylogeny and phylogeography (Huang *et al*., 2000; Robillard & Desutter-Grandcolas, 2006; Oneal, Otte & Knowles, 2010) (Table 2). The molecular data set includes a fragment of the nuclear gene elongation factor alpha (EF1-α, c. 990 bp) and four fragments of mitochondrial genes: cytochrome *c* oxidase subunit 1 (CO1, *c*. 690 bp), cytochrome *b* (Cyt *b*, c. 365 bp), and the large (16S, *c*. 500 bp) and small (12S, *c*. 400 bp) ribosomal sub-units. Ninety-two sequences were newly generated for this study and were deposited in

Table 2. Molecular primers

Gene	Primer	Sequence	Reference	Annealing temperature (°C)
12S	12SF	TACTATGTTACGACTTAT	Kambhampat, 1995	48
	12SR	AAACTAGGATTAGATACCC	Kambhampat, 1995	
16S	16SAG	CGCCTGTTTATCAAAAACATGT	Robillard & Desutter-Grandcolas, 2006	55
	16SBG	AGATCACGTAAGAATTTAATGGTC	Robillard & Desutter-Grandcolas, 2006	
Cytb	427F	YTWGTWCAATGARTMTGAGG	Robillard & Desutter-Grandcolas, 2006	48
	800R	CCYARTTTATTAGGAATTGATCG	Robillard & Desutter-Grandcolas, 2006	
CO1	L2	GCAACGATGATTATTTTCCACT	Nattier *et al*. submitted	49
	H2	CCTGGTAAAATTAGAATGTAAACTTCTG	Nattier *et al*. submitted	
EF1α	RN-M51F	CTTCAGGATGTRTACAAAATTGGTG	Nattier *et al*. submitted	54
	RN-M53-R	GCAATATGAGCWGTGTGGCA	Nattier *et al*. submitted	
	RN-M1F	GCWGCGGGTACTGGTGAR	Nattier *et al*. submitted	54
	RN-M1R	ACACCWGTTTCAACWCGRCC	Nattier *et al*. submitted	

Table 3. Molecular sequences and GenBank accession numbers

Taxa	16S	12S	Cytb	COI	EF1α
Acheta domesticus	AF248698	ADZ97611	AF248682	JF419327	GQ886692
Nisitrus vittatus	AY905314	AY905284	AY905369	–	JN887883
Itaropsis RFC 42	JN411851	–	JN411888	–	–
Itaropsis RFC 53	JN411852	JN411835	JN411889	JN411874	JN411895
Itaropsis RFC 69	JN411853	JN411833	JN411890	JN411870	JN411902
Itaropsis RFC 94	JN411854	JN411831	JN411891	JN411871	JN411905
Itaropsis RFC 99	JN411856	JN411825	JN411893	JN411869	JN411904
Itaropsis RFC 96	JN411855	JN411823	JN411892	JN411873	JN411910
Itaropsis RFC 100	JN411838	JN411826	JN411875	JN411857	JN411903
Itaropsis RFC 142	JN411838	JN411824	JN411876	JN411858	JN411900
Itaropsis RFC 150	JN411840	JN411836	JN411877	JN411859	JN411901
Itaropsis RFC 159	JN411841	JN411822	JN411878	JN411860	JN411899
Itaropsis RFC 167	JN411842	JN411821	JN411879	JN411861	JN411896
Itaropsis RFC 212	JN411843	JN411820	JN411880	JN411862	JN411907
Itaropsis RFC 213	JN411844	JN411827	JN411881	JN411863	JN411908
Itaropsis RFC 214	JN411845	JN411832	JN411882	JN411864	JN411909
Itaropsis RFC 216	JN411846	JN411828	JN411883	JN411865	JN411906
Itaropsis RFC 236	JN411847	JN411834	JN411884	JN411866	JN411898
Itaropsis RFC 237	JN411848	JN411829	JN411885	JN411872	–
Itaropsis RFC 239	JN411849	JN411837	JN411886	**JN411867**	JN411894
Itaropsis RFC 241	JN411850	JN411830	JN411887	**JN411868**	JN411897

GenBank, and seven sequences were downloaded from GenBank (Table 3).

Phylogenetic analyses

Morphological characters

Character data were edited and viewed using Winclada ver. 1.00.08 (Nixon, 1999–2002). All transformations were equally weighted and all characters are non-additive. Autapomorphic characters were excluded from the analyses because they are non-informative phylogenetically.

Characters were polarized using the outgroup comparison (e.g. Nixon & Carpenter, 1993) and two outgroup taxa were considered, namely *Acheta domesticus* (Linné, 1758) (Gryllidae, Gryllinae)

Table 4. Song features of *Itaropsis* from four different localities in peninsular India

Sampling site	Syllable period (ms)	Syllable duration (ms)	Call duration (s)	Dominant frequency (kHz)	No. of individuals
Bombay	81.5 (2.1)	32.9 (2.8)	8.514 (6.81)	7.5 (0.5)	8
Bangalore	100 (2.4)	40.2 (0.9)	9.206 (3.17)	6.6 (0.4)	7
Kadari (Triller)	41.8 (0.9)	26.8 (0.6)	28.241 (24.89)	7.5 (0.3)	7
Kadari (Chirper)	42.5 (2.3)	22.3 (1.2)	0.421 (0.17)	7.5 (0.3)	5
Valparai	54.8 (1.3)	26.4 (0.8)	0.297 (0.05)	6.4 (0.2)	7

Values are means (SE).

and *Nisitrus vittatus* (Haan, 1842) (Eneopteridae, Eneopterinae).

Cladistic analyses of the data matrix were implemented in NONA version 2.0 (Goloboff, 1999) run under Winclada. Heuristic searches were performed using the 'mult*max*N' command with 10 000 replicates, and the 'hold1000' and 'hold/100' options of NONA. Consistency index (CI: Kluge & Farris, 1969) and retention index (RI: Farris, 1989) were calculated with NONA, emulated by Winclada.

Molecular and combined characters

DNA sequences were aligned using MUSCLE (Edgar, 2004) with default parameters. Recent studies have identified potential problems with concatenation of multiple loci for phylogenetic analysis and use of mitochondrial genes only for reconstructing the relationships between closely related taxa (Kubatko & Degnan, 2007; Song *et al*., 2008; Moulton, Song & Whiting, 2010). We thus made separate analyses of each data partition (mitochondrial data, nuclear data, morphology). All data sets produced similar trees (see Results below) and we concatenated all molecular data, and molecular data with morphology, to use all available evidence (Kluge, 1989, 1997; Nixon & Carpenter, 1996).

All the analyses were performed using Bayesian inference and unweighted parsimony. The parsimony analyses were performed under TNT (Goloboff *et al*., 2008) with 5000 replications of Random Addition Sequence and Tree Bisection and Reconnection (TBR). The substitution model of evolution was estimated using the program jMODELTEST v 0.1.1 (Posada, 2008), and the Akaike Information Criteria (AIC; Akaike, 1973, 1974) was used to select the GTR+I+G model. Bayesian analyses were conducted with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Four Markov chains were run simultaneously for sampling of 15 million generations every 100 generations to ensure the independence of samples. The first 37 500 generated trees were discarded as burn-in (25%). The remaining trees were used to construct the 50% majority-rule consensus trees. Two independent runs were performed to check whether convergence on the same posterior distribution was reached and if final trees converged on the same topology. The statistical confidence in nodes was evaluated by posterior probabilities.

RESULTS

Acoustics

Comparisons of the four song features, namely syllable duration, syllable period, call duration, and carrier frequency, revealed striking differences in structure between the songs recorded at the four sites (Table 4). The mean syllable periods and durations of the songs recorded from Bangalore and Bombay were much longer (almost double) than those recorded from Kadari and Valparai. The carrier frequencies of songs from Bombay and Kadari were about 1 kHz higher than those recorded from Bangalore and Valparai (Table 4). In addition, the songs in Bombay and Bangalore were produced as relatively long trills of several seconds duration, whereas the songs of the males from Valparai consisted of short, stereotyped chirps, with a mean duration of only 0.3 s. The males from Valparai were also the only ones to produce amplitude-modulated calls (Fig. 2). In Kadari, encountered males produced either chirps of short duration, typically less than 0.7 s in length, or long, uninterrupted trills, some of which extended for over a minute. We therefore classified the songs into five types, four from the four different localities, with two song types from Kadari, based on the difference in call duration.

Each of the four song features exhibited significant overall differences between the five song types (one-way ANOVA, $P < 0.0001$ in all cases). Post-hoc pairwise comparisons between all the song types (Tukey's HSD test) reinforced the idea that there exist five distinct song types in the four sampling sites (Table 5).

For syllable period more specifically, all pairwise comparisons between the four sites revealed significant differences. This alone suggested the existence of

Table 5. Results of pairwise comparison between song types showing significant differences in different acoustic features

	Bombay	Bangalore	Kadari triller	Kadari chirper	Valparai
Bombay	–				
Bangalore	S**D*F**	–			
Kadari triller	S**D*	S**D**F**	–		
Kadari chirper	S**D*C**	S**D**C**F**	C**	–	
Valparai	S**D**C**F**	S**D**C**	S**C**F**	S**C*F**	–

**$P < 0.005$, *$P < 0.05$. C, call duration; D, syllable duration; F, carrier frequency; S, syllable period.

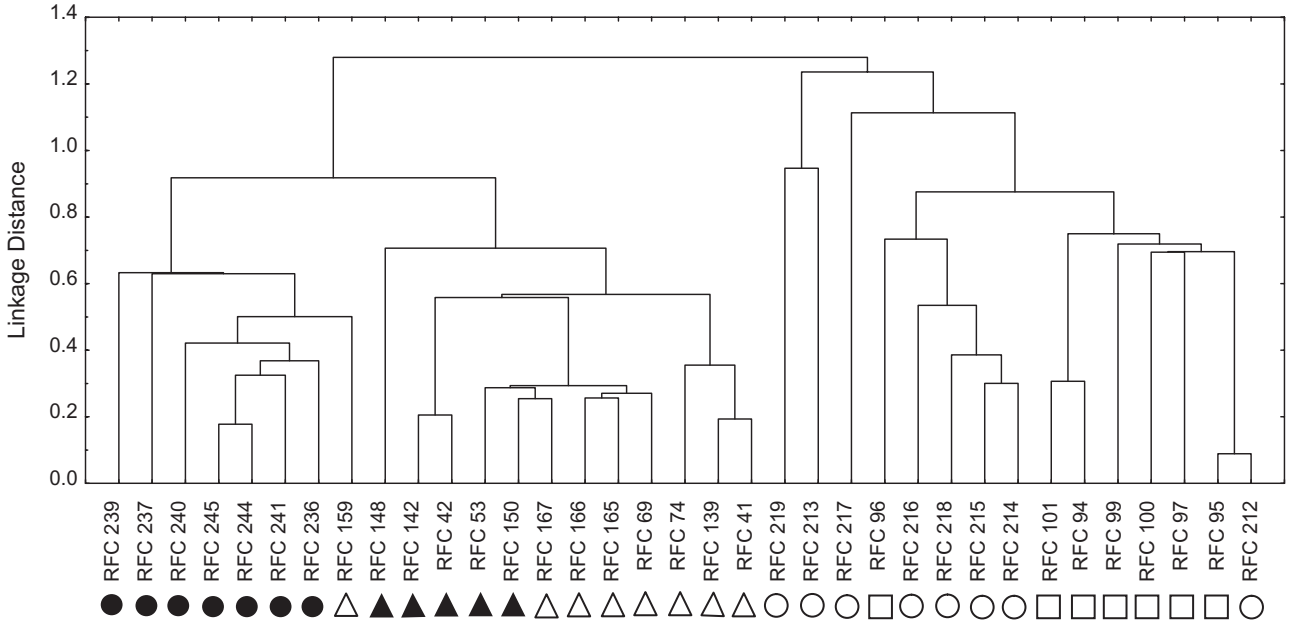

Figure 3. Dendrogram based on four song features: call duration, syllable duration, syllable period and dominant frequency for individuals of the five song types of *Itaropsis*. Closed circles, Valparai chirper; closed triangles, Kadari chirper; open triangles, Kadari triller; open squares, Bangalore triller; open circles, Bombay triller.

four species. The songs from Bombay and Valparai (the northernmost and southernmost sampling sites) differed significantly in all four call features (Table 5), strongly suggesting different species. In addition, the songs from Bangalore differed from those of Bombay and Kadari in syllable period, syllable duration, and carrier frequency (Table 5). Within the Kadari sample, however, there were no significant differences between the short-duration chirping calls and the long-duration trills in syllable period, syllable duration or carrier frequency (Tables 4, 5), suggesting that these calls may in fact be variations of one specific call. Finally, the songs from Kadari (both chirps and trills) differed significantly from those of Valparai in both syllable period and carrier frequency (Table 5). Taken together, the results suggested a conservative estimate of four species, one from each locality, instead of five.

A cluster analysis of the entire data set based on overall similarity using the four song features (Fig. 3) revealed three, rather than four, clusters. Interestingly, the songs from Bangalore and Bombay were grouped together in one cluster, suggesting that individuals from the two groups may constitute one species. The second cluster consisted of the songs from Kadari, including both chirpers and trillers. The third cluster consisted of the songs from Valparai. There were two outliers, which were trilling individuals from Kadari, whose songs were of exceptionally long duration (about 1 min). Qualitatively similar results were obtained when the analysis was repeated without using call duration as a feature by both cluster analysis and multidimensional scaling (results not shown). Thus, the results of the multivariate analysis suggested the existence of only three acoustic entities, which could represent three distinct species

of *Itaropsis*, one widely distributed and extending from Bombay to Bangalore, a second in Kadari (with large variation in call duration), and a third from Valparai.

Morphology

Regular taxonomic analyses, based on male genitalic structures and forewing venation, allowed us to separate the specimens from Valparai only. All the other studied males presented very similar although slightly variable structures, without clear-cut differences, whatever their songs.

For the phylogenetic analyses, we defined 40 characters describing forewing venation ($n = 18$), general morphology (6), coloration (1), and male genitalia (15) (see online Supporting Information, Appendix S1). The number of characters for each structure depends on the amount of variation observed. Most characters are binary, but 11 characters are multistate; four characters are hierarchical, and six characters are shown to be polymorphic (Table 6).

The phylogenetic analysis of morphological characters resulted in one tree (Fig. 4A, 120 steps, CI 41, RI 51). *Itaropsis* was found to be monophyletic and three main clades were obtained. The first clade comprised mostly specimens from Valparai, the second clade comprised the Bombay and Bangalore specimens plus one specimen from Valparai (RFC236), and the third clade consisted of the specimens from Kadari, both chirpers and trillers, in addition to one specimen from Bangalore (RFC99) and one from Valparai (RFC239).

The acoustic clusters were not completely recovered by morphological characters, even though there was a clear congruence between acoustic clusters and morphological clades. Additionally, within the Bombay–Bangalore clade, the specimens from Bombay are clearly separate from those originating from Bangalore, which seem more variable. Within the Kadari clade, no clear separation occurs between the chirpers and the trillers, even though trillers are mostly basal and chirpers mostly apical in position. Finally the Valparai cluster is the most heterogeneous cluster within *Itaropsis*.

Molecular data

For each data set, parsimony and Bayesian analyses gave identical results, and only Bayesian trees are presented here. As shown in Figure 4, the three acoustic clusters (Fig. 3) are broadly recovered, although not always as monophyletic groups, and with high posterior probabilities. Only the Bombay–Bangalore cluster is in fact monophyletic with both mitochondrial (Fig. 4B) and nuclear (Fig. 4C) data sets. The Kadari cluster is monophyletic with mitochondrial genes, but polyphyletic with the nuclear gene, and the Valparai cluster is always polyphyletic and in basal positions.

Combined analysis

In the combined analyses of all data sets, *Itaropsis* was always recovered as monophyletic, as were the Bombay–Bangalore and the Kadari clusters (Fig. 5). The Valparai cluster was not monophyletic, only three of the four specimens being grouped together. The relationship between these clades is unclear (polyfurcation in consensus tree). Contrary to the morphological tree, the Bombay and Bangalore specimens are not sorted according to their origin in the Bombay–Bangalore clade. In the same way, chirpers and trillers from Kadari are not separate according to their songs.

The acoustic clusters found above (Fig. 3) are thus better recovered in the combined analyses, which support the separation of three distinct morphologically cryptic lineages (Fig. 5).

Song evolution and variation

Optimization of the four song features (syllable period, syllable duration, call duration and dominant frequency) on the phylogenetic tree revealed the pattern of song evolution in this genus. The estimated ancestral song of *Itaropsis* consisted of long trills, with intermediate syllable durations and periods (Fig. 6A, B) and a dominant frequency of about 7 kHz (Fig. 6D). From this state, the Bombay–Bangalore clade appears to have evolved exceptionally long syllable durations (32–40 ms) and periods (80–100 ms), whereas they have become significantly shorter (22–26 ms durations and 55–40 ms periods) in the other two clades, to the extent that the syllable durations and periods of the Kadari clade are almost half those of the Bombay–Bangalore clade (Table 4). Call duration (Fig. 6D) was retained in the ancestral state of long trills in the Bombay–Bangalore clade but evolved into short, amplitude-modulated chirps in the Valparai clade. The Kadari clade revealed interesting variation in this regard, with some individuals producing unusually long trills, and others very short chirps. Whether these represent song polymorphisms can only be determined by further detailed analyses of individual male songs over time and of female phonotaxis to the chirp and trill variants.

DISCUSSION

Song as a criterion to delineate species boundaries

We have explored the use of song as a tool to delineate species boundaries. Although songs are to a large

Table 6. Data matrix for 40 morphological characters for 20 *Itaropsis* specimens and two outgroups (see Table 1, Appendix S1)

	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Nisitrus vittatus	0	1	0	0	0	0	0	?	0	2	0	a	a	2	?	?	3	0	0	0	0	0	?	0	?	0	?	?	?	0	?	0	?	1	0	0	?	?	?	?
Acheta domesticus	0	2	0	0	3	1	a	0	a	2	0	1	2	2	?	?	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	0	?	2	0	1	0	0	?	?
RFC42	1	1	1	1	1	2	1	0	1	1	1	?	1	0	0	?	0	1	1	1	1	1	0	0	?	1	1	1	1	1	1	1	1	0	0	1	1	1	1	?
RFC53	1	1	1	1	3	1	1	1	1	1	1	?	1	0	0	?	0	1	1	1	1	1	0	0	?	1	1	1	0	1	1	1	0	0	0	1	1	1	0	0
RFC142	1	1	1	1	1	2	0	?	1	0	1	?	1	a	0	1	0	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0	0	1	1	1	1	?
RFC150	1	1	1	1	2	1	1	1	1	1	1	?	1	0	0	?	0	1	1	1	1	0	?	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0	0
RFC212	1	1	1	0	3	1	1	0	1	0	0	0	1	0	1	?	2	1	1	0	1	1	0	0	?	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0
RFC213	1	1	1	0	3	2	1	0	1	2	0	0	1	0	0	?	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	1	1	0	0
RFC214	1	1	1	0	3	1	1	?	1	2	1	?	2	1	?	0	2	1	1	0	1	1	0	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0
RFC216	1	1	1	1	3	2	1	0	1	2	1	?	1	b	?	1	2	1	1	0	1	0	?	0	?	1	1	0	1	1	1	1	0	0	0	1	1	1	0	0
RFC159	1	1	1	0	2	1	1	1	1	1	1	?	2	0	0	?	1	1	1	0	1	0	?	1	1	1	1	0	0	1	1	1	0	0	0	1	1	1	0	0
RFC167	1	1	1	0	2	2	1	1	1	0	1	?	1	0	0	?	0	1	1	1	1	0	?	0	?	1	1	0	1	1	1	1	1	0	0	1	1	1	0	0
RFC41	1	1	1	0	3	1	1	0	1	2	1	?	1	0	0	?	0	1	1	1	1	1	?	1	?	1	1	1	0	1	1	1	0	1	0	1	1	1	0	0
RFC69	1	1	1	0	2	2	1	1	1	0	1	?	1	0	0	?	0	1	1	1	1	0	?	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	0	0
RFC94	1	1	1	0	2	2	1	1	1	0	1	?	1	1	?	0	2	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0
RFC96	1	1	1	0	2	1	1	1	1	2	0	1	1	c	0	?	2	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	0	1
RFC99	1	1	1	0	2	1	1	1	1	1	1	?	1	0	a	?	2	1	1	1	1	0	?	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	1
RFC100	1	1	1	0	2	1	0	?	1	0	0	1	0	0	0	?	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	1	1	0	0	1	1	1	0	1
RFC236	1	1	1	0	2	1	1	0	0	2	0	0	1	1	?	0	0	1	1	1	1	1	0	0	?	1	1	0	1	1	1	1	0	1	1	1	1	1	0	2
RFC237	1	1	1	0	2	2	0	?	0	2	1	?	0	0	0	?	0	1	1	1	1	0	?	1	0	1	1	0	1	1	0	1	?	0	0	1	1	1	0	0
RFC239	1	1	1	0	2	2	1	0	1	1	0	1	1	1	?	0	0	1	1	0	1	0	?	1	0	1	1	0	1	1	1	1	1	0	0	1	1	1	0	0
RFC241	1	1	1	1	2	2	0	?	0	2	0	0	1	0	0	?	0	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	0	1	1	1	1	?

Polymorphism codes: a, [01]; b, [12]; c, [02].

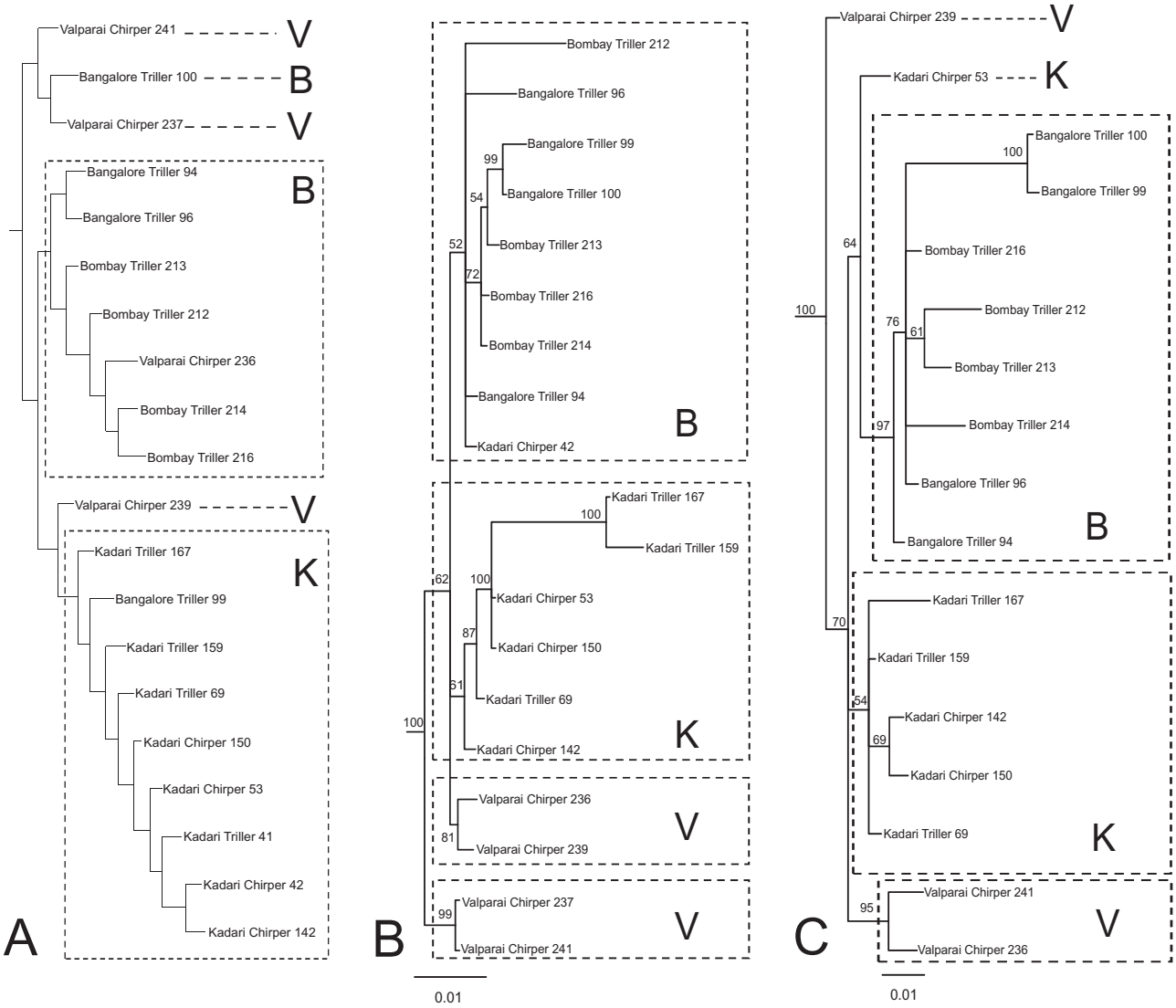

Figure 4. Phylogenetic trees of *Itaropsis* resulting from analysis of morphological and molecular data. A, cladogram obtained with morphological characters (one tree, length 120 steps, CI 41, RI 51); B, phylogenetic tree obtained from Bayesian analysis of mitochondrial data sets for 19 terminals; C, phylogenetic tree obtained from Bayesian analysis of nuclear data sets for 17 terminals. Abbreviations: B, Bombay–Bangalore cluster; K, Kadari cluster; V, Valparai cluster.

extent species-specific in crickets, it is unclear in what manner their features may be used to successfully define species boundaries. Our results show a mismatch between the possible numbers of species obtained by feature-wise statistical analyses and those obtained using measures of overall acoustic similarity. Feature-wise analysis suggested the existence of four or five species, whereas cluster analysis yielded three major groups.

By its rationale, cluster analysis integrates the information which summarizes the global similarity for all four acoustic characters, and not only the raw differences for each feature separately. It also avoids the a priori delimitation of putative acoustic groups common to ANOVA and basic statistics. These approaches may then differ greatly in their ability to differentiate putative species boundaries. Applied to both acoustic and morphological characters, cluster analysis has been previously shown to successfully delineate species boundaries among four classically defined species of the tree cricket genus *Oecanthus* (Metrani & Balakrishnan, 2005). In the same way, a recent study (Popple, Walter & Raghu, 2008) on the songs and host plants of the cicada species *Pauropsalta annulata* over a broad geographical range revealed four song types by cluster analysis, and

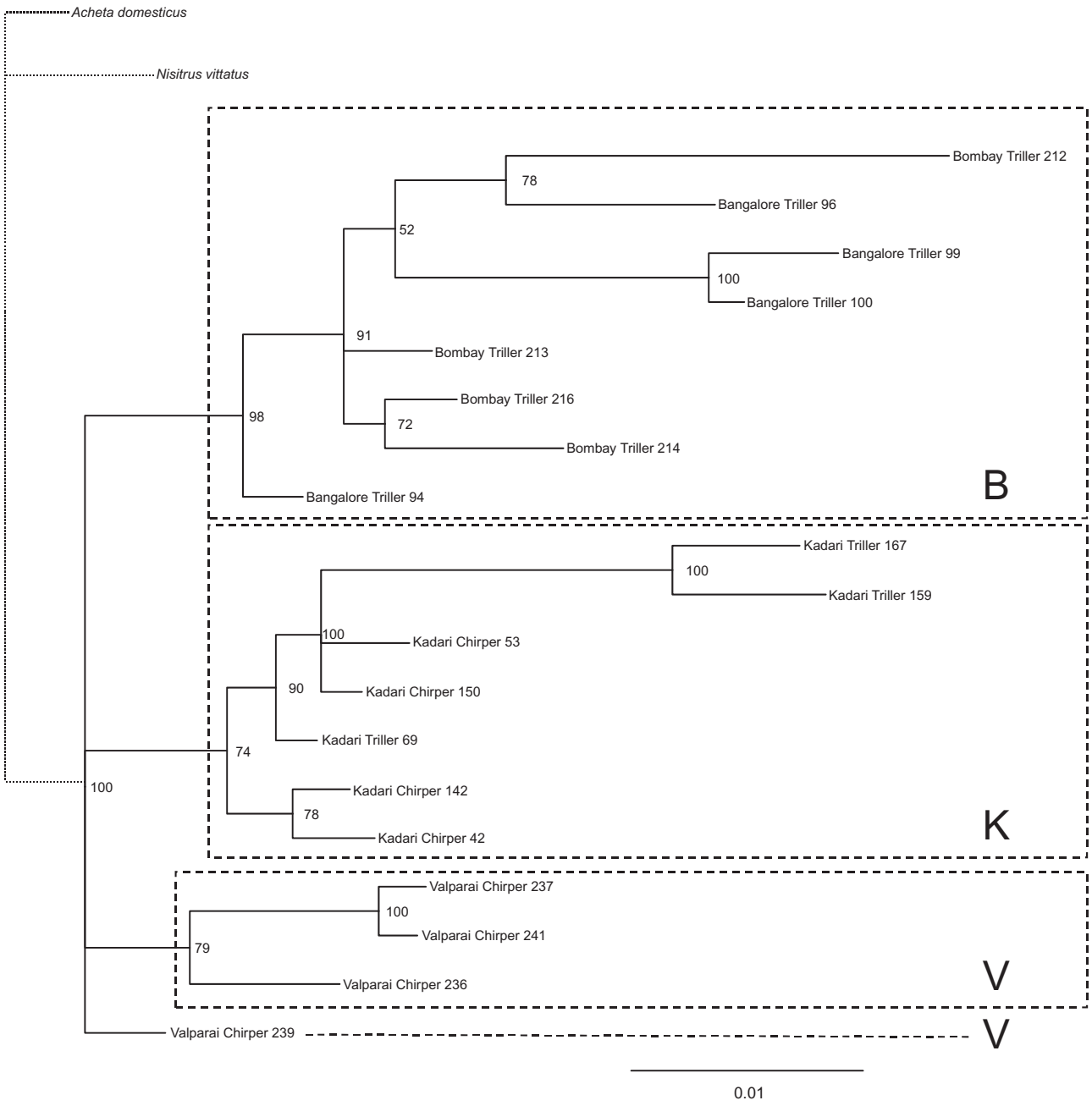

Figure 5. Phylogeny of *Itaropsis* obtained from Bayesian analysis of combined morphological, mitochondrial, and nuclear data sets. Abbreviations as in Figure 4.

these song types were concordant with different host–plant associations of the four groups, suggesting the existence of four cryptic lineages. All these results support the value of calling songs as a good indicator of potential species boundaries, but call attention to their sensitivity to the method used to characterize acoustic units (here, cluster analysis versus feature-wise analysis). In our study, even the acoustic features which are most important for female phonotaxis in crickets (syllable period and carrier frequency) were inadequate for species delimitation when taken separately.

Concordance between acoustic clusters and phylogenetic lineages, and taxonomic levels

Once clusters have been obtained, the question is whether they correspond to independent lineages.

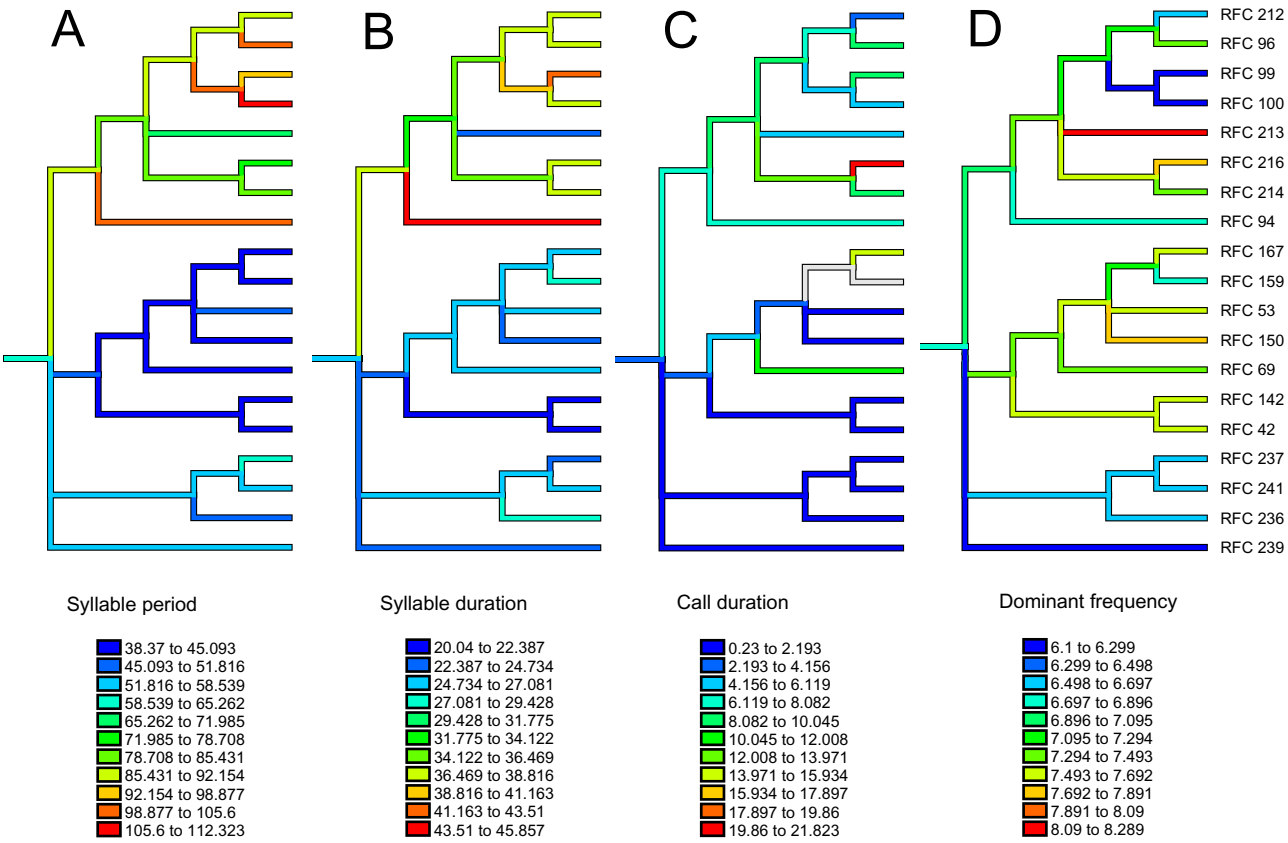

Figure 6. Patterns of song evolution in *Itaropsis* resulting from the optimization of song feature values on the phylogenetic tree obtained from combined data sets for 19 terminals. A, syllable duration; B, syllable period; C, call duration; D, dominant frequency. Symbols as in Figure 3.

Our phylogenetic analyses largely support the acoustic clusters. Two lineages were almost recovered with morphological, molecular, and combined data sets: the Bombay–Bangalore cluster on the one hand, and the Kadari cluster on the other, whatever the heterogeneity of individual songs in the latter lineage. The Valparai cluster was never recovered as monophyletic, and in every phylogenetic analysis there was always at least one terminal which was not placed within the corresponding acoustic cluster.

Even though we did not obtain a complete congruence between the analysed data sets, our phylogenetic line of evidence, based on the congruent signal provided by four mitochondrial genes and one nuclear gene, strongly attested a divergence between *Itaropsis* populations (Fig. 4), and the phylogenetic lineages generated by the combined data sets (Fig. 5) were largely concordant with the acoustic clusters (Fig. 3), which also proved to be geographically consistent. Three morphologically cryptic lineages of *Itaropsis* can clearly be suspected in peninsular India.

These results are half way between those obtained by several authors with similar data sets and analyses. Lougheed *et al*. (2006) analysed seven populations of the hylid frog *Hyla leucophyllata* to infer the relative importance of morphology, molecular sequences (16S RNA) and acoustics in predicting the early stages of population differentiation. They observed a lack of correspondence between molecular data versus morphology and acoustics, and concluded that call variation could not be explained by population history: the populations were ecotypes of one species, resulting from selective variations of morphology (mostly size) related to call frequency. By contrast, a mitochondrial gene-based phylogenetic delimitation of species boundaries in trilling chorus frogs of the genus *Pseudacris* (Lemmon *et al*., 2007) was largely concordant with a previous taxonomic delimitation of these species based on a combination of morphological and acoustic characters. Manier (2004) tested the morphological differentiation of four colubrid subspecies (Colubridae: *Rhinocheilus lecontei*), three from mainland western North America, and one from peninsular Baja California. He found no diagnostic characters for the continental subspecies, which were synonymized with the nominal subspe-

cies, whereas the peninsular subspecies was characterized by slight morphological differences: owing to this and to its geographical distribution, it was maintained as a separate subspecies. At a higher level of differentiation, Padial *et al*. (2009) combined morphological, acoustic, and molecular data to test the status of 15 nominal species of *Pristimantis* frogs. They found a full congruence for only four species, although all of the 15 species were supported by at least one data set. In the *Itaropsis* case study, even though we observed a strong phylogenetic divergence concordant with acoustic clusters, we failed to get an absolute concordance between the data sets, and no lineage was fully characterized by at least one data set. As a consequence we cannot consider the three lineages as distinct species.

The more reasonable conclusion in our case study is then to consider the three *Itaropsis* lineages as incipient species, which have not yet completed their diversification process. In particular, the lack of a diagnostic character, either morphological or acoustic, would invalidate separate distinct species of Indian *Itaropsis* from the practical point of view of a useful and straightforward taxonomy (Manier, 2004; Mulcahy, 2008). This conclusion is also supported by the fact that the studied populations are allopatric and live in open, often man-made environments, while most sibling cryptic species tend to be sympatric and forest dwelling (Bickford *et al*., 2007). Additionally, divergence in mate recognition systems is known to occur most often in sympatric, rather than allopatric, populations (Mendelson & Shaw, 2005; Jang & Gerhardt, 2006).

Itaropsis taxonomy and distribution

The three morphologically cryptic lineages of *Itaropsis* show exclusive distributions: one is widely distributed and extends from Bombay to Bangalore, a second is located in the Western Ghats and coastal areas of southern Karnataka, and the third has been found in Valparai, south of the Palghat Gap. Our first clade could correspond to the nominal *Itaropsis parviceps*, as these were collected from the type locality of the species. The type specimen is a male, and identification will have to be confirmed by specimen comparison. On the other hand, the type of *I. tenella* is a female: preliminary observations show that female genitalia differ geographically, but the extent and significance of this variation have to be checked on a larger scale. As a working hypothesis, and to test the synonymy proposed by Chopard (1969), we propose the existence of two distinct species in the genus *Itaropsis*: *Itaropsis tenella* from Sri Lanka and *Itaropsis parviceps* from western continental India, the latter subdivided into three subspecies, one in the Bombay–Bangalore area, one north of the Palghat gap in the Western Ghats of Karnataka, and one south of the Palghat Gap in the Anaimalais (Valparai).

From a biogeographical point of view, these findings are in agreement with studies on other taxa in the Western Ghats. An extensive study (Bossuyt *et al*., 2004) on the historical biogeography of six taxonomic groups (tree frogs, caecilians, uropeltid snakes, freshwater fishes, crabs, and shrimps) distributed in India and Sri Lanka revealed that the Palk Strait has acted as an effective barrier to gene flow in spite of sea-level changes in geological time that should have allowed species to cross this barrier.

Further, the Palghat Gap, which is a 40-km-wide break in the almost continuous chain of the Western Ghats, has also been proposed to be a biogeographical barrier, based on studies of species distributions and genetic structure (Ripley & Beehler, 1990). Recent studies on the population genetic structure of two large, vagile vertebrate species, the Asian elephant (*Elephas maximus*) and the white-bellied shortwing (*Brachypterys major*), a montane forest bird, have revealed large genetic differences between populations north and south of the Palghat Gap (Vidya *et al*., 2005; Robin, Sinha & Ramakrishnan, 2010). In the case of the shortwing, the genetic differences are sufficiently large to warrant dividing it into two species (Robin *et al*., 2010). Our finding that *Itaropsis* specimens south of the Palghat Gap (from Valparai) differ in song and molecular data from those north of the Palghat Gap (Kadari) is thus not surprising.

The low differentiation of *Itaropsis* phylogenetic lineages could indicate that this genus has differentiated only recently, as most species distributed both in the Western Ghats and in Sri Lanka. The open habitat of *Itaropsis* could lend support to this hypothesis: up to now, areas of endemism have always been delimited and documented for forest living species; these species inhabit the series of hills that make the Western Ghats and may have evolved in isolation during sea level rise (Inger *et al*., 1987). The pattern of distribution of *Itaropsis* lineages show that areas of endemism could also apply to the open habitat biotas occurring in these regions (Das *et al*., 2006), but perhaps that the time of biotic differentiation may be delayed.

Song evolution and variation in *Itaropsis* crickets

At the species level, there have been a number of studies demonstrating the concordance between differences in acoustic features and reproductive

isolation (reviewed in Gerhardt & Huber, 2002). The infraspecific level has received less attention, and this is the level at which our study could help by analysing the role of advertisement signals in cricket diversification.

Our results show a broad congruence between studied lines of evidence: acoustic clusters and the clades resulting from morphological, molecular (both mitochondrial and nuclear), and combined analyses are broadly similar, and all the data sets seem to have evolved jointly. Incongruence in character evolution is often related to faster divergence in some characters than in others (Orr & Smith, 1998), especially characters involved in prezygotic reproductive isolation such as calling songs (Padial *et al*., 2009; Gonzalez, Ornelas & Gutiérrez-Rodriguez, 2011). The lack of incongruence in our results does not support the hypothesis that acoustic signals could have directed lineage diversification in *Itaropsis*, especially at such a low level of diversification. An alternative hypothesis would consider that allopatric populations of *Itaropsis* diverge for all their characters in the same time, by genetic drift for example.

The striking variation in temporal features in this genus, extending from short chirps to extremely long trills, and from the relatively short syllables typical of field crickets to exceptionally long syllables, is not matched by the changes in carrier frequency. The carrier frequency of the ancestral form is estimated at about 7 kHz: the Valparai clade has, on average, somewhat lower frequencies (6–7 kHz), and the Kadari clade, somewhat higher frequencies (7–8 kHz), while the Bombay–Bangalore clade shows no distinct pattern and is characterized by high variation. The song divergence in this genus is thus largely explained through changes in temporal rather than spectral features, as usually observed in crickets (Alexander, 1962; Otte, 1992; Robillard & Desutter-Grandcolas, 2011) even though diversification in frequency values and spectra has been documented in some clades (Robillard, Grandcolas & Desutter-Grandcolas, 2007).

Itaropsis is also characterized by considerable within-clade variation in songs: the Bombay–Bangalore clade in particular shows high inter-individual variation in both carrier frequency and syllable period, two song features that are usually critical for species recognition in crickets (Gerhardt & Huber, 2002). It would be interesting to examine whether female responses in this clade are relatively broadly tuned. Similarly, the Kadari clade shows striking variation in call duration: this raises the possibility of polymorphisms within this clade, although the identical syllable repetition rates and carrier frequencies throughout this clade suggest that females may be evaluating these two features and ignoring the others. The call duration could also be a dynamic feature (*sensu* Gerhardt, 1991) that is under sexual selection. This population also needs to be studied in greater detail to examine the causes and consequences of high song variation within clades.

CONCLUSION

Clearly there is no absolute rule to decide which data set is more accurate in delineating species boundaries. It therefore seems reasonable to rely upon the data which play a major role in the biology and reproduction of the studied species. Behavioural or ecological data may be particularly useful in characterizing reliable signatures of species identity, but they gained from being compared with other data sets in a phylogenetic context to check that they correspond to independent evolutionary lineages (Padial *et al*., 2009). No arbitrary threshold exists for species delimitation (Padial *et al*., 2009 *contra* Fouquet *et al*., 2007), and the large benefits of integrative taxonomy is to consider the information content of contrasted data sets, keeping in mind that taxonomy is fundamentally a practical tool. Even if the lack of a diagnostic character prevents the separation of distinct species, it may reveal lineages that are worth studying deeper, either with other lines of evidence [e.g. female phonotaxis in the case of acoustic animals (Lehtinen, Wojtowicz & Hailey, 2011)], or sampling the geographical distribution with more numerous populations.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of morphological characters used to study the phylogeny of 20 *Itaropsis* terminals and two outgroups.

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