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Source: The Auk, 133(3) : 497-511

Published By: American Ornithological Society

URL: https://doi.org/10.1642/AUK-15-233.1

The Auk
Ornithological Advances
AmericanOrnithology.org

Volume 133, 2016, pp. 497–511
DOI: 10.1642/AUK-15-233.1

RESEARCH ARTICLE

Phenotypic and genetic analysis support distinct species status of the Red-backed Woodpecker (Lesser Sri Lanka Flameback: *Dinopium psarodes*) of Sri Lanka

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Submitted December 20, 2015; Accepted March 23, 2016; Published June 22, 2016

ABSTRACT

Hybridization has challenged taxonomy, since hybridizing forms could be stable evolutionary entities or ephemeral forms that are blending together. The island of Sri Lanka has 2 subspecies of the flameback woodpecker *D. benghalense*: *D. b. jaffnense* in the north and *D. b. psarodes* in the south. Red plumage separates the endemic phenotype *D. b. psarodes* from other subspecies of *D. benghalense*. Despite these differences, intermediate phenotypes in north-central Sri Lanka discouraged the elevation of *D. b. psarodes* into a full species. The recent HBW and BirdLife International checklist, however, has elevated *D. b. psarodes* to a full species (*D. psarodes*), primarily based on its plumage. To objectively evaluate whether this taxonomic elevation is warranted, we examined the phenotypic and genetic affinities of *D. psarodes* within the *D. benghalense* cluster. In doing that we provide the first quantitative phenotypic and genetic analysis across a hybrid zone for an Old World woodpecker group. We sampled woodpeckers along a line transect across the island and measured body shape/size, plumage, and genetic variation in a mitochondrial gene (Cytb). Plumage color ranged from red in the south to yellow in the north, with varying proportions of orange in north-central Sri Lanka (an area of ~66 km). Morphology (body shape/size) and plumage characters showed a clear separation. There are 2 mitochondrial haplotype groups, one in the north and one in the south. A mixture of north and south haplotypes were seen in north-central Sri Lanka. Width of the hybrid zone suggests that some form of selection limits the spread of hybrids into the range of parental forms. Morphological, plumage, and genetic traits are all indicative of limited hybridization in a narrow zone between the 2 taxa, supporting the treatment of *D. psarodes* as a distinct species. This study provides an illustrative example of extensive hybridization between stable taxonomic entities, discouraging the practice of merging hybridizing forms as single species.

Keywords: *Dinopium benghalense*, island endemicity, flameback woodpeckers, hybridization, hybrid zone, speciation, Sri Lanka

Análisis fenotípicos y genéticos apoyan el estatus de especie de *Dinopium psarodes* en Sri Lanka

RESUMEN

La hibridación es desafiante para la taxonomía puesto que las formas que hibridan pueden ser entidades evolutivamente estables o formas efímeras que se están mezclando. La isla de Sri Lanka tiene dos subespecies de *Dinopium benghalense*; *D. b. jaffnense* en el norte y *D. b. psarodes* en el sur. El plumaje rojo separa el fenotipo endémico de *D. b. psarodes* del de otras subespecies de *D. benghalense*. A pesar de estas diferencias, la existencia de fenotipos intermedios en el centro-norte de Sri Lanka previno la elevación de *D. b. psarodes* al estatus de especie. Sin embargo, la lista más reciente del Handbook of Birds of the World y Birdlife International elevó a *D. b. psarodes* al rango de especie (*D. psarodes*) basándose principalmente en el plumaje. Para evaluar objetivamente si se justifica este cambio taxonómico, examinamos las afinidades genéticas y fenotípicas de *D. psarodes* en el grupo de *D. benghalense*. Al hacerlo, presentamos el primer análisis cuantitativo fenotípico y genético a través de una zona de hibridación de un carpintero del viejo mundo. Muestreamos carpinteros a lo largo de un transecto que atravesaba la isla y medimos el tamaño y la forma del cuerpo, el plumaje y la variación genética en un gen mitocondrial (Cytb). El color del plumaje varió de rojo en el sur a amarillo en el norte, con naranja en diferentes proporciones en el centro-norte de Sri Lanka (un área de ~66 km). Los caracteres morfológicos (tamaño y forma del cuerpo) y del plumaje mostraron una separación clara. Existen dos grupos de haplotipos mitocondriales, uno en el norte y otro en el sur. Se vio una mezcla de haplotipos del norte y del sur en el centro-norte de Sri Lanka. El ancho de la zona de hibridación sugiere que alguna forma de selección limita la dispersión de los híbridos hacia la zona de distribución de las formas parentales. Todos los rasgos morfológicos, genéticos y del plumaje indican que existe hibridación limitada entre los dos taxones en una

zona estrecha, lo que apoya el estatus de especie de *D. psarodes*. Este estudio presenta un ejemplo ilustrativo de hibridación extensa entre entidades taxonómicas estables, lo que desaconseja la práctica de unir formas que hibridan en una sola especie.

Palabras clave: Dinopium benghalense, endémicos de islas, especiación, hibridación, Sri Lanka, zona híbrida

INTRODUCTION

The woodpecker genus *Dinopium* has historically consisted of 5 species from South and Southeast Asia, namely *Dinopium rattlesii*, *D. shorii*, *D. javanense*, *D. everetti*, and *D. benghalense* (Winkler et al. 1995, Winkler and Christie 2002, Clements et al. 2012). They are commonly called flamebacks due to their golden-colored mantle, scapulars, and folded wings. Among *Dinopium, D. benghalense* consists of 6 subspecies or races, distributed across South Asia (Gill and Donsker 2014), with 2 in Sri Lanka: *D. b. jaffnense* (Golden-backed Woodpecker) in the north and *D. b. psarodes* (Red-backed Woodpecker) in the south (Rasmussen and Anderton 2012, Gorman 2014). Many color variations have been observed within the Sri Lankan forms (e.g., Legge 1880, Baker 1927, Wait 1931, Whister 1944, Philips 1953, Henry 1971, Rasmussen and Anderton 2012, Lamsfuss 2013, Gorman 2014, Fernando and Seneviratne 2015, Freed et al. 2015).

The red-backed form of the *Dinopium* complex had long been considered as endemic to Sri Lanka. Layard (1853) classified the red-backed form as *Brachypternus ceylonus* and the golden-backed form (*D. b. jaffnense*) as *B. aurantius*. Legge (1880) reported the Red-backed Woodpecker *B. ceylonus* as an endemic species to Sri Lanka. Both Legge (1880) and Wait (1931) commented on the possible hybridization of the 2 island forms. Intermediate phenotypes of *Dinopium* were reported as far back as 1868–1877 (Legge 1880). As a result, after Wait (1931), all authors except del Hoyo et al. (2014) continued to adopt the same taxonomic treatment of the 2 Sri Lankan forms as members of *D. benghalense* (Baker 1927, Whistler 1944, Ripley 1946, Ripley 1949, Philips 1953, Ali and Ripley 1983, Ripley and Beehler 1990, Rasmussen and Anderton 2012).

The recent checklist of Birdlife International (del Hoyo et al. 2014) elevated *D. b. psarodes* to a full species based on a system proposed by Tobias et al. (2010). In this system, a taxon is considered a separate species if an overall differentiation score reaches 7. The overall score is a sum of 2 elements: the quantification of differentiation in 3 main traits (morphology, voice, and biometrics) and 1 subsidiary trait (ecology and behavior), and the quantification of geographical relationship (allopatry or parapatry and the nature of the hybrid zone). In elevating the subspecies *D. b. psarodes* into a full species, del Hoyo et al. (2014) used the differences in its coloration (shining scarlet on back shading to dull red on wings, lack of black in carpal area), measurements (longer bill, wings, and tail) and vocalization (louder and higher-pitched call) compared to *D. b. jaffnense*.

Based on observations of plumage of *Dinopium* in the wild, a recent paper (Freed et al. 2015) has described 8 intermediate plumage types, and assumed that these types are of hybrid origin. Furthermore, Freed et al. (2015) showed that these intermediate types are found in the north-central part of the island. However, a quantitative analysis of these different plumage patterns in the *Dinopium* complex has not been attempted. Here we provide the first comprehensive examination of phenotypic (body shape/size and plumage) and genotypic variation across the 2 taxa of the *Dinopium benghalense* complex in the island to determine how well the criteria proposed by Tobias et al. (2010) for avian taxonomy apply to this species complex and to shed light on the complex biogeographic patterns displayed in *Dinopium* in the South Asian region (de Silva et al. 2014). We hypothesized that (1) *D. psarodes* is genetically differentiated from the *D. benghalense* cluster in a way corresponding to their phenotypic difference; (2) hybridization occurs in the contact zone, resulting in hybrids that have intermediate phenotypes and genotypes; and (3) partial reproductive isolation is achieved through a narrow hybrid zone maintained by selection against hybrids; if so, *D. psarodes* is best treated as a separate species.

METHODS

Field Sampling

From April to December 2013, we captured flamebacks using mist nets and dip nets along a 430 km transect extending from the range of *D. psarodes* (Southern Province; 06.04°N, 79.55°E) into the range of *D. b. jaffnense* (Northern Province; 09.46°N, 80.07°E). Sites were surveyed in intervals of ~50 km in allopatry and ~10 km in sympatry (i.e. across the transition zone between the 2 forms) using optical and acoustic cues, and playback of drumming calls. When a bird was located, call playbacks and a decoy (a life-size replica made out of plastic resembling a *Dinopium* male) were used to entice the bird to fly to the mist net (Seneviratne et al. 2012). From captured birds we measured morphology and plumage, obtained a blood sample (~50 μL), a set of photographs (to be maintained as a reference collection for later phenotypic analysis), and determined the sex (males have red crowns, females have white spots on the forward part of their crowns). We geo-referenced the capture site of each bird.

Museum Specimen Sampling

We examined 55 skins from different collections in the National Museum of Sri Lanka (Appendix Table 6). Similar to our treatment of live birds in the field, we measured phenotypic characters (see below) and obtained a toe pad for tissue sample (5 mg) and photographs from each skin. A bench-mounted white light source was used to illuminate specimens for photographs.

Morphology and Plumage Measurements

We measured 8 variables: culmen length, bill height and bill width to calculate the bill breadth (sum of bill height and width divided by 2); head length and head width to calculate the size of the head (sum of head length and width divided by 2); and flattened wing length, tail length, length of hind reduced digit, first claw length, and tarsus length (Baldwin et al. 1931, Seneviratne et al. 2012). A single observer (S.F.) measured all the characters in both field and museum specimens.

We evaluated 21 color-based characters: color of the forehead, crown, mustache (malar) band, supercilium, face region (between mustache and supercilium bands), chin, breast, scapulars, back (mantle), rump, upper tail coverts, tail, belly, primaries, secondaries, middle coverts, and upper and lower wing coverts. Color analyses were based on Munsell (1976) color charts. We measured 3 plumage metric characters using a dial caliper (0.01 mm): mustache width (measured ventral to the eye), width of the face region, and width of the supercilium. A single observer (S.F.) evaluated the intensity of carotenoid-based color expression (black 0%, golden yellow 17%, yellow 33%, orange 67%, red orange 83%, red 100%) in different plumage patches. We also compared RGB (Red, Green, Blue) and CMYK (Cyan, Magenta, Yellow, Black) values for these above color patches and recorded percentages of each color value (Fernando and Seneviratne 2015).

Amplification and Sequencing of Genes

We stored blood and toe pad samples in Queen's lysis buffer (Seutin et al. 1991) and extracted DNA using standard phenol-chloroform DNA extraction. We followed the same procedures that we used to amplify and sequence DNA (Seneviratne et al. 2012) with several modifications. At least 4 samples per taxon were sequenced for all flameback taxa presented in this study. Other sequences were obtained from GenBank to construct a consensus sequence for each species and identify species-diagnostic variation (Appendix Table 7 and Appendix Table 8). To estimate genetic relationships, we amplified and sequenced the cytochrome *b* (Cytb) gene (see below) of the mitochondrial genome.

Mitochondrial Genome: Cytochrome *b* (Cytb; 481bp)

We amplified a 481 base pair (bp) region of Cytb, using the primer combination *Cyto_b_F* and *Cyto_b_R* (Benz et al. 2006; Appendix Table 7). The PCR reaction mix was as follows: 1X PCR buffer (Life Technologies, Carlsbad, California, USA), 1.5 mM MgCl (Life Technologies), 0.2 mM dNTP mix (Promega, Madison, Wisconsin, USA), 0.5 mM *Cyto_b_F* and *Cyto_b_R* primers, 0.04 units/μL Taq DNA polymerase (Life Technologies), and 2.5 ng/μL template DNA, in a total volume of 10 μL. Thermal cycler conditions for Cytb included an initial 3 min at 94°C, followed by 35 cycles of 20 s at 94°C, 15 s at 53°C, 60 s at 72°C, followed by a 7 min final extension at 72°C.

Sequence Alignment: Genotyping and Phylogenetic Analysis

Sequences for other species of *Dinopium* and the outgroup *Picus* from a neighboring biogeographic region were downloaded from NCBI GenBank and all the sequences were multiple aligned with clustalW (MBL-EBI, Wellcome Trust Genome Campus, Cambridgeshire, UK) in Geneious 6.1.6 (Biomatters Limited, Auckland, New Zealand). Maximum-likelihood (ML) trees were generated using RaxML (Exelixis Lab, Scientific Computing Group, Heidelberg Institute for Theoretical Studies, Heidelberg, Germany), using rapid bootstrap, for 1,000 replicates with the GTR+G model using different outgroup species (Appendix Table 7).

Multiple sequences of Cytb of allopatric *D. psarodes* and *D. b. jaffnense* were then aligned with the Zebra Finch (*Taeniopygia guttata*) mitochondrial genome (Genebank accession number NC_007897; our sequences aligning within the range from 14246 to 14727 bp of the genome) to identify nucleotide sites with differences between the 2 forms, such that those informative markers could be used to genotype intermediate forms and estimate their ancestry. A total of 16 haplotypes was identified, which include 15 polymorphic sites. Analyses were done using DnaSP program (http://www.ub.edu/dnasp). A taxonomically informative G/A polymorphism was observed at 14391 bp, with allopatric *D. b. jaffnense* having nucleotide G and allopatric *D. psarodes* having nucleotide A.

Cline Construction

We collapsed latitude and longitude data of sampled birds to a single dimension by measuring the shortest geographic distance from the capture location of each bird to the southeastern coast of mainland India (as shown in Figure 1A; we call this distance the "Geographic Distance"). We used the program CFit-7 (Gay et al. 2008, Lenormand and Gay 2008) to estimate parameters for the best-fitting cline for the Cytb marker (as in Seneviratne et al. 2012). The cline was estimated as a simple sigmoidal curve across the contact zone. We treated the genetic trait as a simple two-allele system with each individual carrying a single Cytb allele.

We also used CFit-7 (with the unimodal model) to fit clines for PC1 scores of morphological and plumage

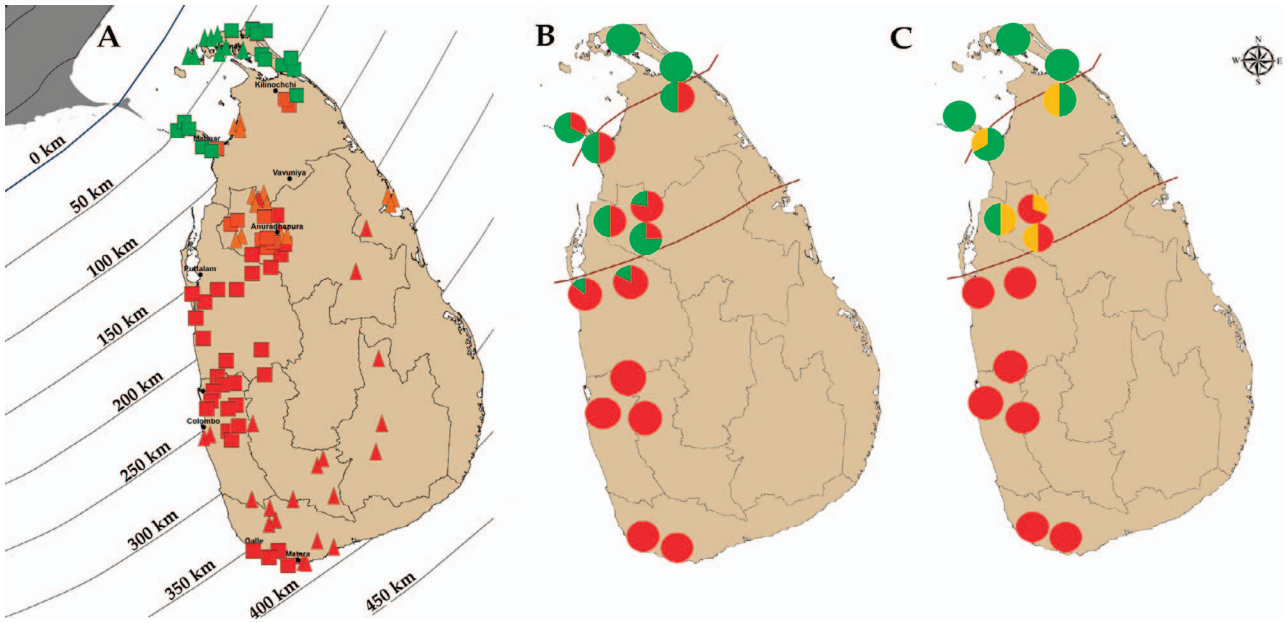

FIGURE 1. Allopatric distribution of *D. b. jaffnense* and *D. psarodes* and the existence of a hybrid zone between the 2 forms as seen from the capture locations of *Dinopium* flamebacks, frequency distribution of mitochondrial haplotype (Cytb), and the frequency distribution of plumage color in Sri Lanka. **(A)** Capture locations of field and museum specimens. The lines indicate the distance from the southeastern coast (Dhanushkodi) of mainland India. Squares denote field capture locations and triangles denote the locations of museum specimens: green = *D. b. jaffnense*, orange = *Dinopium* intermediate, red = *D. psarodes*. **(B)** Frequency distribution of mitochondrial haplotype (Cytb): green = northern haplotype, red = southern haplotype. **(C)** Frequency distribution of plumage color: green = *D. b. jaffnense*, yellow/ red and yellow = *Dinopium* intermediate in the hybrid zone, red = *D. psarodes*. The 2 lines indicate the northern and southern borders of the hybrid zone. The province boundaries are also shown.

characters. However, to better visualize the clinal variation of those phenotypic traits, we also fit cubic splines in program R (R Development Core Team 2010). We used CFit-7 to test whether the plumage, morphology, and genetic clines are coincident (i.e. have the same location) and concordant (i.e. have the same width); differences in location and width can be due to different patterns of selection, dispersal, inheritance, and associations between genes on these traits (Barton and Hewitt 1985). We compared each of the 4 combinations (unconstrained, centers constrained, slopes constrained, and both center and slope constrained) of the 3 phenotypic and genetic clines. The likelihood-ratio test and Akaike information criterion (Akaike 1974) were used to determine the best model with varying sets of constraints on cline centers and slopes.

Testing Whether Selection Maintains the Hybrid Zone

To test whether selection maintains the zone width, we compared the width obtained through cline analysis of field samples across the hybrid zone with the expected width of a neutrally expanding cline: $w = 1.68\sigma\sqrt{T}$ (Endler 1977, Brelsford and Irwin 2009), where w is the width of the neutral cline, σ is the root mean square dispersal per generation (distance between parent and offspring breeding sites), and T is the time in generations since secondary contact (Endler 1977). A narrow hybrid zone compared to neutral expectations would suggest that some form of selection maintains the zone (Barton and Hewitt 1989, Jiggins and Mallet 2000).

No estimates of dispersal distance are available for *Dinopium* or other Asian woodpeckers. Therefore we used estimates of generation time and dispersal of a New World woodpecker, the Northern Flicker (*Colaptes auratus*). Root mean square dispersal (σ) was estimated as 100 km per generation (estimates for *C. auratus*; Moore and Buchanan 1985). Generation time was estimated as 1.9 years, calculated as in Milá et al. (2007) based on the annual survival rate (0.41–0.53; Wiebe 2006), geometric population growth (assuming the population is in demographic equilibrium $\lambda = 1$; Milá et al. 2007), and the age of first breeding (1 year; Walters et al. 2002a, 2002b). We alternatively used a highly conservative estimate of root mean square dispersal (σ) based on North American songbirds (10 km per generation; Paradis et al. 1998).

We considered 2 levels of time since secondary contact of the parental taxa: 10,000 years and 160 years. The climate and other biogeographic characteristics of Sri Lanka remained more or less stable since the Miocene period (Bossuyet et al. 2004). Therefore we assume the secondary contact of *D. psarodes* and *D. b. jaffnense* took

TABLE 1. Comparison of del Hoyo et al. (2014) analysis for splitting *Dinopium psarodes* as a full species with present data shows a similar conclusion. "Scores" = introduced by del Hoyo et al. (2014).

	del Hoyo et al. (2014)		Our study	
Taxonomic characters	Minor characters	Scores	Minor characters (*D. psarodes* vs. *D. b. jaffnense*)	Scores
Biometrics	Longer billed Longer winged Longer tailed Effective size = 2.92	2	Longer billed (34.1 mm vs. 32.5 mm) Longer winged (138.5 mm vs. 133.6 mm) Longer tailed (94.7 mm vs. 86.9 mm) Effective size = 4.21	2
Acoustics	Screechy call (louder and higher-pitched call)	1	Not quantitatively analyzed (louder and higher-pitched call)	1
Plumage and bare parts	Scarlet central upper parts shading to dull red on wings vs. golden yellow shading to dull yellow Lack of black on carpal Effect size not reported	3 1	Contrasting difference in color Red vs. golden yellow Lack of black on carpal Effective size = 6.73	3 1
Ecology and behavior	Non-overlapping differences in foraging/ breeding habitat	1	Non-overlapping differences in foraging/ breeding habitat	1
Geographical relationship	Narrow hybrid zone	2	Narrow hybrid zone (hybridization between *D. psarodes* and *D. b. jaffnense* occurs over a range < 200 km at its maximum point	2
Total scores		10		10

place during the last complete exposure of the Adams Bridge (the land bridge that connects Sri Lanka to the mainland India) due to the drop of sea level ~10,000 years ago (Ripley 1949, Hopkins 1967). However the secondary contact may be more recent (de Silva et al. 2014). We used 160 years as our most conservative estimate of secondary contact based on the time where the species was first described to science in 1853 by the British ornithologist E. L. Layard (note that intermediate phenotypes of *Dinopium* were common in northern Sri Lanka even in 1868–1877; Legge 1880).

Analysis of the Phenotype

To summarize patterns of variation between plumage and size, we carried out separate principal components analyses (PCA) using correlation matrices, first for the 8 morphological characters and second for the 21 plumage characters. The principal axis method was used to extract the components without rotation. The majority of variation was captured by the first component (PC1) in both analyses (morphometric and plumage) and only the first 2 components displayed eigenvalues greater than 1. Therefore we used the first 2 principal components for the rest of the analysis. To examine the relationship between the parental genotypes with parental phenotypes, we used one-way ANOVA with Tukey's family error post hoc test (Sokal and Rohlf 1995). Since the residuals were not normally distributed we performed a rank-based normalization with Tukey's formula. Shapiro–Wilk's test was executed to confirm normalization ($P > 0.05$; Sokal and Rohlf 1995). Significance level was set at $P < 0.05$. All statistical analyses were performed with SPSS 20.0 (IBM Corporation, Armonk, New York, USA).

Evaluation of del Hoyo et al. (2014) Criteria of Splitting *D. benghalense*

We compared our dataset with the data used by del Hoyo et al. (2014) to split *D. psarodes* as a full species following the criteria proposed by Tobias et al. (2010; Table 1). Tobias et al. (2010) used the strength of differentiation in various characters according to effect sizes computed from the means and standard deviations. Effect sizes are most commonly presented as the Cohen's d statistic [$d = X_1 - X_2/S_{\text{pooled}}$, where X = mean of species 1 and 2, S = standard deviation, and $S_{\text{pooled}} = \sqrt{\{[(n_1 - 1) \cdot S_1^2 + (n_2 - 1) \cdot S_2^2]/(n_1 + n_2)\}}$ where n = number of individuals sampled in species 1 and 2], which expresses the difference in means in terms of the amount of within-group variation (Tobias et al. 2010). On the basis of the distribution of effect sizes produced by empirical tests of divergence in undisputed species, they have scored character differences with an effect size of 0.2–2 as minor, 2–5 as medium, 5–10 as major, and >10 as exceptional.

RESULTS

We sampled 70 individuals across the island along a 430 km transect; based on plumage these were identified as 41 *D. psarodes*, 17 *D. b. jaffnense*, and 12 intermediate forms of varied levels of orange plumage. These birds included 32 males and 38 females (sex was determined using plumage; see Methods). We did not observe any sexual differences in

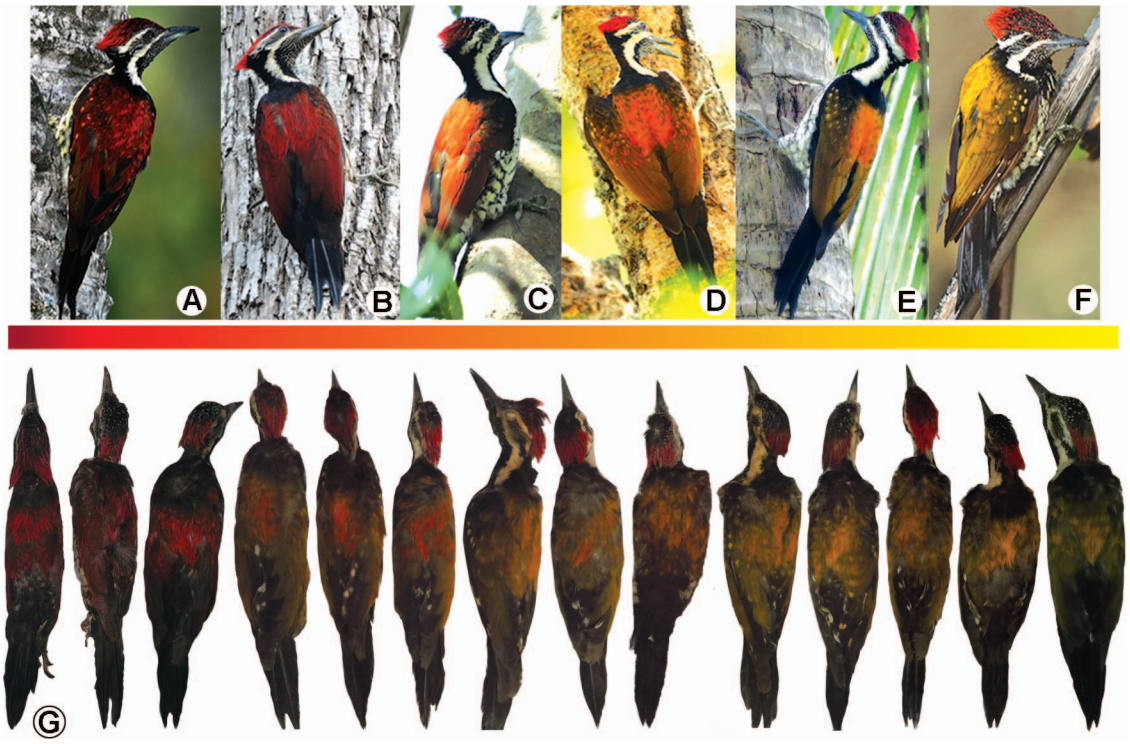

FIGURE 2. Color variation of *Dinopium* flamebacks in Sri Lanka: **(A)** crimson-red plumage (*D. psarodes*) (image courtesy of V. Weeratunge), **(B)** reddish-orange form, **(C)** orange form, **(D)** orange-yellow form, **(E)** orange/reddish mantle, **(F)** golden-yellow plumage (*D. b. jaffnense*) (image courtesy of V. Weeratunge), and **(G)** variation of back color in *Dinopium* flamebacks in the national collection (image courtesy of the Department of National Museums, Sri Lanka).

morphological traits in either species (one-way ANOVA, $F = 0.40$, df = 1 and 70, $P = 0.53$), other than the slight color difference in forehead plumage. From the national collection we sampled 25 *D. psarodes*, 8 *D. b. jaffnense*, and 22 intermediate types (Figure 2G). Since museum and field data originated from different sampling locations (Figure 1A), we analyzed them separately.

Plumage color of the back ranges from red to yellow, with many intermediate colors (Figure 2) as suggested by previous authors (e.g., Fernando and Seneviratne 2015, Freed et al. 2015). A clinal change of plumage characters was observed along the line transect from Jaffna (northern tip of the island: 9.46°N, 80.07°E) to Matara (southern tip of the island: 6.04°N, 80.38°E; Figures 1C, 3C). All individuals captured from Jaffna peninsula had yellow backs (Figure 1A). All individuals captured from the core of the wet zone (southern parts of the island) had crimson-red backs (Figure 1A). The intermediate plumage colors (orange) were found in varying proportions in the north-central dry zone, from the southern border of Wilpathu National Park (08.37°N, 80.07°E) up to Kilinochchi (9.22°N, 80.25°E).

A clinal change of genetic characters (in Cytb) was observed in the line transect across north-central Sri Lanka (Table 2; Figures 1B, 3A). Allopatric *D. b. jaffnense* has a G nucleotide and allopatric *D. psarodes* has an A at the diagnostic polymorphic site in Cytb gene. In the sympatric area a mixture of these Cytb types was found among the intermediate phenotypes.

Our Cfit analysis positioned cline centers 83–149 km (mean center = 115 km) from the southeastern coast of mainland India (Table 3). The Cytb-based genetic cline and the center- and slope-constrained cline based on all traits positioned the cline center at 115–117 km from the coast of

TABLE 2. A composition of genotypes of flameback woodpeckers at Cytb gene along the transect line showing a mixture of genotypes among the intermediate phenotypes in the sympatric area.

		Cytb gene	
Phenotype	Individuals	Northern allele	Southern allele
Northern allopatric area			
D. b. jaffnense	10	10	
Sympatric area			
D. b. jaffnense	6	4	2
D. psarodes	13	10	3
Dinopium intermediates	8	4	4
Southern allopatric area			
D. psarodes	31		31

TABLE 3. Comparison of genetic, plumage, and morphometric clines based on maximum log-likelihood estimates obtained from Cfit-7. Cline centers are in kilometers from the nearest coast of mainland India. Likelihood ratio tests and Akaike Information Criterion (AIC) indicate that a single cline (Model 4) best represents variation in all of these traits. *Asterisks indicate similar centers or widths due to constrained models.

	Allele (Cytb)		Plumage		Morphometric				Comparison		
Model	Center (km)	Width (km)	Center (km)	Width (km)	Center (km)	Width (km)	Log-likelihood	AIC	115/112	χ^2	*P*
1. No constraint	115	142	149	82	83	18	−188.36	408.72			
2. Center constrained	73	250	*	138	*	4	−190.56	417.84	1 and 2	4.4	0.89
3. Slope constrained	116	114	117	*	63	*	−191.63	411.26	1 and 3	6.54	0.96
4. Center and slope constrained	117	118	*	*	*	*	−191.85	407.7	1 and 4	6.98	0.86
									2 and 4	2.58	0.73
									3 and 4	0.44	0.20

India (Table 3; Figure 3A). The widths of these clines range from 18 to 142 km among different traits. The constrained clines, which Akaike Information Criterion (AIC) values indicate should be taken as the best model, gave the center as 117 km and width as 118 km (Table 3). Cubic splines of both morphological and plumage traits showed clinal variation in the same area that of Cytb: ∼117 km from the southeast coast of India (line north of Putlum to Vavunia; Figures 1C & 3). These 3 measured traits are generally concordant in their centers and widths (Figure 3).

When we compare the empirical clines with hypothetical neutral clines unaffected by selection, the empirical clines were always narrower than the neutral clines. Assuming that the secondary contact of *D. psarodes* and *D. b. jaffnense* took place during the last complete exposure of the Adams Bridge due to the drop of sea level (∼10,000 years ago: Ripley 1949, Hopkins 1967), and given the dispersal rate and generation time of *C. auratus*, a neutral cline would have now reached a width of ∼1,700 km. A highly conservative (i.e. minimal) estimate of time since recent contact between taxa (160 years, based on Layard's [1853] original description of the red form and dispersal rate of songbirds) would give a width of 213 km. Just 15 years after this first description came the documentation of intermediate phenotypes between *D. psarodes* and *D. b. jaffnense* (Legge 1880). Assuming 1868 as the time of secondary contact would give a cline width of 192 km—still a much wider cline compared to the observed clines (112 km). The narrowness of the empirical clines suggests some form of selection constrains the width of the cline; such selection can be considered a form of partial reproductive isolation that hinders the blending of the 2 forms and stabilizes them as distinct entities (Barton and Hewitt 1985, 1989).

Dinopium flamebacks in Sri Lanka all have red crowns, and plain black napes and upper mantles (Figure 2A–F). However, their backs have 2 predominant color types (Figure 2A–F), crimson-red and olive-yellow, corresponding to the 2 forms. The intermediate color forms range from golden yellow (Figure 2F) to orange (Figure 2C). The orange color is sometimes confined to the mantle, and sometimes extends more prominently into the wings. Secondaries and coverts show olive-yellow, golden yellow, orange, and crimson red (Figure 2A–F). *D. psarodes* in the extreme south has crimson-red shading in the primaries and in the otherwise black rump as well (Figure 2A). Similarly, the amount of black and white plumes on the facial region and upper wing coverts shows slight variation along the north–south transect. The northern (dry-zone) birds of all color types have broader white supercilium and malar stripes (mustache). Birds in the south (wet-zone) have narrower white facial stripes (hence darker faces; Figure 2A). Upper wing coverts are dotted with 2 rows of white spots in northern birds (Figure 2E, 2F); these spots are less prominent in the southern birds (Figure 2A). In all

TABLE 4. Morphological and plumage traits measured in *Dinopium* flamebacks showing that beak, wing, and tail length of *D. psarodes* are longer and supercilium and mustache width are narrower in *D. psarodes* than in *D. b. jaffnense* (mean ± SE, with data range in parentheses).

	Field sample ($n = 70$)		
	D. psarodes ($n = 41$)	*D. b. jaffnense* ($n = 17$)	Intermediate ($n = 12$)
Morphometric traits (mm)			
Head length	29.66 ± 1.93 (21.90–32.30)	30.52 ± 0.97 (29.30–32.20)	30.42 ± 1.67 (27.30–32.00)
Head width	21.82 ± 0.76 (19.51–23.60)	21.26 ± 0.55 (20.21–22.20)	21.28 ± 0.90 (19.00–22.30)
Beak length	34.15 ± 1.63* (30.61–36.70)	33.50 ± 2.07 (30.60–37.22)	32.61 ± 2.50 (29.80–36.90)
Bill height	8.96 ± 1.00 (8.22–10.50)	8.91 ± 0.54 (7.90–10.30)	8.76 ± 0.85 (7.60–9.80)
Bill width	9.11 ± 0.81 (7.42–11.00)	8.36 ± 0.81 (6.72–9.55)	9.16 ± 0.70 (7.90–10.10)
Flat-wing length	138.59 ± 6.22* (130.52–142.20)	133.60 ± 2.82 (129.00–138.00)	131.64 ± 2.34 (129.00–135.00)
Tarsus length	27.50 ± 1.99 (22.45–30.94)	24.14 ± 1.01 (23.00–26.90)	25.90 ± 2.03 (22.80–28.70)
Tail length	94.70 ± 1.48* (76.00–139.00)	82.92 ± 3.10 (79.00–91.00)	86.80 ± 0.83 (74.00–98.00)
1st claw length	8.94 ± 0.25 (8.30–9.65)	8.96 ± 0.33 (8.40–9.50)	8.97 ± 0.54 (8.10–9.80)
Length of the reduced hind digit	4.40 ± 0.98 (2.50–6.60)	4.16 ± 0.66 (2.80–5.42)	4.68 ± 0.94 (3.30–6.30)
Plumage traits**			
Supercilium width	2.69 ± 0.69* (1.30–4.32)	3.37 ± 0.98 (1.50–6.12)	3.00 ± 0.89 (1.80–4.20)
Face width	7.33 ± 1.11 (4.55–9.20)	7.00 ± 0.50 (6.32–8.00)	6.99 ± 1.18 (5.20–8.54)
Mustache width	3.17 ± 1.18* (1.40–8.92)	3.68 ± 0.80 (2.00–5.00)	3.24 ± 0.66 (2.40–4.40)

* $P < 0.05$ compared to *D. b. jaffnense* (ANOVA with Tukey's family error post hoc test).
** Length or width measured in millimeters (mm).

forms along the transect the throat is black, bill blackish grey, iris chestnut, breast and belly have dark chevrons and streaks, and legs are gray.

Bill length (i.e. total culmen), wing length, and tail length are smaller in *D. b. jaffnense* than in *D. psarodes* and intermediates (Table 4). In the morphology-based PCA, PC1 explained 64.6% of the variation and described size differences between the species (Table 5, Figure 4A). In the morphological PCA, many traits contributed heavily to PC1 (Table 5) and there was a clear separation of *D. psarodes* and *D. b. jaffnense* in PC1 (ANOVA; $P < 0.001$). In the plumage PCA, PC1 captured 66.2% of the variation, correlating well with all characters that tend to differ between *D. psarodes* and *D. b. jaffnense* (ANOVA; $P < 0.001$; Table 5 and Figure 4B).

Both Sri Lankan and Indian yellow forms of *D. benghalense* are clustered together, and form a sister group to Sri Lankan red form (*D. psarodes*) at Cytb (Figure 5).

Our analysis of biometrics following Tobias et al. (2010) and del Hoyo et al. (2014) is consistent with the latter analysis (which resulted in splitting the *D. psarodes* into a full species) but with a greater effect size (4.21 rather than 2.92). Likewise, the quantitative analysis of plumage and bare parts (effect size 6.73), ecology, behavior, and geographical relationship too are in accordance with del Hoyo et al. (2014) (Table 1).

DISCUSSION

We examined phenotypic and genotypic relationships within 2 distinct forms of *Dinopium* flamebacks in Sri Lanka to test the validity of the newly elevated species *D. psarodes*, the Red-backed Woodpecker or the Lesser Sri Lanka Flameback (del Hoyo et al. 2014), a distinct form endemic to the island of Sri Lanka. We documented clear phenotypic and genetic evidence for a hybrid zone, and find that this hybrid zone is narrower (112 km in width) than that expected for a neutrally expanding zone. The narrow hybrid zone suggests some form of selection acts against hybrids; such selection is considered a form of partial reproductive isolation (Barton and Hewitt 1985, 1989, AOU 1998, Brelsford and Irwin 2009).

Is *D. psarodes* a good species under the biological species concept? We argue that despite extensive hybridization in sympatry, there are several key factors suggesting that *D. psarodes* is best considered a distinct species. It is phenotypically (Figures 2 and 4) and genetically (Figures 3A and 5) a distinct entity. Except for hybrids in this narrow contact zone (Table 3), it does not show intermediate phenotypes in the rest of its range, and breeding areas of *D. psarodes* and *D. b. jaffnense* remain largely separated from each other. When our data is evaluated under the species-delimiting criteria used in Tobias et al. (2010), we find strong support for the species status of *D. psarodes*.

Phylogenetic affinities based on Cytb support *D. psarodes*'s separation from *D. benghalense* cluster (Figure 5). A much broader phylogenetic analysis on this group using very large amount of genomic sequence (152,311bp) generated by Restriction Site Associated DNA (RAD) sequencing also revealed a similar pattern (de Silva et al. 2014), where *D. b. jaffnense* cluster with the Indian forms

TABLE 4. Extended.

Museum skins (n =55)		
D. psarodes (n = 25)	*D. b. jaffnense* (n = 8)	Intermediate (n = 22)
29.87 ± 2.69 (25.60–35.90)	28.8 ± 3.78 (25.80–36.20)	29.85 ± 2.38 (24.80–36.20)
22.63 ± 1.14 (20.00–25.00)	21.51 ± 2.04 (20.00–25.40)	22.04 ± 1.98 (18.20–25.40)
32.05 ± 3.25 (21.80–37.80)	30.76 ± 1.39 (29.10–33.10)	30.83 ± 3.17 (29.10–31.30)
9.07 ± 0.61 (8.00–10.80)	8.65 ± 0.58 (8.10–9.70)	8.56 ± 0.69 (8.10–9.70)
8.07 ± 0.64 (6.90–9.40)	7.40 ± 0.48 (6.82–8.11)	7.61 ± 0.70 (6.80–8.10)
129.33 ± 2.56 (108.00–142.00)	130.83 ± 5.27 (125.00–138.00)	127.71 ± 7.87 (125.00–138.00)
24.15 ± 1.77 (21.50–28.20)	22.48 ± 1.01 (21.50–24.00)	22.10 ± 1.86 (21.50–24.00)
97.25 ± 5.14 (89.00–111.00)	97.17 ± 3.37 (92.00–102.00)	97.72 ± 1.95 (92.00–102.00)
9.72 ± 1.28 (7.00–12.00)	9.30 ± 0.98 (7.60–10.40)	9.14 ± 0.84 (7.70–10.40)
–	–	–
2.47 ± 0.58 (1.40–3.90)	3.00 ± 0.62 (2.10–3.80)	2.90 ± 0.55 (1.80–3.80)
6.54 ± 1.10 (5.00–9.20)	6.10 ± 1.30 (4.50–8.30)	6.49 ± 1.02 (4.80–8.30)
3.05 ± 0.59 (2.00–4.90)	3.71 ± 0.51 (2.80–4.10)	3.55 ± 0.84 (2.10–4.10)

and the *D. psarodes* separates out as the sister group with posterior probability of close to 1 (de Silva et al. 2014).

Our multivariate analysis further showed that *D. psarodes* is a distinct phenotypic entity diagnosed by crimson back, black rump and upper tail coverts, and slightly longer tail and tarsus. Intermediate phenotypes were clustered between the 2 parental phenotypes (Figures 3 and 4). The intermediates were found in the contact area between the parental groups, in a narrow band north of Puttalum to Killinochi in the north-central and north-western parts of Sri Lanka (Figure 1). The center of the hybrid zone lies roughly in the middle of this band (~112 km from the mainland; Figure 3). We found crimson-red, red, orange-red, orange, golden yellow, and olive-yellow phenotypes of *Dinopium* in this area (Figure 2). Legge (1880) and several others over the past century have made similar observations (see Introduction). We encountered heterospecific pairs at the center of the hybrid zone as well. For example, in Wilpattu National Park in northwestern Sri Lanka (08.37°N, 80.08°E), we observed a nest with nestlings where the parent male was a *D. psarodes* and the female was a *D. b. jaffnense*. A previous observer also has noted similar heterospecific pairs in this same region (Freed et al. 2015).

Despite the plumage differences, these woodpeckers are vocally very similar (Rasmussen and Anderton 2012). We observed only subtle differences in the frequency in their calls (S. Seneviratne personal observation). In fact both species and their hybrids respond to each other's playback calls.

Sri Lanka has 2 main climatic zones. The southern one-third of the island is the wet zone with over 2,500 mm annual rainfall. The rest of the island, including the

TABLE 5. Variance explained and factor loading of the first 2 principal components (PC) produced in principal components analyses (PCAs) of phenotypic traits: A) morphological traits and B) plumage traits**.** In the morphological PCA, PC1 explained 64.6% of the variation and described size differences between the species, with each standardized morphological variable being roughly equally correlated with PC1 (Figure 4). PC1 scores of morphological traits separate the 2 species. The PC1 of plumage captured 66.2% of the variation. PC2 of both morphological and plumage traits captured minor amounts of variation.

	Field samples (n =70)	
	PC1	**PC2**
(A) Morphometric traits		
Eigenvalue	2.67	0.80
Variance explained	64.6%	10.5%
Factor loadings		
Tail length	0.53	−0.30
Tarsus	0.34	−0.49
Head width	0.54	0.23
1st claw length	0.43	−0.30
Exposed culmen	0.64	−0.11
Hind reduced digit	0.04	0.55
Flat-wing length	0.44	0.14
Head size	0.38	0.64
Head length	0.15	−0.63
Bill height (mm)	0.34	0.050
Bill breadth	0.29	−0.07
Bill width (mm)	0.11	0.04
(B) Plumage traits		
Eigenvalue	3.82	1.17
Variance explained	66.2%	20.7%
Factor loadings		
Back color	0.67	0.19
Rump color	0.86	0.15
Secondary coverts color	0.67	0.16
Supercilium width	−0.43	0.66
Face width	0.23	−0.59
Mustache width	−0.26	0.74

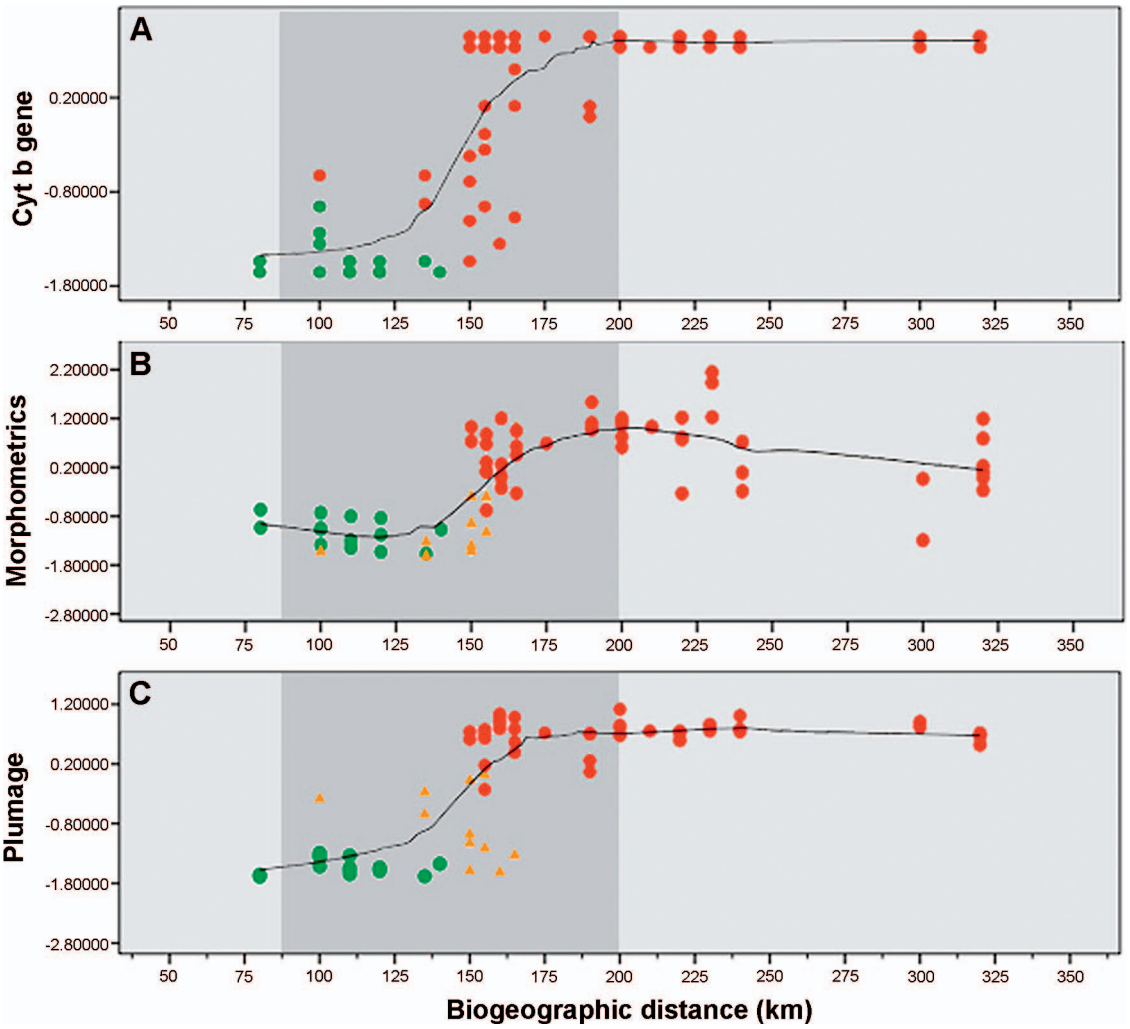

FIGURE 3. Clinal variation of allele frequency, morphology, and plumage of *Dinopium* along an axis representing distance from the southeastern coast of mainland India showing the allopatric distribution of *D. b. jaffnense* and *D. psarodes* and the presence of the hybrid zone. Zero kilometers represents the nearest southeastern coastal line of India (Figure 1A). Points represent mitochondrial haplotype frequency (red indicates southern Cytb haplotype and green indicates northern haplotype) in groups of local individuals **(A)** or PC1 scores (morphological and plumage characters) of individuals (**B**–**C**: red dots indicate red forms, orange triangles indicate intermediate forms, and green dots indicate yellow forms). The lines represent the best-fitting clines generated by CFit-7. Dark gray area indicates the width of the genetic cline. **(A)** Cytb gene, **(B)** Morphology, **(C)** Plumage.

northern parts near mainland India, is the dry-zone with seasonal rainfall averaging ~1,500 mm (Ashton et al. 1997). The Sri Lankan endemic *D. psarodes* is bigger, and has longer bill breadth and length, than *D. b. jaffnense*. A possible reason is that the trees in the wet zone forests are much bigger, such that the bigger bill of *D. psarodes* can better excavate the thick bark. The supercilium and mustache width of *D. psarodes* are narrower than that of *D. b. jaffnense* (Table 4). The darker plumage coloration and the longer beak are general patterns shown by the birds in the wet zone of Sri Lanka compared to their dry-zone and Indian counterparts (Ripley 1946, 1949, Ripley and Bheeler 1990). This fits the general pattern of higher humidity being associated with darker and bigger individuals (Prum et al. 2012, Fernando and Seneviratne 2015). Mainland India and northern Sri Lanka are similar in humidity, and a similar plumage can likewise be found in both the mainland and the island (Fernando and Seneviratne 2015). *D. psarodes* is found in the dry zone of Sri Lanka as well, but there they are found closer to water bodies such as lakes and rivers, which are abundant in the dry zone. This may indicate that *D. psarodes* prefers humid habitats compared to its dry-zone counterpart.

Here we have provided the first comprehensive analysis incorporating both phenotypic and genetic variation across a hybrid zone for an Old World woodpecker group. We

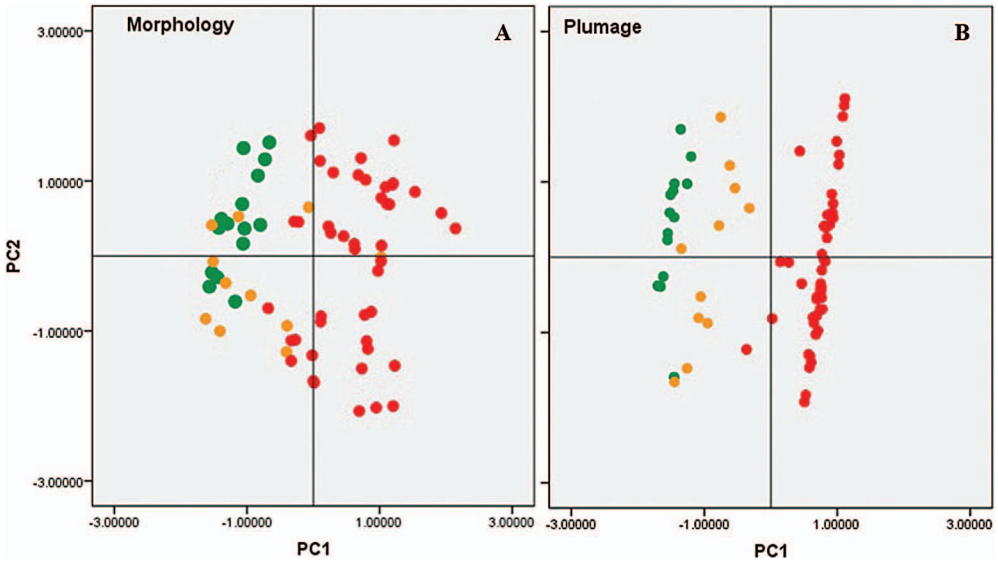

FIGURE 4. Variation of morphological (**A**) and plumage (**B**) characters in adult *D. b. jaffnense* and *D. psarodes* showing clear separation of *D. psarodes* only in plumage. Red = *D. psarodes,* orange = intermediate, green = *D. b. jaffnense*.

conclude that *D. psarodes* of Sri Lanka is both genetically and phenotypically a distinct entity that warrants full species status. Our study provides an example in which the phenotype-based criteria proposed by Tobias et al. (2010) for avian taxonomy are consistent with conclusions arrived at through genetic and cline-based analyses. Hence the conclusion of del Hoyo et al. (2014) to elevate *D. psarodes* to a distinct species. Detailed studies on the amount of introgression in the context of hybridization, the stability of the hybrid zone and the fate of its hybrids, and the ecology of the new species (*D. psarodes*) would shed more light into this charismatic group of poorly known Asian woodpeckers.

ACKNOWLEDGMENTS

Prof. Gaya Ranawaka of the Open University of Sri Lanka, Prof. Sarath Kotagama and Prof. Preethi Udagama of University of Colombo provided assistance in various stages. We thank Kelum Manupriya and numerous other field assistants for their assistance in the field. We thank Janaka Perera, Indrika Kaggodaarachchi, Nelum Wickramasighe, and Manori Goonathilaka for their assistance both in the field and at the National Museum. Three anonymous reviewers and Kristen Ruegg gave valuable comments to early versions of the manuscript. The Department of National Museums provided the needed permission to sample the national collection. The Department of Wildlife Conservation (permit nos. WL/3/2/

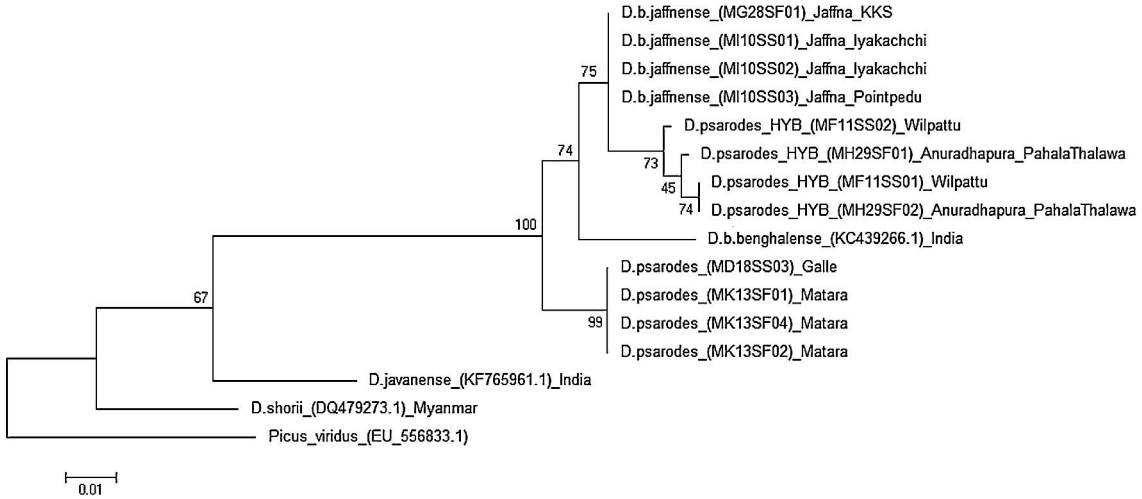

FIGURE 5. Molecular phylogenetic analysis by maximum-likelihood method for Cytb gene.

19/13 and WL/3/2/41/14) and Forest Department (permit no. RE/RES/NFSRC/13) provided the permits to carry out the study in the field.

Funding statement: A Collaborative Research Grant of the University of Colombo to S.S.S. (AP/3/2012/CG/30) funded this study. Our funder had no influence on the content of the submitted or published manuscript and our funder did not require approval of the final manuscript to be published.

Ethics statement: This study has been approved by the Research Committees of the Department of Wildlife Conservation of Sri Lanka (permit nos. WL/3/2/19/13 and WL/3/2/41/14), the Forest Department of Sri Lanka (permit no. RE/RES/NFSRC/13), and the Higher Degrees Committee of the Faculty of Science, University of Colombo.

Author contributions: S.P.F. designed and conducted fieldwork and laboratory experiments, performed the analysis, and wrote the paper. D.E.I. assisted with formulating the research question and writing the paper. S.S.S. conceived the idea, formulated the research question, designed field and laboratory experiments, provided funding, and wrote the paper.

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APPENDIX

APPENDIX TABLE 6. Summary of the sampled specimens at the National Collection, National Museum, Colombo, Sri Lanka.

Museum number	Collected date	Species	Sex
127-2	March 23, 1969	*D. psarodes*	Male
127-V	March 3, 1934	*D. psarodes*	Female
127-I	August 14, 1913	*D. psarodes*	Male
127-Q	November 7, 1923	*D. psarodes*	Female
127-B	August 21, 1905	*D. psarodes*	Female
127	August 22, 1905	*D. psarodes*	Male
127-R	November 27, 1933	*D. psarodes*	Male
127-T	March 3, 1934	*D. psarodes*	Male
127-Z	May 16, 1961	*D. psarodes*	Female
127-P	May 22, 1932	*D. psarodes*	Male
129-Z	January 31, 1940	*D. psarodes*	Male
129-X	January 17, 1940	*D. psarodes*	Male
129-T	June 30, 1951	*D. psarodes*	Female
127-E	February 9, 1913	*D. psarodes*	Male
127-X	July 31, 1958	*D. psarodes*	Male
127-1	September 13, 1961	*D. psarodes*	Female
127-Y	March 31, 1961	*D. psarodes*	Female
127-A	February 15, 1906	*D. psarodes*	female
127-L	November 8, 1923	*D. psarodes*	female
127-K	August 12, 1913	*D. psarodes*	male
127-M	December 2, 1920	*D. psarodes*	female
127-N	November 20, 1924	*D. psarodes*	male
127-O	November 7, 1923	*D. psarodes*	female
127-D	August 6, 1913	*D. psarodes-HYB*	female
127-H	August 23, 1905	*D. psarodes*	male
126-Z	May 30, 1947	*D. b. jaffnense-HYB*	male
126-1	May 8, 1947	*D. b. jaffnense*	male
126-i	March 14, 1933	*D. b. jaffnense-HYB*	female
126-Y	February 17, 1959	*D. b. jaffnense-HYB*	male
126-F	September 10, 1919	*D. b. jaffnense-HYB*	female
126-P	July 1, 1951	*D. b. jaffnense-HYB*	male
126-T	November 17, 1953	*D. b. jaffnense-HYB*	female
126-D	August 13, 1913	*D. b. jaffnense-HYB*	male
126-W	May 28, 1958	*D. b. jaffnense-HYB*	male
126-O	June 29, 1951	*D. b. jaffnense-HYB*	female
126-Q	June 30, 1951	*D. b. jaffnense-HYB*	male
126	February 15, 1954	*D. b. jaffnense-HYB*	female
126-X	February 15, 1952	*D. b. jaffnense-HYB*	female
126-K	March 21, 1933	*D. b. jaffnense-HYB*	female
126-M	July 23, 1945	*D. b. jaffnense-HYB*	male
126-N	July 1, 1945	*D. b. jaffnense-HYB*	female
126-E	January 23, 1911	*D. b. jaffnense-HYB*	female
126	UNKNOWN	*D. b. jaffnense*	female
126-A	April 15, 1904	*D. b. jaffnense*	male
126-B	April 15, 1904	*D. b. jaffnense-HYB*	male
126-H	March 14, 1933	*D. b. jaffnense-HYB*	male
126-U	February 9, 1956	*D. b. jaffnense*	female
126-F	September 10, 1919	*D. b. jaffnense-HYB*	male
126-L	July 22, 1945	*D. b. jaffnense*	male
126-V	February 14, 1956	*D. b. jaffnense*	female

APPENDIX TABLE 7. List of primers used in the genetic analysis.

Gene	Primer	Sequence (5′-3′)	Reference
Cytochrome b	*DINO_Cyto_b_F*	CGATTCTTCGCTTTACACTTCCTCC	Benz et al. 2006
	DINO_Cyto_b_R	ATGAAGGGATGTTCTACTGGTTG	

APPENDIX TABLE 8. Summary of the samples included in the molecular analysis. Voucher number indicates the catalog numbers of Avian Evolution Node or NCBI Genbank.

Taxon	Country of origin	Voucher number	Accession number	
			Cytb	LDH
In group				
Dinopium shorii	Myanmar	B3291	DQ479273.1	NS
D. javanense	India	NRM20026532	KF765961.1	NS
D. benghalense	India	–	KC439266.1	KJ455248.1
D. psarodes	Sri Lanka	MD18SS03 MK13SF01 MK13SF02 MK13SF04	This study	This study
D. b. jaffnense	Sri Lanka	MG28SF01 MI10SS01 MI10SS02 MI10SS03	This study	This study
D. psarodes/ D. b. jaffnense Intermediate	Sri Lanka	MF11SS01 MF11SS02 MH29SF01 MH29SF02	NS	NS
Out group				
Piculus chrysochloros	–	–	NS	NS
Picus viridis	–	–	EU556833.1	NS
Picus flavinucha	India	NRM:20056713	NS	KJ455282

NS = No sequence obtained