ORIGINAL ARTICLE

Genetic analysis of hybridization between white-handed (*Hylobates lar*) and pileated (*Hylobates pileatus*) gibbons in a contact zone in Khao Yai National Park, Thailand

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Abstract

Natural hybridization has played various roles in the evolutionary history of primates. Its consequences range from genetic introgression between taxa, formation of hybrid zones, and formation of new lineages. *Hylobates lar*, the white-handed gibbon, and *Hylobates pileatus*, the pileated gibbon, are largely allopatric species in Southeast Asia with a narrow contact zone in Khao Yai National Park, Thailand, which contains both parental types and hybrids. Hybrid individuals in the zone are recognizable by their intermediate pelage and vocal patterns, but have not been analyzed genetically. We analyzed mitochondrial and microsatellite DNA of 52 individuals to estimate the relative genetic contributions of the parental species to each individual, and the amount of introgression into the parental species. We obtained fecal samples from 33 *H. lar*, 15 *H. pileatus* and four phenotypically intermediate individuals in the contact zone. Both mitochondrial and microsatellite markers confirmed distinct differences between these taxa. Both *H. lar* and *H. pileatus* contributed to the maternal lineages of the hybrids based on mitochondrial analysis; hybrids were viable and present in socially normal reproductive pairs. The microsatellite analysis identified ten admixed individuals, four F1 hybrids, which corresponded to phenotypic hybrids, and six *H. lar*-like backcrosses. All 15 *H. pileatus* samples were identified as originating from genetically *H. pileatus* individuals with no *H. lar* admixture; hence, backcrossing is biased toward *H. lar*. A relatively low number of phenotypic hybrids and backcrossed individuals along with a high number of parental types indicates a bimodal hybrid zone, which suggests relatively strong bias in mate selection between the species.

Keywords *Hylobates lar* · *Hylobates pileatus* · Hybridization · Contact zone · Genetic introgression · Khao Yai National Park

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Introduction

Interspecific hybridization involves interbreeding between individuals of different species within the same genus, which results in the production of genetically mixed individuals. The viability of hybrids varies from none (sterility), to reduced viability, no influence, or rarely, to hybrid advantage over the parental species. The consequences of natural hybridization have been addressed with regard to various evolutionary outcomes (Arnold and Martin 2010; Zinner et al. 2011; Tung and Barreiro 2017). For example, the production of fertile hybrids can lead to the formation of a new lineage (e.g., Lamichhaney et al. 2018), admixed gene pools in the parental species, and reduced fitness of the parental species (Zinner et al. 2011). Adaptive introgression can occur if the admixed populations receive beneficial

genes from the ancestral lineages (Salazar et al. 2010; Sams et al. 2016).

In primates, natural hybridization has been reported in all major lineages, for example in strepsirrhines (Gligor et al. 2009; Pastorini et al. 2009), in platyrrhines in America (Silva et al. 1992; Cortés-Ortiz et al. 2007), in cercopithecids in Asia and Africa (Detwiler 2019; Tosi et al. 2002; Tung et al. 2008), in hylobatids in Asia (Brockelman and Gittins 1984; Marshall and Sugardjito 1986), and in nonhuman hominids (great apes) (de Manuel et al. 2016). Natural hybridization in non-human primates has resulted in the appearance of new morphological variations (e.g., Brockelman and Gittins 1984; Cortés-Ortiz et al. 2007; Detwiler 2019; Gligor et al. 2009), altered vocal patterns (Brockelman and Schilling 1984; Mather 1992), changes in behavior (Ho et al. 2014), life history (Charpentier et al. 2008), and altered reproductive success (Charpentier et al. 2008). An important question raised by the appearance of hybrid characters is how they might affect fitness and alter gene flow across the contact zone.

Apes of the family Hylobatidae originated in Southeast Asia and diverged into four genera around 5–8.3 million years ago (MYA) (Thinh et al. 2010; Israfil et al. 2011; Carbone et al. 2014). The four genera within the Hylobatidae (*Hylobates*, *Hoolock*, *Nomascus*, and *Symphalangus*) contain 20 recognized species (Thinh et al. 2010; Anandam et al. 2013; Fan et al. 2017). *Hylobates*, a diverse genus containing nine species (*Hylobates abbotti*, *Hylobates agilis*, *Hylobates albibarbis*, *Hylobates funereus*, *Hylobates klossii*, *Hylobates lar*, *Hylobates moloch*, *Hylobates muelleri*, and *Hylobates pileatus*), underwent several radiations during the period between 1.5 and 4 MYA, based on molecular evidence (Thinh et al. 2010; Israfil et al. 2011; Chan et al. 2013). The genus likely originated in the Southeast Asian mainland and expanded southward (Whittaker et al. 2007; Thinh et al. 2010). The divergence of *H. lar* and *H. pileatus*, which may not be sister species, has been estimated at 3.65 MYA (CI 3.05–4.25) (Thinh et al. 2010).

Hylobates spp. are distributed allopatrically over the Sunda Shelf and adjacent mainland, and hybridization has been reported for three contact zones: between *H. lar* and *H. pileatus* in Khao Yai National Park, central Thailand (Marshall et al. 1972; Brockelman and Gittins 1984); between *H. lar* and *H. agilis* in northern West Malaysia (Gittins 1978; Brockelman and Gittins 1984); and between *H. muelleri* and *H. agilis* (later renamed *H. albibarbis*) in central Borneo (Marshall and Sugardjito 1986; Mather 1992). Ecological studies of *H. lar*, *H. pileatus* and their hybrids in Thailand (Brockelman 1978; Brockelman and Gittins 1984; Suwanvecho and Brockelman 2012; Asensio et al. 2017) have revealed that *H. lar* and *H. pileatus* in the contact zone have similar feeding behavior, habitat preferences and home range sizes, suggesting that they have virtually identical ecological niches. The two species

were found to be interspecifically territorial and to have similar intra- and interspecific aggressive encounter rates (Suwanvecho and Brockelman 2012), and treat each other as ecological competitors (Asensio et al. 2017). The steep transition between *H. lar* and *H. pileatus* in the contact zone and the relatively low number of intermediate phenotypes (Brockelman and Gittins 1984) suggest that positive assortative mating might occur to create reproductive barriers between these species. Nevertheless, fertile hybrids and backcrossing to the parental species have been observed in the zone, as recognized by intermediate pelage and song patterns (Brockelman and Gittins 1984; Brockelman and Schilling 1984; Marshall and Brockelman 1986; Marshall and Sugardjito 1986; Asensio et al. 2017), and putative second generation female backcrosses could be recognized by the cadence of notes in their great calls (Brockelman and Schilling 1984). However, later-generation backcrosses may not be recognizable from phenotypic characters, and the extent of gene flow between the species is unknown.

Molecular markers such as mitochondrial DNA (mtDNA), sex-linked markers, and microsatellite DNA have been widely applied to determine hybrid individuals and investigate gene introgression in primates (e.g., Tosi et al. 2002; Cortés-Ortiz et al. 2007; Mourthe et al. 2019). Matsudaira et al. (2013) reported mitochondrial gene introgression between *H. lar* and *H. pileatus* in the *H. lar* side adjacent to the contact zone. The authors examined gibbons with *H. lar* phenotypes, and found that some individuals carried mitochondrial haplotypes of *H. pileatus*, suggesting introgression into *H. lar*. However, determining admixed individuals, especially backcrosses, and investigation of gene introgression have not yet been carried out in the contact zone and in the *H. pileatus*-dominated area.

In this study, we aimed to identify admixed individuals and investigate natural hybridization between *H. lar* and *H. pileatus* in the center of the contact zone, where both parental types coexist, by using mtDNA and nuclear microsatellite markers. mtDNA provides information on the phylogenetic relationship between the taxa and the genetic contribution from the maternal lineages. The nuclear microsatellite markers can determine the genetic proportion contributed by each parental species to each individual. In order to investigate the structure of the contact zone, and in particular the movement of genes across it, we tested the following hypotheses: (1) individuals identified as intermediate phenotypes share nuclear genes from both species; and (2) genetic introgression occurs in both parental species.

Methods

Study site

The study site is the narrow (less than 10 km wide) contact zone between *H. lar* and *H. pileatus* in the Khlong

Sai area of Khao Yai National Park, Thailand (14°25′N, 101°23′E) (Fig. 1). The contact zone is in the area of the headwaters of the Takhong River, where the river is too narrow to form a barrier and tree canopies meet over the river. The forest canopy is continuous throughout most of the area except for some patches of fields and human developments. A few roads passing through the area are spanned by tree canopies that permit crossing by gibbons. There is no physical boundary to the species' ranges, and *H. lar*, *H. pileatus*, and hybrid groups, intermingle in the contact zone (Asensio et al. 2017).

Gibbon groups and phenotypes

All species of *Hylobates* live predominantly in small territorial groups consisting of a monogamous breeding pair of adults and their offspring (but not necessarily the offspring of both adults) (Brockelman et al. 1998; Bartlett 2011). The average group size of *H. lar* in Khao Yai is about 3.8 (range 2–7) individuals (Brockelman 2004). We identified four gibbon group types in the contact zone: *H. lar* groups, *H. pileatus* groups, mixed-species groups in which the main reproductive pair consisted of an adult male and adult female of the different species, and hybrid groups in which at least one of the paired adults was phenotypically

Fig. 1a, b The sample collection sites in Khlong Sai study area, Khao Yai National Park, Thailand. **a** The *hatched square* shows the approximate extent of the contact zone (W. Y. Brockelman, unpublished data), and the *circle* shows our study site. **b** Distribution of the study individuals in the contact zone. Sampling locations are indicated by *open circles* (*Hylobates lar*), *filled circles* (*Hylobates pileatus*) and *triangles* [intermediate hybrids (*Hybrid*)]
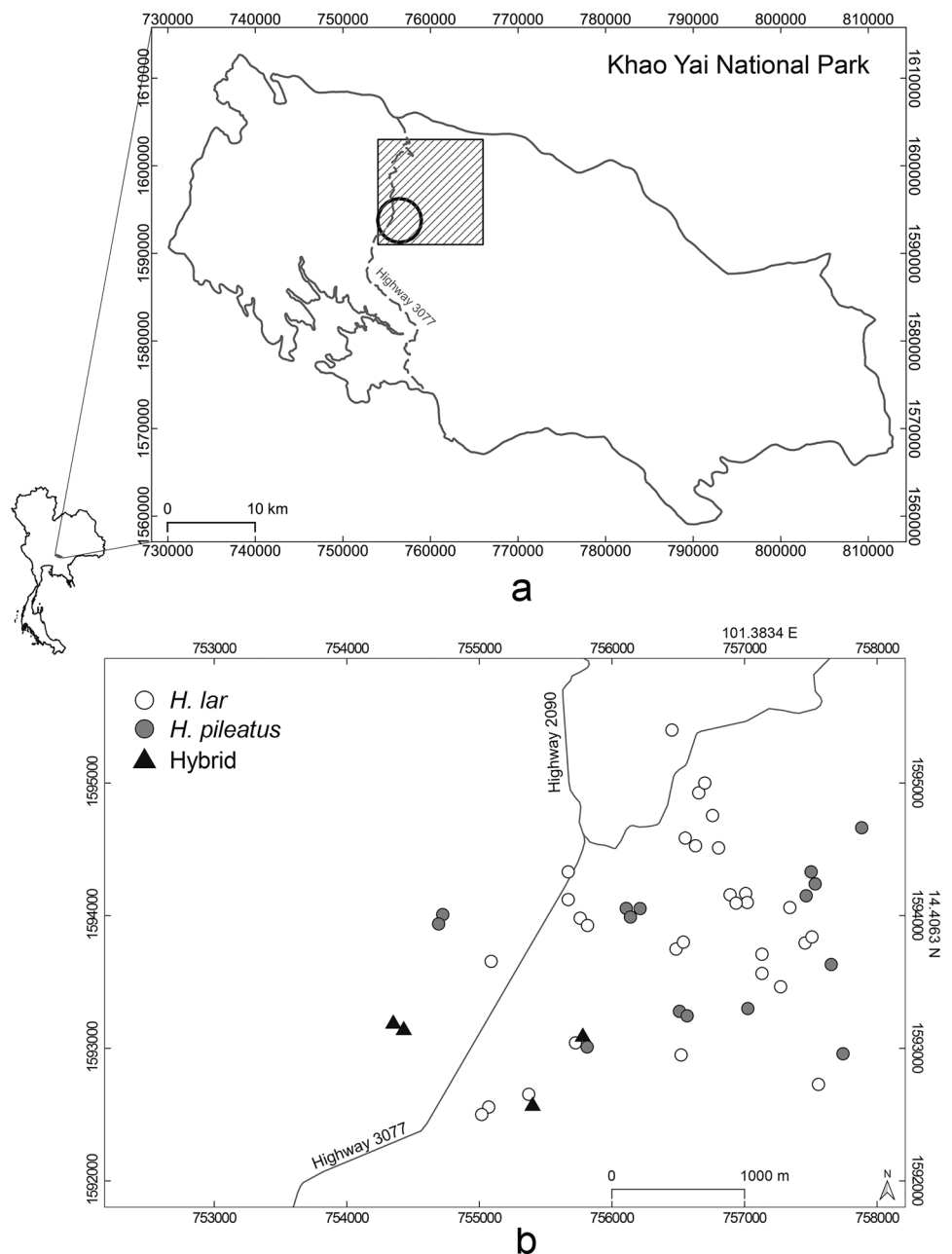
hybrid-like (Brockelman and Gittins 1984; Asensio et al. 2017).

We recognized *H. lar*, *H. pileatus* and their hybrids by pelage and vocalizations (Brockelman and Gittins 1984; Brockelman and Schilling 1984). *H. lar* is asexually dichromatic as it has two color morphs: black (or dark brownish-black), and buff. Both morphs have a complete white face ring and whitish fur on the hands and feet. Body color in *H. lar* appears to be controlled by a single gene in which an allele for black is dominant over an allele for buff coloration (Brockelman 2004). Brockelman (2004) showed that reproductive pairing in *H. lar* is random with respect to color, and that pairs with different color combinations do not differ significantly with respect to their production of offspring. It is unclear how these alleles affect hybrid coloration. *H. pileatus* is sexually dichromatic in pelage coloration (Marshall et al. 1972). The duets of *H. lar* and *H. pileatus* are clearly distinct and easily recognizable from vocal patterns of both sexes (Marshall et al. 1972; Brockelman and Schilling 1984).

Sample collection

The gibbons, which were mostly unhabituated, were followed during daytime from February 2013 to September 2015 to collect fecal samples, whilst attempting not to disturb them by maintaining a cautious distance. The identity of the gibbons was recorded using pelage, color, age-class and sex, whenever possible, along with their group identification and composition following Asensio et al. (2017). In nearly all cases, the defecating individual was recognized (Table 1). For some samples (from two *H. lar* and six *H. pileatus* individuals), however, the collectors were not completely sure which animal had defecated. However, as these unknown samples belonged to groups classified as either *H. lar* or *H. pileatus* (as defined above), the exact identity of the individuals that produced them did not affect the results. We collected approximately 30 g of feces immediately after the gibbons defecated. The fecal samples were preserved in DET buffer (20% dimethyl sulfoxide, 250 mM ethylenediaminetetraacetic acid, 100 mM Tris, pH 7.5 saturated with

Table 1 Individual fecal samples of *Hylobates lar*, *Hylobates pileatus* and intermediate hybrids collected in the contact zone in Khao Yai National Park

Group ID[a]	Group composition[b]	Sample ID[c]
L1	AdF-L, *AdM-L*, SubM-L, *Juv-L*, Inf-L	J32_HL, J33_HL
L2	*AdF-L*, *AdM-L*, *Juv-L*, Inf-L	A23_HL, J02_HL, J08_HL
L5	*AdF-L*, *AdM-L*, *SubM-L*, Juv-L	A13_HL, J10_HL, A03_HL
L8	AdF-L, AdM-L, *SubM-L*, 2Juv-L	A04_HL
L9	*AdF-L*, *AdM-L*, *SubM-L*, Inf-L	J21_HL, A17_HL, J20_HL
L10	AdF-L, AdM-L, SubM-L, Juv-L, Inf-L	A01_HL[d], SA03_HL[d]
L11	*AdF-L*, *AdM-L*, *SubM-L*, Inf-L	A10B_HL, A09A_HL, N02_HL
L12	*AdF-L*, *AdM-L*	J18_HL, J19_HL
L14	*AdF-L*, *AdM-L*, Juv-L, *Inf-L*	J12_HL, A40_HL, J14_HL
L18	AdF-L, *AdM-L*, *SubM-L*, Inf-L	J26_HL, N01_HL
L20	*AdF-L*, AdM-L	J28_HL
SL	A solitary adult male	A08_HL
P1	*AdF-P*, AdM-P, SubM-P, *Juv-P*, Inf-P	J03_HP, SA07_HP, J06_HP[d]
P3	*AdF-P*, AdM-P, 2Juv-P, Inf-P	A10A_HP, A11A_HP[d], J22_HP[d]
P4	AdF-P, AdM-P, SubM-P, 2*Juv-P*	A11B_HP, A12_HP[d], A18_HP[d], A20_HP[d]
P5	*AdF-P*, AdM-P, SubM-P, Juv-P, Inf-P	A28_HP
P6	*AdF-P*, *AdM-P*	A34_HP, A33_HP
P18	*AdF-P*, AdM-P, Inf-P	J29_HP
H1	*AdF-H*, AdM-L, 2*Juv-H*, Inf-H	A06_HB, A05_HB
M2	*AdF-L*, 2*AdM-P*, *Juv-H*, Inf-H	A26_HL, A25_HP, A22_HB
M3	AdF-P, *AdM-L*, Juv-H, *Inf-H*	A14_HL, A15_HB

Individuals identified during sample collection are indicated by *italics*. Some groups (e.g., L8) have two individuals in the same age category. Group identity (*ID*) and group composition follow Asensio et al. (2017)

[a]*L Hylobates lar* group, *SL* solitary *H. lar*, *P Hylobates pileatus* group, *H* hybrid group, *M* mixed-species group

[b]*AdF* Adult female, *AdM* adult male, *SubF* subadult female, *SubM* subadult male, *Juv* juvenile, *Inf* infant, *L H. lar* individual, *P H. pileatus* individual, *H* intermediate hybrid individual

[c]*HL H. lar* phenotype, *HP H. pileatus* phenotype, *HB* intermediate phenotype

[d]Gibbon individuals that were not identified during sample collection

NaCl) in 50-ml polypropylene tubes at ambient temperature in the field, and stored at 4 °C upon arrival at the laboratory until DNA extraction.

There were more *H. lar* than *H. pileatus* individuals at the study site. We obtained a total of 64 fecal samples comprising 40 from *H. lar*, 20 from *H. pileatus*, and four from hybrids that were determined phenotypically. We genotyped all of these samples using mtDNA and microsatellite DNA markers. Based on phenotyping, mtDNA sequencing and microsatellite genotype, ten of the gibbons were found to have yielded two to three samples each. All the duplicate samples yielded the same mitochondrial haplotypes and microsatellite genotypes and were later excluded from the data analyses. Thus, in total, 52 samples were included in the analyses, and comprised those from 33 *H. lar*, 15 *H. pileatus*, and four hybrid individuals.

DNA extraction and amplification

We extracted DNA from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen Hilden, Germany) following the manufacturer's protocol, except for the elution steps. We extended the elution time to 30 min, and eluted the DNA in a 150-µl elution buffer. We amplified a 667- or 668-bp fragment of mitochondrial D-loop containing hypervariable segment I using the primers GIBDLF3 (5′-CTTCACCCTCAGCACCCAAAGC-3′) (Andayani et al. 2001) and HY16092R (5′-AAGACAGATACTGCGACATAGG-3′)

(Matsudaira et al. 2013). We conducted the polymerase chain reactions (PCRs) in a 12.5-µl reaction, containing 1× PCR buffer, 1.25 µg BSA, 2.0 mM of each dNTP, 2.4 mM MgCl$_2$, 0.32 µM of each primer, 0.5 U HotStar *Taq* DNA polymerase (Qiagen) and 2 µl DNA template. The cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. A negative control was included in each amplification to check for contamination during the process. The PCR products were purified by a restriction enzyme digestion method using Exonuclease I and Shrimp Alkaline Phosphatase. The purified products were sequenced from both directions by Macrogen (South Korea) using an ABI 3730XL DNA analyzer (Applied Biosystems).

Since there were no species-specific microsatellite primers for *Hylobates* spp., we selected nine human-derived microsatellite loci (Table 2) which were successfully amplified and polymorphic for both *H. lar* and *H. pileatus*. We amplified each locus separately in a 12.5-µl reaction containing 1× PCR buffer, 1.25 µg BSA, 2.0 mM of each dNTP, 2.4 mM MgCl$_2$, 0.32 µM of fluorescent-labeled forward primer, 0.32 µM reverse primer, 0.5 U of HotStar *Taq* DNA polymerase (Qiagen) and 2 µl DNA template. The cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 1 min, 52–57 °C for 1 min (Table 2) and 72 °C for 1 min, and a final extension at 72 °C for 15 min. A PCR negative control was included

Table 2 The human-derived microsatellite markers used in this study

Locus	Primer name	Primer sequence (5′–3′)	Motif	T_a (°C)	Reference for the primer sequence
D1S1656	D1S1656-F	GTGTTGCTCAAGGGTCAACT	(GATA)$_n$	55	Barelli et al. (2013)
	D1S1656-R	GAGAAATAGAATCACTAGGGAACC			
D2S367	D2S367re-F[a]	TGGTCTAAGGGTCACCATCC	(CA)$_n$	56	Nürnberg et al. (1998)
	D2S367re-R[a]	ATCCACTGGCACCTACTTGG			
D3S1768	D3S1768-F	GGTTGCTGCCAAAGATTAGA	(GATA)$_n$	55	Barelli et al. (2013)
	D3S1768-R	CACTGTGATTTGCTGTTGGA			
D4S243	D4S243-F	TCAGTCTCTCTTTCTCCTTGCA	(GATA)$_n$	57	Barelli et al. (2013)
	D4S243-R	TAGGAGCCTGTGGTCCTGTT			
D5S1457	D5S1457-F	TAGGTTCTGGGCATGTCTGT	(GATA)$_n$	56	Barelli et al. (2013)
	D5S1457-R	TGCTTGGCACACTTCAGG			
D7S817	D7S817-F2	TAAATCTCTTTATGGCTGACTG	(GATA)$_n$	57	Bradley et al. (2000)
	D7S817-R	GGGTTCTGCAGAGAAACAGA			
D10S1432	D10S1432-F	CAGTGGACACTAAACACAATCC	(GATA)$_n$	56	Bradley et al. (2000)
	D10S1432-R	TAGATTATCTAAATGGTGGATTTCC			
D11S2002	D11S2002-F	CATGGCCCTTCTTTTCATAG	(GATA)$_n$	54	Barelli et al. (2013)
	D11S2002G-R	AGCAACCCTCCCACCTTAG			
D13S321	D13S321-F	TACCAACATGTTCATTGTAGATAGA	(GATA)$_n$	52	Barelli et al. (2013)
	D13S321-R	CATACACCTGTGGACCCATC			

T_a Annealing temperature

[a] Redesigned primer

in each amplification. The PCR products were visualized on 2.5% agarose gel. The amplified PCR products from different loci with different dyes were multiplexed and sent for fragment length analysis using an ABI 3730XL DNA analyzer (Applied Biosystems) with a 400HD internal size standard. The allele sizes were analyzed using Peak Scanner Software v1.0 (Applied Biosystems). The procedure was repeated at least three times for each sample at each locus. The consensus genotypes were scored from at least two out of the three results in order to reduce errors caused by noninvasive sampling, such as allelic dropout. Additional repeats were performed if the samples did not show a consensus genotype. All microsatellite genotypes were repeatedly analyzed until two gave the same result. We also checked genotypes of parent–offspring pairs, and no mismatch was found.

Mitochondrial DNA analysis

The mitochondrial sequences were aligned using MUSCLE as implemented in MEGA7 (Kumar et al. 2016). We constructed phylogenetic trees using maximum likelihood (ML) and Bayesian inference (BI) approaches. The ML analysis was performed by using Treefinder (Jobb 2011) with 1000 bootstrap replications. The BI tree construction was performed by using MrBayes 3.2.6 (Ronquist et al. 2012) using four independent Markov Chain Monte Carlo (MCMC) runs for 1,000,000 generations with trees sampled every 200 generations. The first 25% of samples were discarded as burn-in. The sequences from the GenBank database for *H. lar* (accession no. X99256) and *H. pileatus* (AB504749 and HQ622787) were included in the tree construction. The sequences of *Hoolock leuconedys* (KY250071) and *Hoolock hoolock* (NC_033885) were used as outgroups. The BI tree was displayed with the posterior probabilities using FigTree v1.4.3 (Rambaut 2016). The haplotype diversity and nucleotide diversity of the *H. lar* and *H. pileatus* clades were separately analyzed using DnaSP v6 (Rozas et al. 2017). We constructed a haplotype network using PopART v1.7 (Leigh and Bryant 2015) to examine the intraspecific relationships among the haplotypes. We examined genetic differentiation between *H. lar* and *H. pileatus* by analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using the pairwise F_{ST} calculated by conventional F-statistics based on haplotype frequencies and the distance method, implemented in Arlequin v3.5 (Excoffier and Lischer 2010).

Microsatellite DNA analysis

For the microsatellite analyses, we included only the samples that had been successfully genotyped for at least seven loci. The probability of identity (PID), indicating the probability of two random individuals containing the same genotype based on the nine microsatellite loci, was calculated using GenAlEx

v.6.5 (Peakall and Smouse 2012). We evaluated the microsatellite diversity of *H. lar* and *H. pileatus* based on the number of alleles, observed heterozygosity and expected heterozygosity using Microsatellite Toolkit version 3.1 (Park 2001). The presence of null alleles and allelic dropout was examined using Micro-Checker v2.2.3 (van Oosterhout et al. 2004). Deviations from Hardy–Weinberg equilibrium (HWE) at each locus in each species, and linkage disequilibrium (LD) between pairs of loci, were assessed using Genepop v4.7.5 (Raymond and Rousset 1995; Rousset 2008). A sequential Bonferroni correction (Holm 1979) was applied to adjust for the significance levels of the HWE and LD tests for the multiple comparisons among loci at $\alpha = 0.05$. We estimated the pairwise genetic distance between *H. lar* and *H. pileatus* by calculating Wright's F_{ST} (Weir and Cockerham 1984) and Slatkin's R_{ST} (Slatkin 1995; Michalakis and Excoffier 1996). Slatkin's R_{ST} assumes a stepwise mutation model for microsatellite data. We examined genetic differentiation between the two species using AMOVA (Excoffier et al. 1992) implemented in Arlequin v3.5 (Excoffier and Lischer 2010).

Hybridization analysis

We analyzed the admixture model using a Bayesian MCMC method in Structure v2.3.4 (Pritchard et al. 2000). The number of populations (K) was determined as $K = 2$, representing the two parental species. Admixture analysis was performed with 1,000,000 MCMC generations, 20 independent simulations per K, and 100,000 generations burn-in. The estimates of admixture (q-value) were calculated for each individual. The q-value represented the proportion of genetic contribution from the parental lineages in the individual samples. Since we did not collect samples from parental populations outside the zone of contact, we could not determine the exact threshold q-value for distinguishing the pure species and hybrids. We sorted the q-value of the 47 samples from low to high and determined the cut-off q-value at 95%. Threshold values of 0.90–0.95 were reported and applied as in similar, previous, hybridization studies (e.g., Pastorini et al. 2009; Malukiewicz et al. 2015). The q-value ranged from 1.0 for *H. lar* to 0.0 for *H. pileatus*. Individuals with a q-value ≥ 0.95 were considered pure *H. lar* and were assigned to cluster 1, and those with q-values ≤ 0.05 were considered pure *H. pileatus* and were assigned to cluster 2. Individuals with intermediate q-values were considered admixed.

Results

Mitochondrial DNA

The phylogenetic trees constructed by using the ML and BI methods resulted in the same topology. Two distinct

clades, *H. lar* and *H. pileatus*, were obtained with a high Bayesian posterior probability (presented as a percentage; 100%) and high ML bootstrap (100%) nodal supports. All *H. lar*-like samples were placed in the *H. lar* clade, and all *H. pileatus*-like samples were placed in the *H. pileatus* clade (Fig. 2). The three hybrid samples, A05_HB, A06_HB and A22_HB, were placed in the *H. lar* clade,

and the hybrid sample A15_HB was placed in the *H. pileatus* clade.

A total of 15 haplotypes were determined among the 52 samples. Eight haplotypes (Hlar01–Hlar08) belonged to the *H. lar* clade (*n* = 36), and seven haplotypes (Hpil01–Hpil07) belonged to the *H. pileatus* clade (*n* = 16). The previously undocumented haplotypes (Hlar03, Hlar04, Hlar06, Hlar08
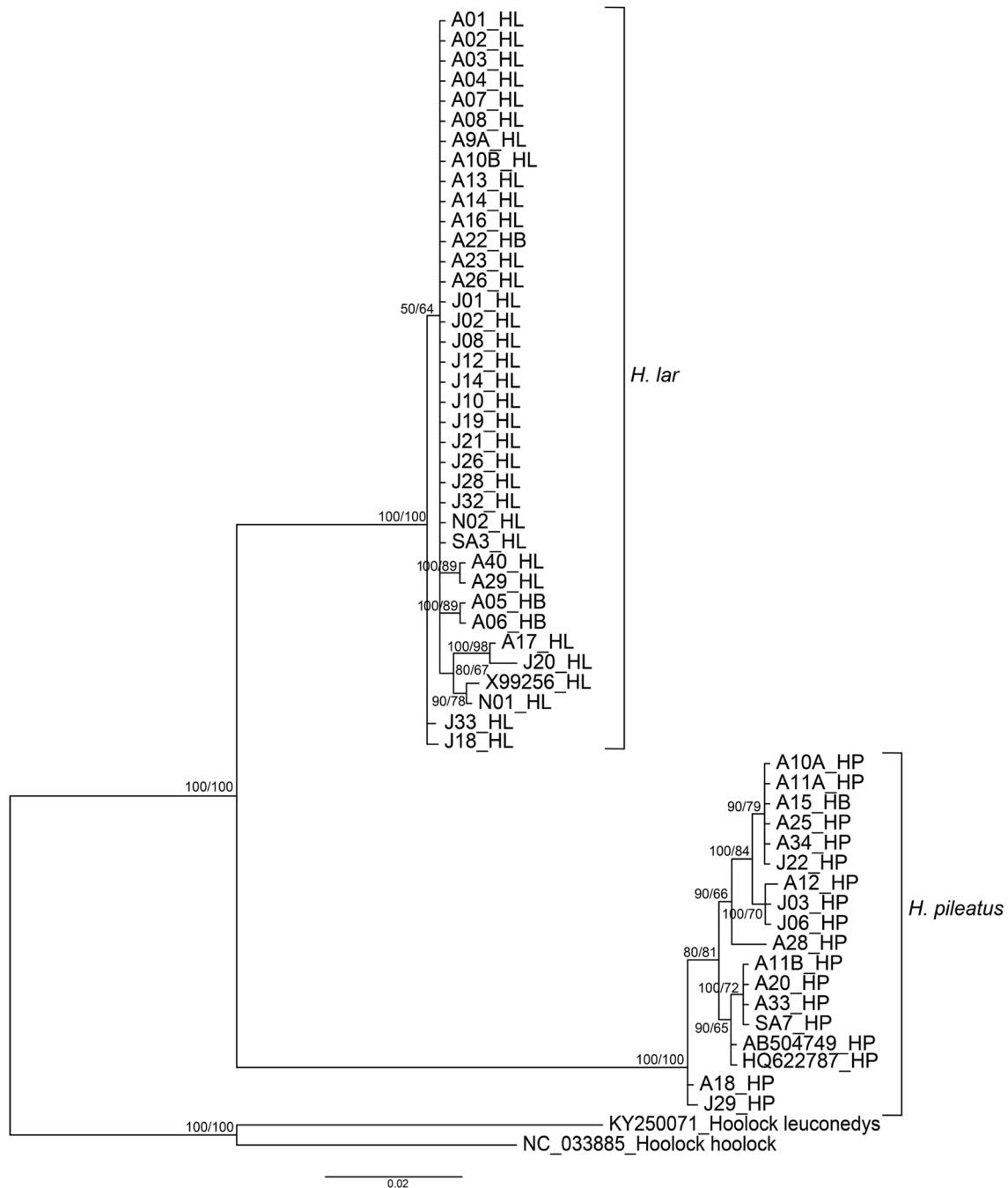

Fig. 2 Phylogenetic tree of *H. lar* and *H. pileatus* inferred from the D-loop gene based on maximum likelihood (ML) and Bayesian inference (BI). Bayesian posterior probabilities (presented as percentages) and maximum-likelihood bootstrap support values are presented on the branches (BI/ML). *HL H. lar* phenotype, *HP H. pileatus* phenotype, *HB* hybrid with intermediate pelage

and Hpil01–Hpil07) were submitted to GenBank (accession nos. MT302850–MT302860). The haplotypes Hlar01, Hlar02, Hlar05 and Hlar07 corresponded to the haplotypes HKY2, HKY9, HKY1 and HKY7, respectively, which were previously identified in the *H. lar* population near the contact zone in Khao Yai National Park by Matsudaira et al. (2013). The haplotype diversity and nucleotide diversity of the *H. lar* clade were 0.5524 ± 0.0968 and 0.0026 ± 0.0017, respectively, and those of the *H. pileatus* clade were 0.8167 ± 0.0729 and 0.0065 ± 0.0038, respectively.

The haplotype network presented two distinct haplogroups, *H. lar* and *H. pileatus* (Fig. 3). The *H. lar* haplogroup showed a star-like shape in which Hlar01 was the most common haplotype, whereas the *H. pileatus* did not show this pattern. The intermediate hybrids contained mtDNA of both *H. lar* and *H. pileatus*. The three hybrids placed in the *H. lar* haplogroup carried haplotypes Hlar05 and Hlar08; the hybrid placed in the *H. pileatus* haplogroup carried haplotype Hpil01.

The pairwise F_{ST} between the *H. lar* and *H. pileatus* clades calculated based on both the conventional *F*-statistic ($F_{ST} = 0.336$) and the distance method ($F_{ST} = 0.968$) were high. Genetic differentiation at the species level based on both F_{ST} calculations was highly significant ($P < 0.001$).

Microsatellite DNA

We excluded five of the 33 *H. lar* samples from the microsatellite analysis because they were successfully genotyped for less than seven loci. Finally, we included 47 samples of 28 *H. lar*, 15 *H. pileatus* and the four intermediate hybrid individuals for the microsatellite analyses. The nine microsatellite loci were polymorphic and highly informative. The PIDs based on the nine loci used were as low as 2.2×10^{-9} in *H. lar* and 6.6×10^{-8} in *H. pileatus*, indicating that this microsatellite panel was suitable for individual identification

and population genetics analyses. The microsatellite diversity of both species was considered moderate to high with the average number of alleles per locus at 7.1 ± 2.1 in *H. lar* and 5.6 ± 1.7 in *H. pileatus*. The average observed heterozygosity was 0.615 ± 0.031 and 0.644 ± 0.042 in *H. lar* and *H. pileatus*, respectively (Table 3). Overlapping allele ranges and shared alleles were observed for all loci, but the allele frequencies of the shared alleles were different between species. A significant deviation from the HWE was detected in locus D11S2002 for *H. lar* with a relatively high value of F_{IS}. This was likely due to nonrandom sampling, since we obtained samples from close relatives in family groups. All loci were found in HWE for *H. pileatus* (Table 3). The presence of null alleles was detected for three loci (D4S243, D11S2002, D13S321) for *H. lar*, and one locus (D1S1656) for *H. pileatus*. We did not detect allelic dropout for any loci for either species. Thus, we included all loci for further analyses. No significant LD was found between pairs of loci in *H. lar* or in *H. pileatus*. The pairwise F_{ST} and R_{ST} between *H. lar* and *H. pileatus* were high. The AMOVA indicated significant genetic differentiation between the species ($F_{ST} = 0.256$, $P < 0.001$; $R_{ST} = 0.579$, $P < 0.001$).

The hybridization analysis revealed evidence of crossing between *H. lar* and *H. pileatus*. All *H. pileatus* phenotype samples were determined as pure species in cluster 2 with the *q*-values 0.009–0.038. Twenty-two *H. lar* samples were considered genetically *H. lar* in cluster 1 with *q*-values of 0.951–0.993. The results from program Structure identified ten admixed individuals, six *H. lar*-like and the four intermediate hybrids, with *q*-values of 0.410–0.939 (Fig. 4). The four hybrids had approximately equal proportions of genetic contribution (*q*-values 0.410–0.562) from each parental species. We observed a distinct cluster of *H. pileatus*, but did not observe a clear boundary in the *H. lar* cluster. We suspect some of the *H. lar*-like individuals might be backcrosses to *H. lar*, and therefore contain a greater genetic

Fig. 3 The haplotype network based on the mitochondrial D-loop of *H. lar* (*Hlar*; *yellow*) and *H. pileatus* (*Hpil*; *blue*). The proportion of hybrids sharing the haplotype is shown in *white*. The *size* of each *circle* is proportional to the number of samples carrying the haplotype. *Black dots* are connection points, representing missing haplotypes. *Perpendicular bars* on the connecting line represent the number of mutational steps between haplotypes
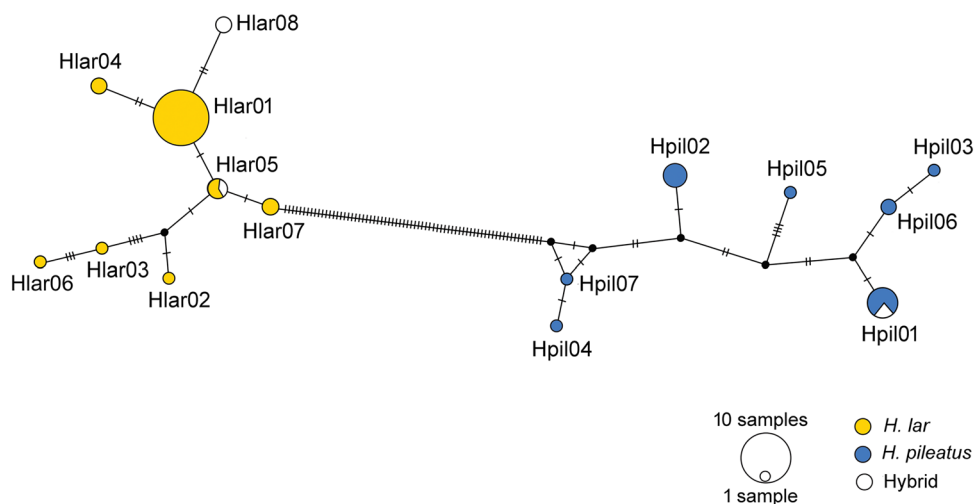
Table 3 Microsatellite diversity of *H. lar* ($n=28$) and *H. pileatus* ($n=15$) from the contact zone

Locus	*H. lar*					*H. pileatus*				
	AR	N_A	H_O	H_E	F_{IS}	AR	N_A	H_O	H_E	F_{IS}
D1S1656	149–185	8	0.778	0.765	−0.018	145–177	8	0.462	0.732	0.379
D2S367	89–97	3	0.179	0.227	0.215	89–97	3	0.267	0.297	0.104
D3S1768	184–204	5	0.741	0.732	−0.013	188–208	6	0.667	0.782	0.152
D4S243	239–271	8	0.607	0.774	0.219	235–263	5	0.600	0.600	0.000
D5S1457	127–167	9	0.704	0.769	0.118	127–163	7	0.667	0.690	0.035
D7S817	113–141	6	0.607	0.692	0.124	113–141	6	0.800	0.667	−0.209
D10S1432	149–201	9	0.786	0.834	0.059	149–165	5	0.733	0.740	0.010
D11S2002	201–233	7	0.500[a]	0.728	0.317	193–229	7	0.933	0.839	−0.117
D13S321	215–247	9	0.630	0.853	0.266	215–239	3	0.667	0.559	−0.202
Mean		7.1	0.615	0.711			5.6	0.644	0.656	

AR Allele size range, N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} Wright's inbreeding coefficient

[a]Indicates deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction

Discussion

Our findings suggest a high degree of genetic differentiation between *H. lar* and *H. pileatus*. Gene flow between the species is limited, resulting in a narrow hybrid zone, which concords with previous findings in the study site (Brockelman and Gittins 1984; Asensio et al. 2017). Gene flow is asymmetrical in the hybrid zone, with greater introgression into the *H. lar* side than into *H. pileatus*.

Hybridization between *H. lar* and *H. pileatus*

The hybridization analysis confirmed that four individuals (8%) with intermediate pelage were hybrids or backcrosses. They were members of mixed-species and hybrid groups. Hybrids A22_HB and A15_HB were offspring of heterospecific groups M2 (female *H. lar* × male *H. pileatus*) and M3 (female *H. pileatus* × male *H. lar*) studied by Asensio et al.

(2017), and are likely to be F1 hybrids. Hybrid A06_HB was the female of group H1 paired with a *H. lar* male. There is no information about the parents of this adult female. She has intermediate pelage and a great call pattern. The *q*-value of A06_HB is close to 0.5; thus, she is likely to be an F1 hybrid or an early generation backcross. Hybrid A05_HB is a juvenile; observations of her behavior and her pelage indicated that she is the offspring of female A06_HB, and hence a recent-generation backcross to *H. lar*. The *q*-values of A05_HB and A06_HB, respectively 0.422 and 0.434, are similar. These findings support our first hypothesis regarding a mixture of nuclear genes from both species in putative hybrids.

Interbreeding between female *H. lar* × male *H. pileatus* and the reciprocal cross produced viable F1 hybrids. Thus, females from both parental species could pass their mitochondrial genes to their hybrid offspring. Nevertheless, we did not find evidence of introgression of *H. pileatus* mitochondrial genes into the *H. lar* population, unlike Matsudaira et al. (2013), who did. All samples of the *H. lar* phenotypes contained *H. lar* mitochondrial haplotypes, as did all samples of the *H. pileatus* phenotypes. The fact that more
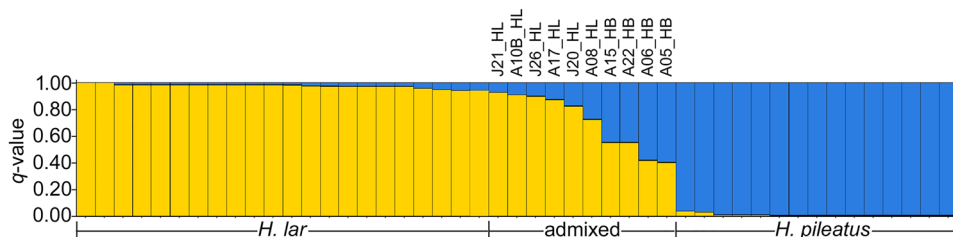

Fig. 4 Admixture analysis of 47 samples of *H. lar*, *H. pileatus* and intermediate hybrids from the contact zone based on microsatellite analysis for the number of populations ($K=2$). *Each bar* represents one individual [*H. lar* genetic admixture (*yellow*), *H. pileatus* genetic admixture (*blue*)]. The *y*-axis shows the *q*-values: *H. lar* (≥ 0.95), *H. pileatus* (≤ 0.05), admixed ($0.05 < q$-value < 0.95). Ten admixed individuals were determined; the sample codes are shown *above the bars*. For abbreviations, see Fig. 2

hybrids had *H. lar* haplotypes (three individuals) than *H. pileatus* haplotypes (one individual) suggests that females of *H. lar* are more prone to mate with the other species than females of *H. pileatus*. This suggestion needs to be further tested with more samples from hybrids.

Matsudaira et al. (2013), who reported introgression into the *H. lar* population located at the Mo Singto study area approximately 5 km northwest of the contact zone, found nine out of 68 *H. lar* individuals carrying an *H. pileatus* haplotype (HKY11). However, we did not find the HKY11 haplotype in our *H. pileatus* samples from the contact zone. Matsudaira et al. (2013) suggested that these nine individuals were possibly third- or later-generation backcrosses. We determined that our four mitochondrial haplotypes of *H. lar* were the same as those reported at Mo Singto in the *H. lar*-dominated area, which suggests gene flow between the two areas. In addition, we observed a female *H. pileatus* in the *H. lar* range at Mo Singto during the period from 1997 to 2010 (she produced no offspring), and in May 2018 another female *H. pileatus* was seen (C. Kongrit, personal observation) but never formed a reproductive pair bond with any *H. lar* male. An adult female *H. pileatus* without a mate could not survive in an area populated by *H. pileatus* groups (to our knowledge, no group has ever been found to contain two adult *H. pileatus* females), but an adult *H. pileatus* female may share the territory of an *H. lar* group without being evicted by residents, even though she would have difficulty in establishing a pair bond. Brockelman and Gittins (1984) found more phenotypic hybrids at the edge of the zone than in the center of the zone. Occasionally, adults dispersing into the area occupied by the other species may pair and breed, but we believe that the most likely way that *H. pileatus* mitochondrial haplotypes move into the *H. lar* population is through repeated backcrossing, as suggested by Matsudaira et al. (2013). After the third or fourth generation backcross, the phenotypes will become indistinguishable from those of *H. lar* (making successive backcross daughter matings easier), while daughters will carry the full complement of maternally inherited *H. pileatus* haplotypes.

The presence of a low number of intermediate hybrids [6% (Brockelman and Gittins 1984); 7% (Asensio et al. 2017); 8% (this study)], and high abundance of parental species in the contact zone exemplify a bimodal hybrid zone (Jiggins and Mallet 2000). Previous studies provided clear evidence of a higher ratio of conspecific pairs than heterospecific pairs and hybrid groups in this contact zone [194 conspecific pairs, 20 heterospecific and hybrid groups (Brockelman and Gittins 1984); 29 conspecific pairs, three heterospecific pairs (Asensio et al. 2017)], resulting in a rarity of F1 hybrids. This contrasts greatly with reports on contact zones between well-marked, more closely related species, such as *H. albibarbis* and *H. muelleri* in Borneo (Mather 1992), and *Eulemur fulvus rufus* and *Eulemur*

albocollaris (Wyner et al. 2002), in which the parental types are separated by a zone of intergrades or hybrid-like individuals. Several other contact zones between primate species have been reported to have > 20% hybrid individuals [*Papio cynocephalus* and *Papio anubis* (Tung et al. 2008); *Alouatta caraya* and *Alouatta guariba clamitans* (Mourthe et al. 2019)].

Although multigenerational backcrosses were found in the present study, F1 hybrids were relatively rare. The rarity of recent-generation hybrids, especially F1, and the existence of the parental species in contact with one another in the center of the hybrid zone indicate highly nonrandom mating and strong reproductive isolation between the species. Such a pattern has been also reported in other primate hybrid zones (e.g., Cortés-Ortiz et al. 2019). The narrow hybrid zone between *H. lar* and *H. pileatus* is likely to be maintained as a tension zone under a dispersal and selection balance, in which parental species disperse into a contact zone and selection acts against hybrids (Barton and Hewitt 1985). As gibbon social groups are highly sedentary, movement into the zone should occur by dispersal of single individuals in search of mates. Hence, all groups within the zone would be the result of pairing of such individuals within the zone and not of the displacement of newly mated pairs or whole groups. Natal dispersal distance is typically only one or two territories in the *H. lar* population in the Mo Singto study site about 5 km from the contact zone (Brockelman et al. 1998). However, Asensio et al. (2017), comparing successfully mated pairs in homospecific, mixed species and hybrid groups, found no significant differences in the number of offspring present per group. However, we need to determine whether hybrids are as successful at finding mates as the parental species, and because every hybrid is genetically unique, we cannot assume that they are uniform in this regard (Arnold 1997).

Microsatellite analysis revealed six admixed *H. lar*-like individuals, suggesting the direction of backcrossing towards *H. lar*. The presence of such backcrosses indicates that a larger sample size would yield more later-generation backcrosses not recognizable by pelage alone. Hence, our second hypothesis is only partially supported as we found no evidence of introgression into *H. pileatus*.

In addition, Brockelman and Gittins (1984) observed that four female *H. lar* had more notes in their great calls than those typical of female *H. lar*, and suspected that they might have been backcrosses. Admixed individuals can show a wide variety of pelage patterns, but quantitative variation in the highly stereotyped great calls appears to be a more sensitive indication of genetic parentage than pelage (Brockelman and Schilling 1984). Whatever its cause, this finding indicates that the hybrid zone might be larger if acoustic cues are taken into account, particularly

as song pattern differences are the clearest way of distinguishing biological species of gibbons (Marshall and Marshall 1976).

Unidirectional gene flow into *H. lar* could be driven by female choice in pair bonding (Wirtz 1999). The duetted song patterns of *H. lar* and *H. pileatus* are species specific (Marshall and Marshall 1976; Brockelman and Schilling 1984; Marshall and Sugardjito 1986), and are related to the maintenance of pair bonds (Geissmann and Orgeldinger 2001). In the *H. pileatus* duet, males wait during the long bubbly trill of the female and add their "coda" during the last part of the trill (Marshall et al. 1972). In contrast, the male *H. lar* adds his coda a second or two after the female great call ends (Marshall et al. 1972; Geissmann 1984; Raemaekers et al. 1984). We speculate that female hybrids that produce an intermediate song type without the *H. pileatus*-like bubbly trill may have a lower chance of coordinating the duet with *H. pileatus* males, but may sing more easily with *H. lar* males. Asensio et al. (2017) found that all adult female intermediate hybrids in the study area were paired with *H. lar* males. It is also possible that the direction of gene flow in the contact zone could be affected by the patterns of dichromatism in the species. For example, *H. pileatus* females might discriminate against light-colored *H. lar* morphs (W. Y. Brockelman, unpublished data).

To summarize our main findings, natural hybridization between *H. lar* and *H. pileatus* produced viable and fertile F1 hybrids. The intermediate phenotypes of the hybrids reflected the contribution of nuclear genes from both parental species. The relative abundance of parental types versus the low number of F1 hybrids and admixed individuals indicates the presence of reproductive barriers between these biological species [sensu Mayr (1963)]. Our findings provide evidence for asymmetrical introgression of nuclear genes into *H. lar*; however, further investigation of the genetic structure of *H. lar* and *H. pileatus* in their allopatric areas is recommended, as this would provide a better understanding of the directions of gene flow between these two parental species.

Author contributions NA, WYB and CK contributed to the research idea, study design and field data collection. The laboratory work and data analysis were performed by DM, CK and EJ. The first draft of the manuscript was written by DM. All the authors commented on and revised the manuscript.

Declarations

Conflict of interest All of the authors declare that they have no conflict of interest.

Ethical statement This research was carried out under animal ethics protocol no. MUSC61-040-442 authorized by Mahidol University-Institute Animal Care and Use Committee (MU-IACUC).

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