Differential introgression suggests candidate beneficial and barrier loci between two parapatric subspecies of Pearson’s horseshoe bat *Rhinolophus pearsoni*

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Abstract

Observations that rates of introgression between taxa can vary across loci are increasingly common. Here, we test for differential locus-wise introgression in 2 parapatric subspecies of Pearson’s horseshoe bat (*Rhinolophus pearsoni chinensis* and *R. p. pearsoni*). To efficiently identify putative speciation genes and/or beneficial genes in our current system, we used a candidate gene approach by including loci from X chromosome that are suggested to be more likely involved in reproductive isolation in other organisms and loci underlying hearing that have been suggested to spread across the hybrid zone in another congeneric species. Phylogenetic and coalescent analyses were performed at 2 X-linked, 4 hearing genes, as well as 2 other autosomal loci individually. Likelihood ratio tests could not reject the model of zero gene flow at 2 X-linked and 2 autosomal genes. In contrast, gene flow was supported at 3 of 4 hearing genes. While this introgression could be adaptive, we cannot rule out stochastic processes. Our results highlight the utility of the candidate gene approach in searching for speciation genes and/or beneficial genes across the species boundary in natural populations.

Key words: gene flow, hybridization, hybrid zone, reproductive isolation.
genomes (Chd1 and Sws1) taken from our previous study (Mao et al. 2010), 2 X-linked genes and 4 hearing genes. Among these loci, Sws1 was suggested to be pseudogenized in Rhinolophus (Zhao et al. 2009), thus, it can be used as a neutral control in the comparison with other loci. We predicted that hearing genes might exhibit higher levels of introgression because of their benefits for animals in adapting to new environments, whereas introgression rates of X-linked genes would be reduced, compared to other autosomal genes.

Materials and Methods
Ethics statement and sampling
All tissues used in this study were sampled from bats for our former project (Mao et al. 2010) (see details in Figure 1 and Table 1). The non-lethal procedure of sampling consisted of taking wing membrane biopsies from bats, and was approved by the National Animal Research Authority, East China Normal University (approval ID 20080209). Bats were initially assigned to R. p. pearsoni or R. p. chinensis on the basis of taxonomy-specific and non-overlapping call frequencies (Zhang et al. 2009; Mao et al. 2010). One congeneric species R. affinis was included as an outgroup.

DNA sequencing
In this study, we amplified and sequenced introns from 2 X-chromosomal genes (Usp9x and Pola1) and 4 hearing genes (Prestin, Tmc1, FoxP2 and Kcnq4) in bats sampled for an earlier study (sample information and primer details are summarized in Table 2). Sequence data from 2 additional genes, Chd1 and Sws1, were taken from our previous study (Mao et al. 2010).

Polymerase chain reactions (PCR) were performed in 50 μl reaction mixtures containing 10–50 ng DNA, 0.25 mM of each primer, and 2.5 μl Premix Taq polymerase (TaKaRa). The thermal profiles for Usp9x, Pola1, FoxP2, Kcnq4, and Prestin have been described previously (Lim et al. 2008; Mao et al. 2014). For Tmc1, we used: 95 °C for 5 min; 34 cycles of 30 s at 94°C, 40 s at 61 °C, 90 s at 72 °C; 72 °C for 10 min. PCRs were carried out on a PTC-220 thermal cycler (Bio-Rad). DNA sequencing was undertaken with either the forward primer for Tmc1, Usp9x, Pola1, and Kcnq4 or both forward and reverse primers for FoxP2 and Prestin. PCR products were analyzed on an ABI PRISM 3700 automated sequencer (Applied Biosystems). When multiple heterozygous sites were present in the sequences, haplotypes were resolved probabilistically using PHASE 2.1 (Stephens et al. 2001) and traditional tree-based phylogenetic methods may be difficult to represent true genealogies (Posada and Crandall 2001). We, therefore, performed network-based phylogenetic reconstructions for each nuclear marker by constructing statistical parsimony networks in the package TCS version 1.21 (Clement et al. 2000).
Gene flow

Shared or closely related haplotypes between R. p. pearsoni (excluding Sichuan) and R. p. chinensis were observed at several nuclear genes (see section ‘Results’), which could have resulted from either introgression or incomplete lineage sorting. To distinguish these 2 processes we ran IM models in the program IMa2 (Hey and Nielsen 2007; Hey 2010). We repeated the IM analysis for each of the 8 loci (Chd1, Sus1, Usp9x, Pola1, Prestin, FoxP2, Kcnq4, and Tmc1) individually. Data for Chd1 and Sus1 were taken from our previous study (Mao et al. 2010). Before performing the IM analysis, for each locus we used DnaSP to test for recombination using the 4-gamete test (Hudson and Kaplan 1985). For loci showing recombination, only those segments without recombination were used in the IM analysis. It was worth pointing out that nonrecombined regions of each marker still showed informative variation between R. p. chinensis and R. p. pearsoni (data not shown). DnaSP was also used to assess neutrality based on the Hudson–Kreitman–Aguade test (HKA, Hudson et al. 1987) and Tajima’s D test (Tajima 1989) whose values were not significant (see Supplementary Table S1). For this reason, and because recent simulations (Strasburg and Rieseberg 2010) have highlighted the robustness of IM models to selection, all of the focal genes were used in the IM models (also see Bull et al. 2006; Pardo-Dieaz et al. 2012). Inheritance scalars were set at 0.75 for 2 X-linked markers (Usp9x and Pola1) and 1 for autosomal markers. For all loci, the Hasegawa-Kishino-Yano (HKY) model was applied. Several preliminary runs were performed to establish upper bounds on prior distributions. To check for the convergence of the Markov chain, the IM analysis was run at least twice using different random seeds. Each run included 200 000 genealogies at every 100 steps after a burn-in of 10⁶ steps including 20 Metropolis-coupled chains with a geometric heating scheme: -hfg -hn20 -ha0.96 -hb0.9. A total of 200 000 genealogies were used to perform likelihood ratio tests of the nested models for migration rates (Hey 2010).

Results

Haplotypes from the 2 X-linked genes (Usp9x and Pola1) were resolved into 3 subnetworks, corresponding to R. p. chinensis, R. p. pearsoni, and a divergent group of R. p. pearsoni from Sichuan (Figure 2A,B). However, 3 of the 4 hearing genes displayed contrasting results to this, with at least 1 haplotype of Prestin, FoxP2, and Tmc1 shared between R. p. pearsoni and R. p. chinensis (Figure 2C–E). It was notable that the shared FoxP2 haplotype between R. p. pearsoni and R. p. chinensis was from populations of their contact zone, Hunan and Fujian. For the fourth hearing gene, Kcnq4, we found a 63-bp deletion in R. p. chinensis compared to R. p. pearsoni (Figure 2F), indicating strong divergence between these 2 taxa at this locus. Like other nuclear genes, networks based on these 4 hearing genes showed that R. p. pearsoni haplotypes from Sichuan were strongly divergent from those from elsewhere. Consequently, individuals of R. p. pearsoni from Sichuan were excluded from estimates of migration rate in the IM analysis.

Two independent IM analysis gave similar posterior probability with the effective sample sizes of ≥ 200 for the migration rate parameter, indicating convergence on the true stationary distribution. To test whether introgression contributed to the observation of shared or closely related haplotypes between R. p. pearsoni excluding Sichuan and R. p. chinensis at several nuclear genes, we compared the fit of models with and without gene flow for all 8 loci individually. Based on likelihood ratio tests, the model with zero...
Table 1. GenBank accessions for all samples used in the molecular analysis. N means the location number as shown in Figure 1

| N | Sample locations       | Coordinates | Code | Prestin | Tmc1 | FoxP2 | Kcnq4 | Usp9x | Pola1 |
|---|------------------------|-------------|------|---------|------|-------|-------|-------|-------|
| 1 | Qingyang               | N30:20:511 | E117:50:128 | AH  | JX02283 | KCS74587, 93 | JX02243 | KCS74518, 20 | JX02378-79 | JX02319, 20,12,33 |
| 2 | Jiaxing                | N30:26:785 | E118:24:783 | AH  | JX02282 | KCS74589, 84,86 | JX02244 | KCS74512, 21 | JX02374-75 | JX02322, 28 |
| 3 | Huanghaijiniao        | N29:45:107 | E118:23:171 | AH  | JX02284 | KCS74603 | JX02245 | KCS74511 | JX02392-93 | JX02329-31 |
| 4 | Huangshaixinning      | N50:23:181 | E118:14:116 | AH  | JX02285 | KCS74585, 88 | JX02246 | KCS74514, 28 | JX02397, 99 | JX02334 |
| 5 | Fuchanshanling        | N29:22:112 | E117:34:324 | JX  | –       | KCS74589-90 | JX02247 | KCS74519 | JX02427-28 | JX02321, 36 |
| 6 | Fuchanqingfeng        | N29:22:262 | E117:39:357 | JX  | –       | KCS74598-600 | JX02248 | KCS74524, 29-30 | JX02422-23 | JX02325-26 |
| 7 | Fuchanquinhui         | N29:22:662 | E117:32:335 | JX  | –       | KCS74602 | JX02249 | KCS74523 | JX02424, 26 | JX02327 |
| 8 | Guwang cave           | N27:42:664 | E117:41:531 | JF  | –       | KCS74591-92 | JX02251 | KCS74513,22 | JX02408, 12 | JX02340-41 |
| 9 | Yanzijiao             | N27:48:511 | E117:42:505 | JX  | –       | KCS74594, 96 | JX02253 | KCS74515, 16,33 | JX02405-07 | JX02338 |
| 10 | Taining               | N26:42:236 | E117:29:867 | FJ  | –       | KCS74595 | JX02252, 54 | KCS74531-32 | JX02403-04 | JX02337 |
| 11 | Liancheng            | N25:12:404 | E117:30:066 | FJ  | –       | KCS74606 | –       | KCS74517 | JX02402 | JX02339 |

Table 2. Primers information for nuclear markers used in this study

| Name of markers | ID | Length (bp) | Primers information | References |
|-----------------|----|-------------|---------------------|------------|
| The nucleosome remodeling factor gene | Chd1 | 556 | F: GATAARTCAGARACAGCCCTTAGA GC R: TTTCGCCCTCAGCTGACTCC | Lim et al. (2008) |
| The short-wavelength-sensitive opsin gene | Sux1 | 645 | F: CACAGGCTATGTGGTCGACCTTC R: GCCCGTGGGATGGCTATTGA | Mao et al. (2014) |
| Prestin intron 4 | Prestin | 536 | F: GAGGAGTAAATGCGACCAA R: ATCCCACTGTACCGCTTTG | Mao et al. (2014) |
| Transmembrane cochlear-expressed gene 1 | Tmc1 | 515 | F: AGACCAAATTTCACTCTATCACCA R: GTTACGAGGAAACCTCTGAATGG | This study |
| The voltage-gated potassium channel subfamily KQT member 4 | Kcnq4 | 646 | F: GCTTACCTCATAACCCCTATCA R: CCTGAGAATAGCAAACTCTGCG | Mao et al. (2014) |
| Ubiquitin specific protease 9 X | Usp9x | 674 | F: GCGGTGTCAGGTGGAGAA R: GCAGGGAGCGGTAATAGAA | Lim et al. (2008) |
| Polymerase (DNA directed) alpha 1 | Pola1 | 549 | F: GAAACTTGTAGAGCCCGGAAGA R: ACCTCCCTTCCTTTTGGAG | Mao et al. (2014) |

Discussion

Patterns of differential introgression have been frequently used to search for putative speciation genes involved in reproductive isolation and/or beneficial genes which can spread across the species boundaries (see Payseur 2010). In this study, results from 2 X-linked markers (Pola1 and Usp9x) suggested no introgression between R. p. pearsoni and R. p. chinesis supporting previous findings from the other 2 nuclear genes Chd1 and Sux1 (Mao et al. 2010). Furthermore, IMa2 analysis based on likelihood ratio tests could not reject the model of zero gene flow at these 4 genes individually.
between these 2 subspecies, perhaps indicating these genes are involved in reproductive isolation either directly or via linkage to other genes.

In contrast to the above patterns, 3 of 4 hearing genes (Prestin, Tmc1, and FoxP2) exhibited shared and/or closely related haplotypes between R. p. pearsoni and R. p. chinensis. While this result could in theory be explained by either incomplete lineage sorting or introgression (Funk and Omland 2003; Ballard and Whitlock 2004), the results of the IMa2 analyses supported the latter scenario, with the rejection of the model of zero gene flow at these 3 hearing genes when analyzed individually based on likelihood ratio tests. This result was consistent with our previous finding that Prestin appeared to

Figure 2. Statistical parsimony networks for each nuclear marker used in this study. Haplotypes representing lineages of R. p. chinensis and R. p. pearsoni are shaded orange and blue, respectively. Each circle represents a single haplotype and the area of circle size is scaled by haplotype frequency. The filled black circles represent missing or unsampled haplotypes. Haplotypes were coded as population identities (AH, JX, FJ, SC, HN, GX, GZ, YN, VN) as shown in Figure 1. The arrow in Kcnq4 network denotes a 63-bp deletion (1 mutational step) between R. p. chinensis and R. p. pearsoni.
show gene flow across the hybrid zone between 2 subspecies of the congeneric species \( R. \ affinis \) (Mao et al. 2014). More horseshoe bat taxa need to be studied to test the generality of this pattern.

Several scenarios can be considered to explain the pattern of increased rates of introgression observed in 3 of 4 hearing genes examined. First, it is possible that these 3 hearing genes in fact provide an adaptive advantage in a heterospecific background (Arnold 2006; Pardo-Diez et al. 2012; Hedrick 2013). Indeed in mice (\( M us \)), genes that function in olfaction are shown to be subject to adaptive introgression across a hybrid zone (Teeter et al. 2008). Our neutrality tests failed to support evidence of selection acting on genes examined here; nonetheless, it is known that strong adaptation can occur in the absence of detectable signatures of selection (e.g., \( M c I r \) gene in mice, Domingues et al. 2012) and therefore we cannot rule this out completely. If introgression of these hearing genes was beneficial, these genes might not be involved in echolocation call frequency. Otherwise, hybrids would be particularly selected against due to quite different call frequency between their parental taxa (\( R. \ pearsoni \) and \( R. \ chinensis \), see Mao et al. 2010). Alternatively, these hearing genes examined may be linked to loci that can cross the species boundaries due to positive selection. Ultimately, functional analysis on additional candidate hearing coding gene sequences from individuals of the 2 focal taxa would be needed to test more thoroughly for adaptive introgression associated with what is likely to be a complex phenotypic trait.

Third, the observed transfer of alleles across taxon boundaries may have arisen via stochastic processes (i.e., genetic drift), and it is often difficult to distinguish the roles of these 2 processes in introgression events (but see Payseur et al. 2004; Teeter et al. 2008; Fitzpatrick et al. 2009). This is especially likely to be the case if these hearing genes under study do not directly impact on echolocation call frequency per se, but rather function in other aspects of this complex trait.

Although not a focus of our study, the observed strong levels of both mitochondrial and nuclear differentiation between \( R. \ pearsoni \) individuals from Sichuan versus those from adjacent populations strongly point to the presence of a cryptic taxon. In addition, published differences in diploid chromosome number and chromosomal rearrangements between \( R. \ pearsoni \) from Sichuan (2N = 46, Wu et al. 2009) and ones from other regions (e.g., 2N = 44 in Guizhou, Mao et al. 2007) also support either different taxa or distinct chromosomal races. Such chromosomal rearrangements are well known to reduce gene flow and thus increase genetic differentiation, for example, by suppressing recombination (Ortiz-Barrientos et al. 2002; Navarro and Barton 2003).

In conclusion, parapatric taxa that undergo genetic exchange offer good opportunities to identify candidate loci that cross taxonomic barriers versus those that resist gene flow and thus might be related to reproductive isolation. By examining patterns of differential introgression among candidate loci, we revealed evidence of increased introgression from \( R. \ chinensis \) to \( R. \ pearsoni \) at 3 of 4 hearing genes and reduced introgression at 2 X-linked and 2 autosomal loci. However, we were unable to explicitly relate gene flow across species barriers to phenotypic differences in the relevant individuals. Although this study is one of the first to test for introgression of sensory genes among different taxa, our statistical power to find effects was limited by our low coverage of the genome. To address this issue, as well as known heterogeneity in genomic divergence (reviewed in Nosil et al. 2009), high-throughput sequencing approaches (e.g., whole-genome resequencing) offer promise for more thoroughly assessing genetic differentiation and introgression in these and other taxa (Twyford and Ennos 2012; Martin et al. 2013).

### Supplementary Material

Supplementary material can be found at [http://www.cz.oxfordjournals.org/](http://www.cz.oxfordjournals.org/)

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