Genetica (2011) 139:511–524
DOI 10.1007/s10709-011-9573-1

Population genetic structure and phylogeographical pattern of rice grasshopper, *Oxya hyla intricata*, across Southeast Asia

Tao Li · Min Zhang · Yanhua Qu · Zhumei Ren · Jianzhen Zhang · Yaping Guo · K. L. Heong · Bong Villareal · Yang Zhong · Enbo Ma

Received: 21 November 2010 / Accepted: 2 April 2011 / Published online: 20 April 2011

Abstract The rice grasshopper, *Oxya hyla intricata*, is a rice pest in Southeast Asia. In this study, population genetic diversity and structure of this *Oxya* species was examined using both DNA sequences and AFLP technology. The samples of 12 populations were collected from four Southeast Asian countries, among which 175 individuals were analysed using mitochondrial DNA cytochrome *c* oxidase subunit I (COI) sequences, and 232 individuals were examined using amplified fragment length polymorphisms (AFLP) to test whether the phylogeographical pattern and population genetics of this species are related to past geological events and/or climatic oscillations. No obvious trend of genetic diversity was found along a latitude/longitude gradient among different geographical groups. Phylogenetic analysis indicated three deep monophyletic clades that approximately correspond to three geographical regions separated by high mountains and a deep strait, and TCS analysis also revealed three disconnected networks, suggesting that spatial and temporal separations by vicariance, which were also supported by AMOVA as a source of the molecular variance presented among groups. Gene flow analysis showed that there had been frequent historical gene flow among local populations in different regions, but the networks exhibited no shared haplotype among populations. In conclusion, the past geological events and climatic fluctuations are the most important factor on the phylogeographical structure and genetic patterns of *O. hyla intricata* in Southeast Asia. Habitat, vegetation, and anthropogenic effect may also contribute to gene flow and introgression of this species. Moreover, temperature, abundant rainfall and a diversity of graminaceous species are beneficial for the migration of *O. hyla intricata*. High haplotype diversity, deep phylogenetic division, negative Fu's F_s values and unimodal and multimodal distribution shapes all suggest a complicated demographic expansion pattern of these *O. hyla intricata* populations, which might have been caused by climatic oscillations during glacial periods in the Quaternary.

Keywords AFLP · COI · *Oxya hyla intricata* · Phylogeography · Population structure · Rice grasshopper

T. Li · M. Zhang · Z. Ren · J. Zhang · Y. Guo · E. Ma (✉)
Institute of Applied Biology, Shanxi University, 030006 Taiyuan, Shanxi, China
e-mail: maenbo2005@126.com

Y. Qu
Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, 100101 Beijing, China

K. L. Heong · B. Villareal
Entomology, CESD, International Rice Research Institute, DAPO Box 7777, 1301 Metro Manila, Philippines

Y. Zhong
Institute of Biodiversity Science and Geobiology, Tibet University, 850000 Lhasa, China

Y. Zhong
School of Life Sciences, Fudan University, 200433 Shanghai, China

Introduction

Scientists have long explored the formation and differentiation of floras and faunas (Avise and Walker 1998; Kuchta and Tan 2005). In 1943, the hypothesis of isolation by distance (IBD) was predicted and indicated that genetic differentiation between populations increased with geographic distance (Wright 1943). However, the correlation

between genetic differentiation and geographic distance is not always simply linear, and it is probably influenced by many other factors (Gruenthal and Burton 2008; Fehlberg and Ranker 2009; Song et al. 2009; Jin and Liu 2010; Rebecca et al. 2010; Zhang et al. 2010). In the past few decades, glacial cycles have been considered as the most important factor shaping population genetic structure and promoting floral and faunal diversification (Zhang et al. 2005; Meng et al. 2007; Qu and Lei 2009; Arana et al. 2010; Borer et al. 2010; Arrigo et al. 2011). The Pleistocene glaciations had important effects on the patterns of spatial distribution and genetic structure of extant species (Avise and Walker 1998; Hofreiter et al. 2004). In particular, during the Quaternary, climatic changes led to recurrent retreats and advances in some species' distribution ranges, which caused different genetic structures and diversity among populations. It is likely that such repeated retreat and recolonization may have increased the rates of species diversification (Liu et al. 2006).

In contrast to Europe and North America, where the effects of recent glacial cycles on genetic diversity have been well studied (Lunt et al. 1998; Tregenza et al. 2000; Horn et al. 2009; Buckley et al. 2009), the genetic legacy of the Pleistocene remains poorly understood for Southeast Asia, where glaciation was not synchronous with the Northern Hemisphere ice sheet maxima (Li et al. 2009). With the exception of the Qiangtang-Tibetan plateau and parts of high montane areas, most of eastern Asia was not covered by ice cap and tundra. Therefore, Southeast Asia has been recognised for having species richness hotspots that would be expected to have provided stable habitats during the ice ages. It has been suggested that there are at least two refugia in China: one is the margin of the Qiangtang-Tibetan plateau (Qu and Lei 2009; Jin and Liu 2010) in northwest China, and the other is Hengduan mountain and its adjacent area (Wang et al. 2010) in southwest China. However, little research has focused on southeast Asia, and only a few studies have shown that geological events and climate changes in late Pleistocene had played a role in intraspecific divergence, bottlenecks and the demographic expansion of extant species (Huang et al. 2007; Zhang et al. 2008a, 2010; Song et al. 2009; Jin and Liu 2010). These studies are mostly related to vertebrates, while such investigations of insects have rarely been reported.

The rice grasshopper, *O. hyla intricata* Stál (Orthoptera: Catantopidae), is one of the most common and widespread grasshoppers in Asia. Its range stretches from northern China to Singapore, from western China to as far east as the Philippines and possibly beyond (Hollis 1971). As its name implies, the rice grasshopper usually feeds on several graminaceous species, especially on rice, and is one of the most important agricultural pests in this region. It is notable that *O. hyla intricata* is not one of highly mobile species such as *Locusta migratoria manilensis* (Zhang et al. 2009), but can fly farther than *Podisma pedestris* (Barton and Hewitt 1982). Therefore, gene flow between populations of different regions is likely to be low, and patterns of genetic differentiation will reflect historical patterns (Lunt et al. 1998).

In recent years, several studies on *O. hyla intricata* have been conducted, but most of these have focused on phylogenetic analysis (Ren et al. 2004) and molecular cytogenetic characterisation (Yoshimura et al. 2006). As a result, the phylogeographical pattern and genetic structure of *O. hyla intricata* remains largely unknown. Most phylogeographical studies have typically been based on haplotype data and occasionally on nuclear markers, such as AFLP, but they rarely combine both techniques. Using different markers with contrasting modes of inheritance and rates of evolution might provide a more accurate and comprehensive understanding this species' history (Flanders et al. 2009). To understand the phylogeographical pattern of *O. hyla intricata* populations characterised by different latitudes, habitats and population life histories, we used AFLP and mitochondrial DNA COI sequences to investigate the genetic diversity and structure of *O.hyla intricata*, and gain insight into the effects of glacial cycles and geological events in the population structure and evolutionary history of this species in southeast Asia.

Materials and methods

Samples

A total of 243 *O. hyla intricata* individuals were collected from 12 locations (Table 1) and three *Gesonula punctifrons* individuals were analysed as outgroups. Six populations were sampled in China, four populations were collected from the Philippines, and one population was collected from each of Malaysia and Singapore, respectively. Samples were preserved in absolute ethanol for genomic DNA extraction.

Genomic DNA was extracted from the femur samples following the method described by Dinesh et al. (1993). Muscle tissue was cut into small pieces and placed in the mixture of 40 µl 10% SDS and 360 µl TES buffer (10 mM Tris–HCl, pH 8.0; 50 mM EDTA, pH 8.0; 200 mM NaCl). After being digested with proteinase K at 55°C for 4 h and RNase A at 37°C for 2 h, DNA was extracted from the resulting solution with phenol and chloroform. Genomic DNA was precipitated with ethanol and dissolved in TE buffer. DNA concentrations were estimated and standardized using known concentrations of λDNA on 1.0% agarose gel. DNA concentration was brought to 50–150 ng/µl. Extractions were stored at −20°C.

Table 1 List of samples, nucleotide diversity, haplotype diversity and population genetics parameters for 12 *Oxya hyla intricata* populations

Locations	Group	Code	Elevation (m)	Latitude	Longitude	Number for COI	Number of haplotype	Haplotype diversity	Nucleotide diversity	Number for AFLP	*P* (%)	N_a	N_e	*H*	*I*
Beijing city	Group 1	BJ	49	39°54′N	116°23′E	10	5	0.800 ± 0.100	0.00263 ± 0.00043	10	25.62	1.2562 ± 0.4370	1.2021 ± 0.3630	0.1102 ± 0.1933	0.1582 ± 0.2748
Yunnan Tengchong	Group 1	TC	1630	25°03′N	98°31′E	15	7	0.781 ± 0.102	0.00187 ± 0.00039	21	52.83	1.5283 ± 0.4998	1.2521 ± 0.3592	0.1466 ± 0.1928	0.2233 ± 0.2725
Guangxi Laibin	Group 1	LB	74	23°38′N	109°15′E	15	5	0.705 ± 0.112	0.00172 ± 0.00050	22	27.21	1.2721 ± 0.4456	1.1954 ± 0.3391	0.1110 ± 0.1865	0.1619 ± 0.2692
Guangdong Gaozhou	Group 1	GZ	22	21°51′N	110°43′E	15	4	0.467 ± 0.148	0.00133 ± 0.00048	21	24.26	1.2426 ± 0.4292	1.1795 ± 0.3373	0.1002 ± 0.1832	0.1452 ± 0.2627
Hainan Wanning	Group 1	WN	128	18°44′N	110°24′E	15	7	0.657 ± 0.138	0.00204 ± 0.00056	20	26.30	1.2630 ± 0.4408	1.1930 ± 0.3460	0.1080 ± 0.1871	0.1569 ± 0.2684
Malaysia Kuala Lumpur	Group 1	ML	74	02°59′N	101°42′E	15	4	0.638 ± 0.093	0.00133 ± 0.00034	19	36.73	1.3673 ± 0.4826	1.1497 ± 0.2681	0.0950 ± 0.1534	0.1505 ± 0.2280
Singapore Punggol	Group 1	SIN	15	01°24′N	103°54′E	15	4	0.467 ± 0.148	0.00160 ± 0.00054	19	20.18	1.2018 ± 0.4018	1.1321 ± 0.2980	0.0747 ± 0.1610	0.1101 ± 0.2314
Tibet Zayu	Group 2	XZ	1530	32°05′N	97°29′E	15	3	0.362 ± 0.145	0.00059 ± 0.00025	20	44.22	1.4422 ± 0.4972	1.2355 ± 0.3437	0.1387 ± 0.1888	0.2096 ± 0.2718
Philippines Balaoan	Group 3	BAL	11	16°49′N	120°24′E	15	5	0.695 ± 0.109	0.00264 ± 0.00079	20	36.05	1.3605 ± 0.4807	1.1607 ± 0.2808	0.1002 ± 0.1602	0.1566 ± 0.2366
Philippines IRRI campus	Group 3	IRRI	10	14°10′N	121°15′E	15	5	0.562 ± 0.143	0.00113 ± 0.00034	20	35.37	1.3537 ± 0.4787	1.2082 ± 0.3349	0.1220 ± 0.1839	0.1829 ± 0.2663
Philippines Iloilo	Group 3	ILO	29	11°16′N	123°03′E	15	3	0.362 ± 0.145	0.00169 ± 0.00077	20	33.11	1.3311 ± 0.4711	1.1817 ± 0.3244	0.1058 ± 0.1767	0.1593 ± 0.2550
Philippines Davao	Group 3	DAV	104	06°47′N	125°11′E	15	4	0.467 ± 0.148	0.00139 ± 0.00052	20	29.93	1.2993 ± 0.4584	1.1055 ± 0.2321	0.0681 ± 0.1326	0.1105 ± 0.1987
Overall						175	56			232	91.61	1.9161 ± 0.2776	1.6335 ± 0.3198	0.3585 ± 0.1528	0.5250 ± 0.2037

P the percentages of polymorphic loci, N_a observed number of alleles, N_e effective number of alleles, *H* gene diversity, *I* Shannon's Information index

COI sequencing

A 643 base pair fragment of the mitochondrial DNA COI gene was amplified in a subset of 175 individuals (Table 1) using specific PCR primers for insects given in Folmer et al. (1994). Reactions were carried out in 50 µl volumes, including 3 µl template DNA, 1.25 U Pfu DNA polymerase (TIANGEN, China), 5 µl 10× PCR buffer, $MgCl_2$ at a final concentration of 4 mM, each dNTP at a final concentration of 4 µM, and 1.5 pmol of each primer. PCR cycling conditions included an initial 2 min 95°C denaturation, followed by 35 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s, plus a final extension at 72°C for 5 min.

The PCR products were purified using OMEGA PCR purification kit (OMEGA, USA) and then were sequenced in both directions on a 3730 semiautomated DNA sequencer (Applied BioSystems) using Perkin-Elmer Prism terminator cycle sequencing kits (Applied BioSystems) with AmpliTaq FS polymerase with BigDye terminators. The sequencing program consisted of 25 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s and extension at 60°C for 4 min. Sequences were compared visually to the original chromatograms to avoid reading errors.

AFLP analysis

To corroborate the phylogeographical information uncovered by mitochondrial data, we used genome-wide AFLP markers, which have been useful in uncovering recent genetic divergences in other arthropods (Rich et al. 2008).

The AFLP analysis was performed following the protocol described by Vos et al. (1995) with a few modifications: 400 ng genomic DNA was digested for 2.5 h with the restriction enzymes *Eco*RI and *Mse*I successively. The ligated DNA was diluted 1:10 with ddH_2O prior to pre-selective amplification. Pre-selective amplification was carried out using 50 ng of each primer in a total final volume of 30 µl with thermal cycling parameters of 30 cycles for 30 s at 94°C, 1 min at 56°C, 1 min at 72°C, and a final hold at 10°C.

Eight primer combinations with three additional selective bases were used for selective amplification. These primer combinations were *Eco*RI–ACA/*Mse*I–CTG, *Eco*RI–ACA/*Mse*I–CTC, *Eco*RI–AAG/*Mse*I–CTC, *Eco*RI–AAG/*Mse*I–CTG, *Eco*RI–ACA/*Mse*I–CTA, *Eco*RI–ACC/*Mse*I–CAC, *Eco*RI–ACT/*Mse*I–CAG, and *Eco*RI–ACT/*Mse*I–CAC. PCR was carried out using the labeled *Eco*RI primer at 0.05 µmol and *Mse*I primer at 0.1 µmol for 13 cycles of 30 s at 95°C, 30 s at 65°C less 0.7°C per cycle, and 1 min at 72°C; followed by 23 cycles of 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C, and a final hold at 10°C.

Samples of selective amplification were electrophoresed in 6% (w/v) denaturing polyacrylamide gel in 1× TBE buffer for 3.5 h at 100 W. The fragments in the gel were silver-stained (Bassam et al. 1991) with minor modifications. The approximate fragment length was estimated by comparison to PBR^{322} DNA/*Msp*I marker (SABC). Clear and unambiguous bands in length ranging from 100 to 600 bp were considered as usable, and the fragment data was transformed into a binary (1/0) data matrix.

Data analysis

Sequences were aligned using CLUSTAL X (Thompson et al. 1997). COI sequence diversity was investigated by comparing population estimates of mitochondrial haplotype diversity (h) and nucleotide diversity (p) using the program DNASP version 4.0 (Rozas et al. 2005). A maximum parsimony network was constructed using TCS 1.21 (Clement et al. 2000) with a 95% connection limit. Loops were resolved following the criteria of Pfenninger and Posada (2002). Neighbour-joining (NJ), Maximum likelihood (ML), and Maximum parsimony (MP) phylogenetic analyses were used to identify major clades and to evaluate the relationships among the haplotypes of the COI sequences. General time reversible + invariable sites + Gamma (GTR + I + G) were selected by the software MRMODELTEST v. 2.3 (Nylander 2004). NJ analyses were performed in MEGA 4.0 using a Kimura 2-parameter model with 1,000 bootstrapping replicates (Saitou and Nei 1987). ML analyses were performed using PHYML with a GTR + I + G model and 1,000 bootstrapping replicates (Guindon and Gascuel 2003). MP analyses were performed in PAUP* 4.10b (Swofford 2002) using a heuristic search with 1,000 random sequence repetitions and tree-bisection-reconnection (TBR) branch-swapping.

MDIV (Nielsen and Wakeley 2001) was used to estimate the divergence time and migration rate between populations. The program uses a Bayesian approach to estimate population divergence times and migration rates simultaneously between pairs of populations that are assumed to have diverged from a common ancestral population. MDIV was run for three times with different random seeds to obtain consistent distributions of results using the following settings: an Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985) with the transition/transversion ratio estimated directly from the data; Markov chain simulation for 10,000,000 steps, of which the first 1,000,000 were discarded as burn-in, and a maximum values of 10 for M and T. The divergence times of splits between phylogroup pairs were estimated using the formula $t_{\text{divergent time}} = T_{\text{pop}} \times (\text{Theta}/2\ \mu\text{k})$ and a generation time of 0.5 years, where T_{pop} is maximum posterior probability of divergence time; Theta is effective population

size; μ is mutation rate per nucleotide; and k is the number of nucleotides assayed. Since there is no direct calibration point for referring to mutation rate of *O. hyla intricata* or its relatives, an average mutation rate of 1.00×10^{-8} per site per year for the *O. hyla intricata* mitochondrial Cytb gene was used in this study according to a number of published literature (e.g. Brown et al. 1979; Fleischer et al. 1998; Song et al. 2009). The mutation rate of COI was then calculated by multiplying the ratio of average net distance for COI sequence *vs* that for Cytb sequence download from GenBank (AY615833–AY615844) using the software MEGA4 (Tamura et al. 2007). Time to most recent common ancestor (TMRCA) was also estimated with the software MDIV.

Mismatch distributions were calculated between the observed and expected mismatch distributions used as a test statistic; *P* values represented the probability of obtaining a simulated sum of squared deviation greater than or equal to the one observed. Values of Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) were calculated from the total number of segregating sites and used to assess the evidence for population expansion, under which negative values are expected (Aris-Brosou and Excoffier 1996). Estimation and testing were done by bootstrap resampling (10,000 replicates) using ARLEQUIN version 3.0.1 (Excoffier et al. 2006).

A coalescent-based Bayesian method was implemented in the program LAMARC 2.0 (Beerli and Felsenstein 1999, 2001; Kuhner 2006) to jointly estimate gene flow between populations. A positive or negative g indicates that the population grew or shrank exponentially, respectively. A GTR + I + C model, maximum-likelihood estimates of substitution rates (A/C: 2.56; A/G: 11.52; A/T: 6.18; C/G: 2.98; C/T: 14.60; and C/T: 1.00) and base frequencies (0.31, 0.18, 0.15, and 0.35 for A, C, G, and T, respectively) were used, as estimated by ModelTest (Posada and Crandall 1998). Default Bayesian priors were used for all parameters. Two independent runs of LAMARC were performed to check the consistency of estimates.

The percentage of polymorphic loci (*P*), observed number of alleles (N_a), effective number of alleles (N_e), gene diversity (H_E) (Nei 1973), Shannon's Information index (*I*), Nei's genetic distance (Nei 1978) were calculated (POPGENE 1.31; Yeh et al. 1999) to examine the population genetic diversity and differentiation of *O. hyla intricata*. POPGENE 1.31 was also used to detect gene flow among populations. Four populations, LB and GZ, were combined as a population (SC), and SIN and ML were combined as another (SM) in gene flow and STRUCTURE analyses. A neighbour-joining (NJ) tree (Saitou and Nei 1987) was generated based on the genetic distance (PHYLIP 3.67; Felsenstein 2004), and the clustering probabilities at each node were calculated by bootstrap resampling 1,000 times. The geographical subdivision among populations was estimated by a hierarchical analysis of molecular variance (AMOVA) in ARLEQUIN (version 3.01; Excoffier et al. 2006); significance of Φ_{ST} was evaluated by comparison to a null distribution of Φ_{ST} values under the hypothesis of no difference between populations. The grouping strategy was that twelve populations were separated into four groups according to obvious geographical isolation. The arrangement with the highest value of among group variation (FCT) in each grouping scheme was inferred as being the most probable geographical subdivision. A Mantel test (Mantel 1967) was performed (NTSYS-pc 2.02; Rohlf 1998) to investigate the correlation between the genetic distance and geographical distance of *O. hyla intricata* populations. Similarity coefficients were also subjected to principal coordinate analysis (PCO) using Matlab version 7.1 (Math Works, Inc.). An assignment test and population structure examination were conducted (STRUCTURE 2; Pritchard et al. 2000) according to the inferred population clusters using a Bayesian approach. Specifically, STRUCTURE placed individuals into *K* subgroups that had distinctive allele frequencies without a priori population information. *K* was chosen and varied from 2 to 13. The parameters were used as followed: correlated allele frequencies and an admixed origin of populations were assumed; burn-in and replication values were set at 50,000 and 300,000, respectively. Each test yielded a log-likelihood value of the data, and the output, Pr(*X*|*K*), can be used as an indication of the most likely number of groups according to Evanno et al. (2005).

Results

COI results

We obtained 643 bp sequences of the partial COI gene from 175 individuals. The COI sequences contained 113 variable sites, of which 98 were parsimony informative, generating 56 haplotypes.

The values of haplotype diversities were from 0.362 to 0.800, and nucleotide diversities were from 0.00059 to 0.00264. High nucleotide diversities were observed in BAL (0.00264) and BJ (0.00263). The lowest haplotype diversity and nucleotide diversity were observed in Singapore (0.362 and 0.00059) (Table 1).

Phylogenetic trees estimated by three methods (MP, ML and NJ) were basically compatible but with a small amount of variance in the relative positions and the bootstrap support values of some branches (Fig. 1). The trees were geographically structured, and haplotypes from neighbouring locations were mostly clustered into three groups,

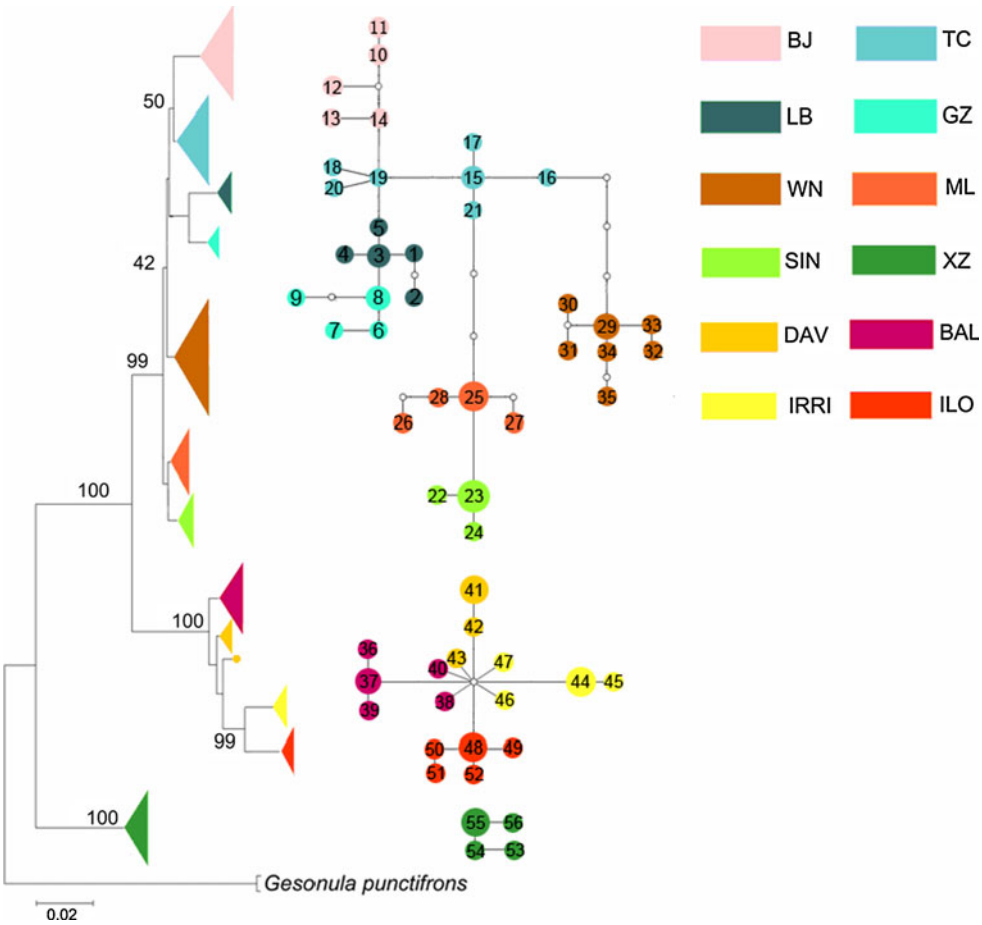

Fig. 1 Neighbor joining (*NJ*) tree and nested clade TCS networks based on COI. Nodal values above the *line* indicate bootstrap supports. *Colors* represent geographical groups and spatterworks stand for locations of sample sites. *Empty circle* indicate undetected intermediate haplotype states separated by one mutational step

Table 2 Analysis of molecular variance (AMOVA) of 12 *O. hyla intricata* populations based on COI sequence data

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	Φ-statistics	*P* value
Among groups	2	1,617.594	15.93850 Va	82.30	$\Phi_{CT} = 0.82302$	0.00000
Among populations within groups	9	383.480	2.90233 Vb	14.99	$\Phi_{SC} = 0.84678$	0.00000
Within populations	163	85.600	0.52515 Vc	2.71	$\Phi_{ST} = 0.97288$	0.00196
Total	174	2,086.674	19.36598			

Φ_{CT} the genetic variation among geographical groups, Φ_{SC} the genetic variation among populations within the geographical group, Φ_{ST} the genetic variation within populations across the entire study area

i.e. group 1 (including most China populations and Singapore and Malaysia populations), group 2 (Tibet), and group 3 (four Philippines populations). However, the TC population clustered with the BJ population first rather than other South China populations.

Of the 56 haplotypes generated by the combined dataset, 29 were singletons, and the other 27 haplotypes were shared between individuals within population. No haplotype was shared among the populations. Results of AMOVA suggested that the extant *O. hyla intricata* populations are highly geographically structured: 82.30% of genetic variation was attributed to the genetic differences among geographical groups ($P < 0.00001$) (Table 2).

Analysis of TCS yielded three unconnected haplotype networks, which were congruent with the topology described in the phylogenetic tree (Fig. 1). There are 43 mutation steps between group 1 and group 2, and 25 mutation steps between group 1 and group 3, when the network was constructed with a 100% connection limit. In the networks, haplotypes from the locations within the same geographical group first linked to each other and were then connected with haplotypes in the neighbouring group. All haplotypes from the Philippines formed network B. Network C included four haplotypes.

The results obtained from the LAMARC analysis (Fig. 2) showed that there was frequent historical gene flow

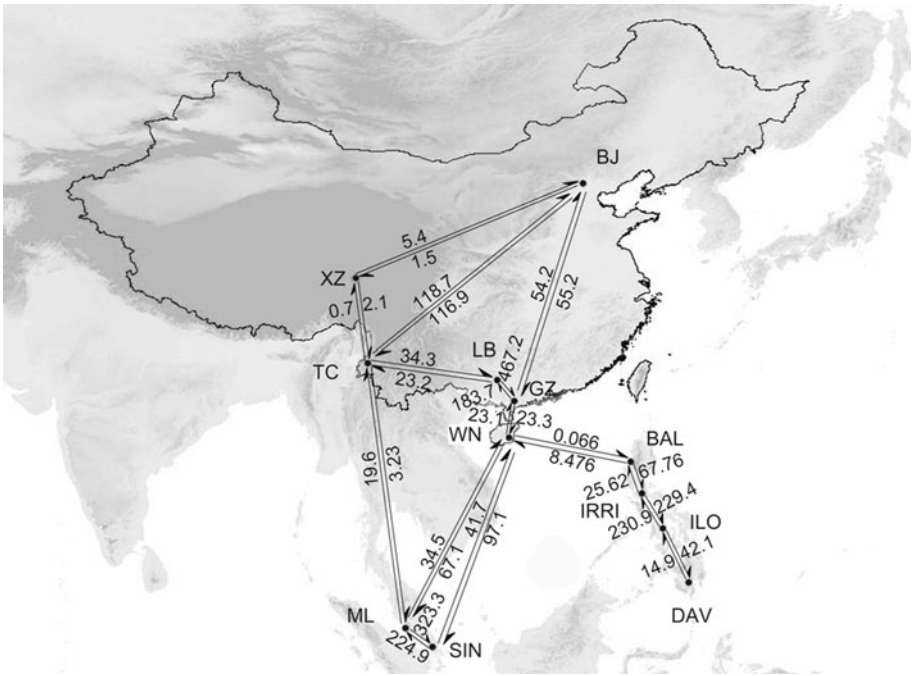

Fig. 2 Gene-flow networks between *O. hyla intricata* populations. Bayesian estimates of historical asymmetrical migration (*Nfem*: the product of effective population size and the proportion of immigrants within each population) between populations were labeled between neighboring populations

Table 3 Mismatch distribution analyses of *O. hyla intricata* based on COI sequences

	Tajima	*P*	Fu's	*P*
Group 1	−0.66453	0.27300	−14.90463	0.00100
Group 2	−0.21115	0.43400	−0.67430	0.19100
Group 3	0.18227	0.64600	−0.30188	0.48200

among local populations of *O. hyla intricata* in different regions. Relatively high *Nfem* values (greater than 180 for both directions) of some populations were found between populations LB and GZ, IRRI and ILO, SIN and ML. XZ had relatively small *Nfem* value for both directions with neighbouring populations. However, small *Nfem* value was also observed between the Philippines populations and mainland populations, e.g., the value of *Nfem* from SIN to ILO was 20.03.

A COI mutation rate of 2.77×10^{-8} was calculated and used to estimated the divergence time. This divergence time was dated to 2.41 Mya between XZ and other populations, while the divergence time between group 1 and group 3 was 2.31 Mya. TMRCA for all haplotypes of *O. hyla intricata* was estimated to 10.03 Mya. Tajima's *D* test showed negative values, except in group 3, and all of these values were not significant. Fu's F_s test showed negative values in all groups, and only group 1 had a significant *P* value indicating significant difference from expectation under neutrality (Table 3). The demographical dynamics of the three geographical groups were inferred from mismatch distributions. The results showed that the mismatch distributions in Group 2 fitted unimodal curves (Fig. 3).

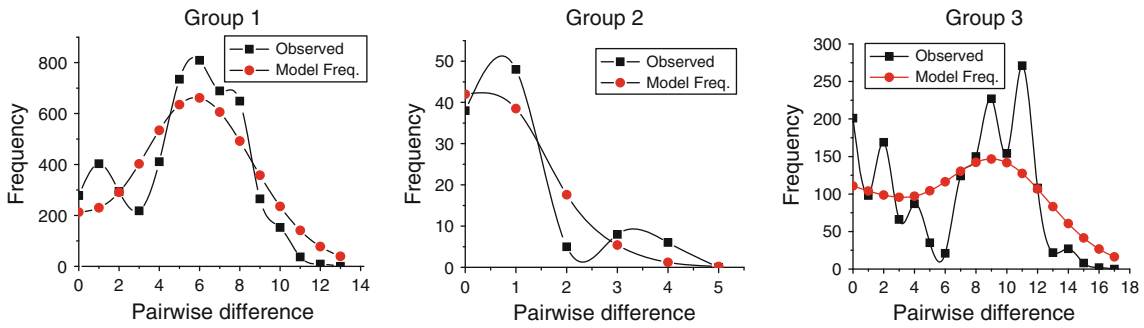

Fig. 3 Mismatch distributions for the regional groups of *O. hyla intricata*. These *curves* represent the frequency distribution of pairwise differences: observed (*black dots*), and model frequency (*red dots*)

Table 4 Population pairwise F_{ST} values (above diagonal) and genetic distance values (below diagonal) (Nei 1978)

Pop.	BJ	WN	LB	GZ	TC	XZ	SIN	ML	BAL	IRRI	ILO	DAV
BJ	****	0.5912	0.5781	0.6372	0.5846	0.6491	0.6633	0.5781	0.6112	0.5949	0.6152	0.6589
WN	0.3113	****	0.6146	0.6493	0.6118	0.6813	0.6997	0.6098	0.6718	0.6324	0.6515	0.6909
LB	0.3029	0.3271	****	0.5795	0.5860	0.6619	0.6916	0.6080	0.6602	0.6516	0.6704	0.7058
GZ	0.3762	0.3593	0.2644	****	0.6163	0.6791	0.6797	0.6122	0.6874	0.6627	0.6985	0.7405
TC	0.3476	0.3610	0.3291	0.3541	****	0.6129	0.6281	0.5658	0.6039	0.5679	0.5978	0.6488
XZ	0.3945	0.4214	0.3940	0.3966	0.3430	****	0.6938	0.6299	0.5657	0.5527	0.5830	0.6424
SIN	0.3724	0.4051	0.4043	0.3436	0.3258	0.3648	****	0.5121	0.6991	0.6631	0.6981	0.7473
ML	0.3467	0.3686	0.3787	0.3581	0.3406	0.3865	0.1953	****	0.6454	0.6212	0.6546	0.6880
BAL	0.3372	0.4169	0.4073	0.4348	0.3407	0.2306	0.3947	0.4366	****	0.3392	0.4571	0.4955
IRRI	0.3456	0.3779	0.4353	0.4258	0.3165	0.2423	0.3663	0.4262	0.0969	****	0.2819	0.3696
ILO	0.3441	0.3730	0.4311	0.4644	0.3305	0.2499	0.3924	0.4597	0.1485	0.0732	****	0.3680
DAV	0.3572	0.3778	0.4314	0.4858	0.3566	0.2724	0.4193	0.4601	0.1457	0.0939	0.0837	****

Table 5 Pairwise gene flow (N_m) of 10 *O. hyla intricata* populations based on AFLP data

	BJ	WN	SC	TC	XZ	SM	BAL	DAV	ILO	IRRI
BJ	****									
WN	0.4465	****								
SC	0.7944	0.7444	****							
TC	0.4899	0.4731	0.9182	****						
XZ	0.3235	0.3141	0.6672	0.4685	****					
SM	0.8309	0.7454	1.3861	0.9861	0.8313	****				
BAL	0.4036	0.3360	0.8169	0.5432	0.6756	0.9090	****			
DAV	0.4420	0.4394	0.8635	0.6303	0.7329	0.9982	1.1271	****		
ILO	0.4410	0.4218	0.7491	0.5610	0.6485	0.8589	0.6929	1.4629	****	
IRRI	0.3157	0.3223	0.6005	0.4356	0.4418	0.7239	0.5563	0.5563	1.0167	****

AFLP results

A total of 441 bands were identified using eight AFLP primer combinations from 232 *O. hyla intricata* individuals collected from 12 populations, among which 404 bands were polymorphic. The values of N_a were from 1.2018 to 1.5283, and values of N_e were from 1.1321 to 1.2521 (Table 1). The TC population had the highest genetic diversity ($P = 52.83\%$, $H_E = 0.1466$), while the SIN population had the lowest diversity ($P = 20.18\%$, $H_E = 0.0747$).

The F_{ST} values estimated between the populations ranged from 0.2819 to 0.7473 (Table 4). The highest F_{ST} value was 0.7473, between the DAV and SIN populations, and the lowest F_{ST} value was 0.4172, between the IRRI and ILO populations. Nei's genetic distances among twelve populations were from 0.0732 to 0.4858 (Table 4). The greatest genetic distance was 0.4858, between the GZ and DAV populations, and the lowest value was 0.0732, between the LB and GZ populations.

Gene flow analysis based on AFLP data was also performed, indicating a low level of gene flow ($N_m = 0.4337$) among the *O. hyla intricata* populations. In the pair-wise comparison (Table 5), most values of N_m between populations were <1, but few >1, e.g., that between SC and SM, BAL and DAV, DAV and ILO, ILO and IRRI.

The Mantel test detected no significant correlation between genetic distances and geographic distances among populations ($r = 0.31103$; $P = 0.9904$). Four Philippines populations formed a distinct clade with a high bootstrap value of 1,000 in the unrooted NJ tree (Fig. 4) due to their geographical isolation from the other mainland populations. SIN and ML clustered together later with most of the China populations. The XZ population was located in the middle of the phylogeny tree, which was different from trees based on COI sequence data. AMOVA analysis demonstrated a high level of genetic structure, with 16.56% ($P < 0.00001$) of variation apportioned among groups (Table 6).

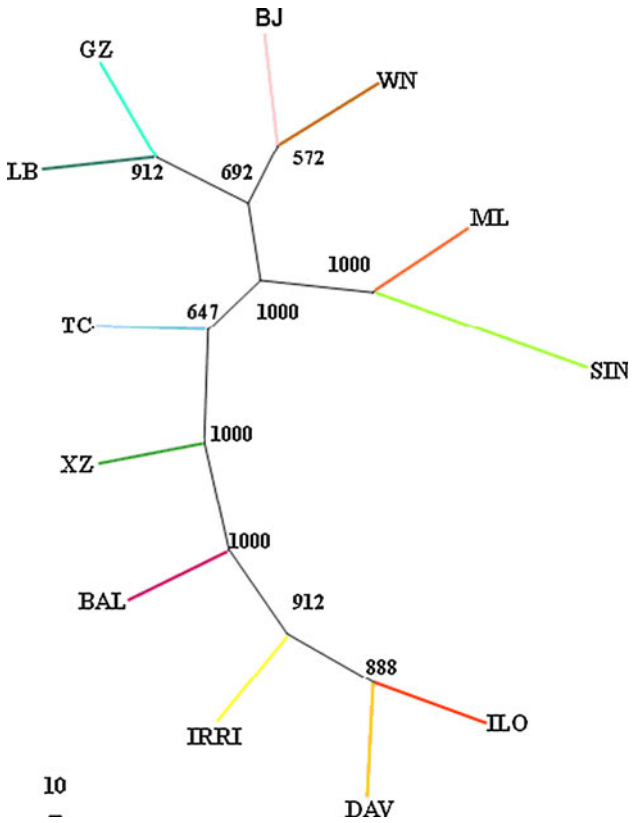

Fig. 4 NJ trees of Nei's genetic distances from AFLP of twelve *O. hyla intricata* populations, nodal values indicate bootstrap supports

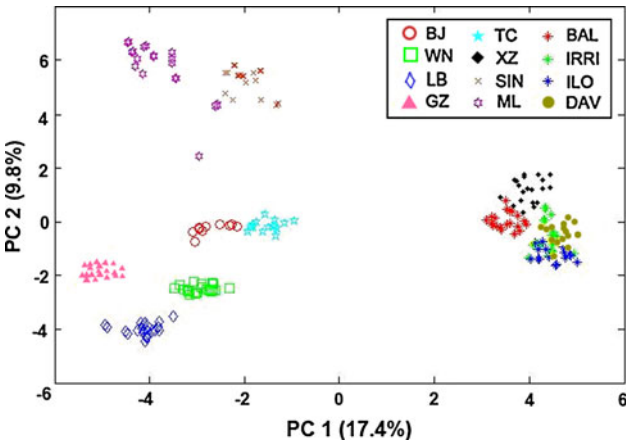

Fig. 5 Two-dimensional plot of the principal coordinate analysis based on the AFLP data set

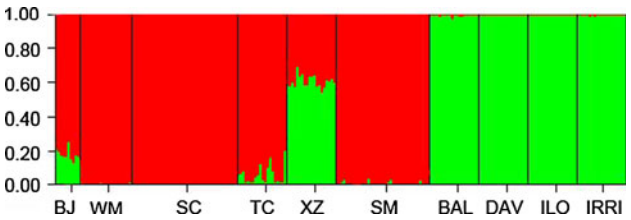

Fig. 6 Estimated genetic structure for $K = 2$ obtained with the structure program. Each individual is represented by a line

The PCO result showed that the first two axes explained 17.4 and 9.4% of the variation in the data, respectively (Fig. 5). The two-dimensional plot of the first two axes (26.8% total variation explained) showed that most sampled individuals of *O. hyla intricata* clustered together as their own populations, except for three individuals from ML population mixed into the SIN population. Three main groups were formed: XZ with four Philippines populations, the rest of China populations, and SIN and ML populations.

To test for possible gene introgression among different populations, we used a Bayesian population clustering approach implemented in the program STRUCTURE to infer the population admixture of the *O. hyla intricata* populations. We obtained a value of $K = 2$ with the support of Pr ($X|K$) results, indicating that all of the individuals from the mainland, except for XZ population, were included in one group, whereas all of the individuals from the Philippines were in another distinct group, with only a weak signal of introgression between the BJ and TC populations (Fig. 6). XZ population exhibited a strong signal of introgression from Philippines populations and mainland populations.

Discussion

Genetic diversity of *O. hyla intricata* based on COI sequence data and AFLP data

No obvious trend of genetic diversity was found along the lat/longitude gradient among geographical groups. From

Table 6 Analysis of molecular variance (AMOVA) of 12 *O. hyla intricata* populations based on AFLP data

Source of variation	*df*	Variance components	Percentage of variation (%)	Φ-statistics	*P* value
Among groups	2	15.64094	16.56	$\Phi_{CT} = 0.16556$	0.00000
Among populations within groups	9	51.15639	54.15	$\Phi_{SC} = 0.64895$	0.00000
Within populations	220	27.67335	29.29	$\Phi_{ST} = 0.70707$	0.00000
Total	231	100.80209			

Φ_{CT} the genetic variation among geographical groups, Φ_{SC} the genetic variation among populations within the geographical group, Φ_{ST} the genetic variation within populations across the entire study area

the combined AFLP and DNA data, the TC population was the population with relatively high diversity, with high levels of haplotype diversity, polymorphic loci and gene diversity, while SIN was observed as the population with the lowest diversity.

Tengchong is located in the Hengduan Mountains, which form a dramatic series of north–south trending valleys and ridges extending from 1,000 to over 6,000 meters in elevation (Peng et al. 2000), and it has been found to be a centre of active speciation and is identified as one of the global biodiversity "hotspots" (Myers et al. 2000). It is considered that this region is located among the southern temperate and tropical regions in China, which have been suggested to have acted as refugia for various animals and plants during Pleistocene glacial periods (Wu 1987; Wang and Liu 1994; Li et al. 2005; Long et al. 2006; Zhang et al. 2010). Due to deep valleys coupled with recently uplifted mountains in this region, a high level of diversity has appeared there. Zhang et al. (2008b) and Liu et al. (2009) studied the genetic diversity of six *Oxya japonica* populations and five *O. hyla intricata* populations across China, and found that one population of *O. japonica* and one population of *O. hyla intricata* (both collected from Hengduan Mountains) present the highest genetic diversity, respectively. We hypothesised that the complicated topology of the Hengduan Mountains might be the reason for the high genetic diversity of the TC population. During the long glacial episodes of the Quaternary, *O. hyla intricata* might have experienced frequent retreats or expansions along the altitudinal gradient of the mountains, and temperature changes in glacial and interglacial periods could have been the major factor leading to frequent gene exchanges among populations inhabiting different altitudes.

Many wild species have experienced population declines as a result of commercial overexploitation and habitat destruction (Pang et al. 2003; Hrbek et al. 2005), including *O. hyla intricata*. Singapore's high rate of urbanisation might be a cause of the low population diversity observed there. The samples of this population were collected below a small hill in the northeast of Singapore where there were a number of buildings, but only a small area of bush and weeds, and the natural environment appeared to have been destroyed. Therefore, with the dramatically decreased effective population size, the genetic differentiation in this population is gradually disappearing, which may explain the low genetic diversity in this region. In addition, the low genetic variability within SIN population than other populations may be caused by its biogeographic position, e.g., isolation by strait, and at the end of Malaysian peninsula with less connection to other populations (Walker et al. 2008; Marina et al. 2011).

Although Hainan Island is isolated from the mainland by the Qiongzhou Strait, its population diversity has not been decreased. On one side of the strait, Hainan Island, which is the sole tropical island in China and exhibited a tropical maritime climate, is warm and wet all year round. Coupled with the large area of rice cultivation on Hainan Island, the humid climate makes it especially suitable for *O. hyla intricata* survival.

There was no significant differentiation in the genetic diversity observed between the Philippines group and the mainland group. The COI sequences obtained indicated that mainland populations had a higher haplotype diversity and nucleotide diversity than those in the Philippines, while the AFLP data showed the opposite results. This phenomenon might be caused by using different molecular markers in the two analyses. In contrast to what was observed for Hainan Island, the Philippines exhibited obvious isolation due to the presence of its associated straits in both glacial and interglacial periods. The Philippines are composed of many islands connected to each other by the continental shelf. Rainfall, vegetation and farming on the larger islands are similar to those on Hainan Island, thus providing an appropriate environment for the survival of *O. hyla intricata*. Therefore, the effective population size in this group did not decrease due to the isolation of the islands.

Biogeography and population genetic structure

In the present study, isolation due to geographical barriers appeared to be the factor causing the current population genetic structure of *O. hyla intricata*. Although the position of the XZ population varied in the phylogeny trees, the topology from COI and AFLP results both revealed that 12 populations were separated into three distinct clades: a mainland group, the XZ population and a Philippines group. The deep genetic structure recovered within *O. hyla intricata* was consistent with geographical isolation.

A Mantel test detected no significant correlation between the determined genetic distances and geographic distances, indicating that the isolation by distance pattern might not be accepted absolutely. The phylogenetic results suggested that the observed population structure was related to geographical distance, and the F_{ST} value and genetic distance results also supported this hypothesis. Due to the short geographic distances between GZ and LB and between ILO and IRRI, the genetic distance between them was particularly low, and these pairs of populations were the first two to cluster in the phylogenetic tree, respectively. However, some other populations did not fit with the hypothesis described above. For example, if the population structure was formed only under isolation by distance scenario, the genetic distance between XZ and BJ would be smaller than those between BJ, SIN and ML; therefore, we suggest other factors may explain this genetic structure.

TCS yielded three unconnected haplotype networks, which were congruent with the topology described in the

phylogenetic trees, thus suggesting a long history of allopatric separation. The distinct pattern observed and the estimated divergence time suggested spatial and temporal separations coinciding with climatic and paleogeographic changes following the uplift of the Tibetan plateau from the late Miocene to mid Pliocene and isolation by the barrier of the Bashi Channel. This isolation might also be caused by the poor dispersal capability of *O. hyla intricata*, which likely impeded gene flow among populations with a distant range.

Therefore, some of the observed pattern might be related to limited gene exchange among geographical groups. Apart from anthropogenic influences, isolation by distance and/or geographic isolation could play an important role in the evolutionary history of this low vagility species (Kuchta and Tan 2005; Huang et al. 2007). Geographical barriers seem to have important effects on population divergences. It could be imagined that the complicated topology of this region played an important role in initiating phylogeographic differentiation and further sculpting preexisting phylogeographic variety during glacial oscillations.

The uplifted mountains following the collision of the Indian subcontinent and the mainland of Asia created climatic conditions that were complex and diverse, both attitudinally and latitudinally (Yang 1991; Zhao 1999), thus resulting in the particular genetic pattern of the XZ population. The XZ population was located in southwest China, and the special topography of its habitat in the southern Tibetan plateau is affected by warm air from the Indian Ocean. The southern Tibetan plateau has a higher temperature than other high altitude locations, which likely allowed the XZ population to survive. However, the complex terrain in the region created limited gene flow, which caused the isolation of the XZ population, resulting in the formation of its unique genetic structure, as it could only contact other populations through the southern migration route.

Due to the deep sea between the Philippines and China, no channel existed during glacial and interglacial periods to allow migration. After a long period of evolution, this isolation resulted in the unique population structure of the Philippines population, which exhibited long-term population diversity. In addition, four populations from the Philippines were collected from three islands; however, there is no deep sea between these islands, and the distance between them is short. Therefore, gene flow existed among these populations (Table 5; Fig. 2).

Since the genetic differentiation among populations could not be explained by the IBD model (Alvarez et al. 2007), we deduced that other two factors may contribute to gene flow and introgression in our investigated populations. First of all, as the well known pest of domesticated rice, these *Oxya* populations in managed habitats could be directly dispersed by humans when they are transported along with conveyances (Hafner et al. 2001; Alvarez et al. 2007; Castoe et al. 2007). This anthropogenic effect on dispersal might well explain some unexpected gene flow and genetic patterns. The WN population was separated from the mainland by the barrier of a strait, but the restricted gene flow detected among the WN population and others were significant. This might be explained by translocation via human introduction, such as accidental introduction via agricultural transportation. The *Nfem* value between SIN and ILO (20.06) may be also explained by this reason. Besides, as *O. hyla intricata* is an oligophagous insect restricted to relatively moist habitats, it can only migrate across limited distances (Ma et al. 2010), particularly within the complicated topology of South China, thus resulting in low levels of gene flow among populations separated by long geographic distances. The F_{ST} value and AMOVA showed that significant genetic differentiation was detected among these populations.

The star-like phylogeny of the Philippines populations might suggest the possible geographical origins of regional populations and the occurrence of short distance migrations. In the network, there was no shared haplotype between populations. This type of distribution might be interpreted as being the result of population isolation due to specific habitat requirements. These specific habitat requirements would limit the rate of dispersal and choice of migration routes of this species (Zhang et al. 2010). LAMARC results also showed that the TC population exhibited apparent gene flow, not only with each Chinese population, but also with the ML and SIN populations. In particular, for the LB and GZ populations, in southern China, and for SIN and ML, mild temperatures, abundant rainfall and a great number of graminaceous species have been beneficial for the migration of *O. hyla intricata*.

Evolutionary history of *O. hyla intricata*

Phylogenetic branching patterns could suggest the dispersal direction and the origin location of a population (Zhang et al. 2010), which has been validated successfully in studies of amphibians and reptiles with limited dispersal capabilities (Carranza et al. 2000; Fu et al. 2005; Huang et al. 2007; Zhang et al. 2010).

Our results showed that the mismatch distributions in the XZ group fitted unimodal curves and three singletons were connected to the central haplotype by only one mutational step, which might suggested that this population had passed through recent demographic expansions following a bottleneck. The STRUCTURE results also indicated that the XZ population exhibited a strong signal of introgression with the Philippines group and the mainland

group ($K = 2$) (Fig. 6). The unsymmetrical campanulate unimodal curve of the mismatch distributions and the leftwards shift of the peak also suggested that the XZ population was a young population with low genetic diversity that originated after the uplift of Tibetan plateau.

Any expansion event might be affiliated with past geological events and climatological changes (Jin and Liu 2010). Dating such expansions has always been a focus of phylogeographers, even though molecular clocks vary in their rates to some extent (Thorpe et al. 2005). Although most of China has never been covered by ice sheets, this area might have experienced cooler and drier climates within the last 15-million-year period (Axelrod et al. 1996). The tremendous climatic changes during this period might have influenced the distribution and evolution of many plants and animals in China and its neighbouring areas (Wang and Ge 2006). In the present study, a generally west to east dispersal trend of *O. hyla intricata* populations could be suggested by our phylogenetic tree and TCS analysis. It is proposed that *O. hyla intricata* might have colonised a wide area from southwest China to other warm, low altitude regions. We postulate that these dispersal events occurred in the most recent Pleistocene glacial period, when lower temperatures would have accommodated southward and westward dispersal and range expansion. It should be noted that the high haplotype diversity, deep phylogenetic division, negative Fu's F_s values and the unimodal and multimodal distribution shapes all indicate past demographic expansion of these *O. hyla intricata* populations. This might have been caused by climatic oscillations during glacial periods in the Quaternary.

Acknowledgments We would like to thank Dr. Wenjuan Wang and Dr. Liyan Zeng for assistance with data analysis. This project was supported by Funds for International Cooperation and Exchange of the National Natural Science Foundation of China (Grant No. 30810103907), Natural Science Foundation of China (31000974).

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