Mountain ridge and sea: Geographic-barrier effects on genetic diversity and differentiation of the Hong Kong newt (*Paramesotriton hongkongensis*) revealed by AFLP

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The Hong Kong newt (*Paramesotriton hongkongensis*) has a restricted distribution in Hong Kong and its adjacent coastal area in China. We employed amplified fragment length polymorphism (AFLP) DNA markers to examine the genetic diversity and differentiation of *P. hongkongensis* populations (101 individuals) from three streams. Two mainland populations are separated by Tai Mo Shan, the highest peak in Hong Kong. On Hong Kong Island, there is only one population. We investigated whether the marine barrier would affect the population genetic structure and diversification of Hong Kong newts more than an upland peak barrier. With seven selected primer combinations, 476 AFLP markers with 102 polymorphic loci indicated that newt populations exhibited considerable genetic differentiation. Our results indicate that the two mainland populations, separated by the mountain ridge, were genetically more distant from each other than from the island population separated by the sea barrier that was created about 6000 years ago. The terrestrial biogeographic barrier appears to be crucial in influencing genetic divergence of Hong Kong newt populations.

Introduction

Amphibian populations are in decline worldwide due to a complex of local, regional, and global causes (Lips *et al*. 2005, Storfer *et al*. 2009). Habitat fragmentation and loss are among the leading causes of amphibian population decline and extinction (Blaustein *et al*. 1994). The genetic consequences of habitat fragmentation are an important component of population extinction risk assessment for threatened and endangered species. Isolated small populations in fragmented habitats may undergo genetic drift and inbreeding, resulting in a decrease of allelic

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heterozygosity and loss of rare alleles, and this loss of genetic diversity will increase the extinction risk of small populations (Frankham 2005). Consequently, the estimation of genetic diversity is critical to management and conservation initiatives of concerned and threatened species and populations (Hughes *et al.* 2009).

The Hong Kong newt, *Paramesotriton hongkongensis* (Myers & Leviton 1962) is endemic to Hong Kong and the coastal Guangdong Province, China (Miller 2004), within the northern fringes of the Tropic of Cancer. As the only member of the largely non-tropical order Caudata (tailed amphibians) among the 23 amphibian species found in Hong Kong, *Paramesotriton hongkongensis* is one of the very few Old-World tropical tailed amphibians. Formerly, the Hong Kong newt was treated as a subspecies of *P. chinensis* (Fei 1999), but its rather distinct physical appearance (larger, and more robust) supports its classification into an independent species (Myers & Leviton 1962). Anthropogenic disturbances, such as habitat alteration, stream channelization, water pollution, and pet-trade collection led to the designation of *P. hongkongensis* as a protected species by the Agriculture, Fisheries and Conservation Department of the Hong Kong Special Administrative Region (SAR), China. The newt is categorized as globally Near Threatened on the IUCN Red List of endangered species (IUCN Red List 2010), and might be listed as Threatened in the near future. Thus, conservation and ecosystem management plans for maintaining existing populations of *P. hongkongensis* are needed to prevent further decline. However, knowledge about the ecology and population biology of *P. hongkongensis* is highly limited, because of no such studies of the genus *Paramesotriton* in Asia. Moreover, nothing is known about the genetic diversity and gene flow among *P. hongkongensis* populations inhabiting habitats with different landscape features, such as in a mainland or on islands.

Landscape genetics integrates specific landscape features with population genetics for understanding genetic processes, such as gene flow and genetic drift to shape genetic variation and genetic connectivity, which can provide insights into ecological and evolutionary processes (Manel *et al.* 2003). Landscape features, such as islands and mountains, can influence dispersal and gene flow among populations (Holderegger & Wagner 2008). Using molecular markers to identify special barriers to dispersal and gene flow, quantify genetic diversity, and locate genetic discontinuity patterns of populations between contemporary and historic landscapes is a key theme of landscape genetics, because of their high information content (Storfer *et al.* 2007). A number of molecular markers have been recently developed and their applications in conservation genetics have been remarkably productive and successful (DeSalle & Amato 2004). Amplified fragment length polymorphism (AFLP) DNA markers are a popular tool of genetic analysis for evolutionary genetics and conservation ecology because of its high information content, reliability, polymorphism, and wide genomic coverage (Vos *et al.* 1995, Bensch & Akesson 2005, Meudt & Clarke 2007, Storfer *et al.* 2009), in which AFLPs have been more frequently used in plants than animals (Storfer *et al.* 2010). Additionally, restriction fragments can be visualized by PCR without the knowledge of nucleotide sequence (Ajmone-Marsan *et al.* 1997, Mock *et al.* 2002). In this study, using AFLP markers, we evaluated genetic diversity and differentiation among three populations of *P. hongkongensis* in Hong Kong, two on the mainland and one on an island. Our main objective was to provide baseline information on genetic diversity and population genetic structure of *P. hongkongensis* for the conservation and protection of this species. We predicted that geographic barriers relating to mountain ridges and islands would play an important role in generating population genetic diversification. We speculated that the effects of a marine barrier between island and mainland populations on the population genetic structure of the Hong Kong newts is more important than the effects of an upland barrier between mainland populations.

Material and methods

Paramesotriton hongkongensis is restrictively distributed in several isolated locations on the mainland portion of Hong Kong SAR, as well as

Fig. 1. Locations of the three sampling streams in Hong Kong, China. PNS: Pak Ngau Shek Stream; TSS: Tai Shing Stream; and TTT: Tai Tam Tuk Stream. Tai Mo Shan is the highest mountain in Hong Kong (958 m a.s.l.).
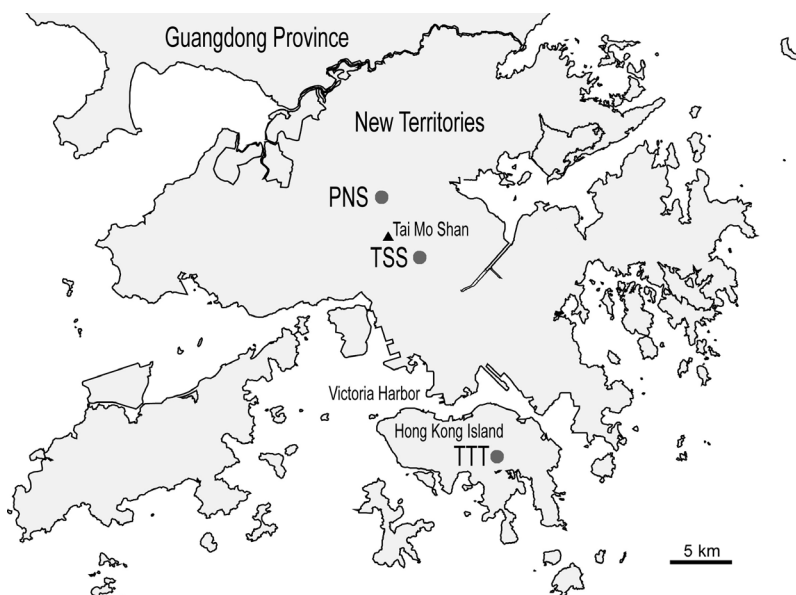
on Hong Kong Island (Karsen *et al.* 1998, Lau & Dudgeon 1999). The mainland New Territories and Hong Kong Island are separated by Victoria Harbor, part of the South China Sea (Fig. 1). The newts occur in pools of stony hillstreams with cobbles, boulders and riparian vegetation; they are predators that feed on larval invertebrates, shrimps, snails, fishes and tadpoles (Karsen *et al.* 1998, Dudgeon & Corlett 2004, Zhang, personal observation). The Hong Kong Newts were collected from breeding sites in streams on the mainland and Hong Kong Island. Pak Ngau Shek Stream (PNS, UTM grid reference: 50Q KK036844) and Tai Shing Stream (TSS, UTM: 50Q KK065796) were located on the mainland New Territories. These streams were separated by Tai Mo Shan, a rugged peak that is the highest point (958 m a.s.l.) in Hong Kong. TSS is on the southeastern slope of Tai Mo Shan, while PNS is on the northern slope. Although the Hong Kong newt occurs on both the east and the west sides of Tai Mo Shan, only one population was found on the east side of Tai Mo Shan, which was located in TSS. The samples of Hong Kong newts from PNS and TSS were collected at 80 m a.s.l. and 200 m a.s.l., respectively. Those two populations were not connected due to the rugged high peak of Tai Mo Shan. The only population of Hong Kong newts distributed on

Hong Kong Island was in the Tai Tam Tuk Stream (TTT, UTM: 50Q KK123638) at 210 m a.s.l. (Lau & Dudgeon 1999).

Sample collection

A total of 101 *P. hongkongensis* individuals were live captured using a hand net from the three study sites (34 in PNS, 30 in TSS, and 37 in TTT) in November 2005, which was the early phase of the breeding period when adults congregated in pools. The tail tip about 1 cm long was non-lethally clipped from each captured individual, and the newts were immediately released to the streams. Tail tissue samples were placed individually in 5.0 ml plastic sampling tubes stored in liquid nitrogen and then frozen at –80 °C until DNA extraction. Our capture and handling procedures were approved by the Agriculture, Fisheries and Conservation Department of Hong Kong Special Administration Region.

DNA extraction

Total genomic DNA was extracted from tail-tip tissue samples using the Aljanabi and Martinez (1997) method, modified in Wang *et al.* (2001).

The tissue samples were ground into powder in liquid nitrogen in a 1.5 ml centrifuge tube. 400 μl extraction buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 2% SDS [w/v], 20 μg ml^{-1} RNAse) and 20 μl proteinase-K (400 μg ml^{-1} final concentration) were added and tubes were incubated at 60–65 °C for 1 h whereupon 300 μl of 6 M NaCl was added to each sample. Samples were vortexed for 30 s at the maximum speed and centrifuged for 30 min at 10 000 g. DNA was precipitated with isopropanol, rinsed with 70% ethanol, dried in air, and then resuspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Five tubes without tissue samples were treated in the same way as described above in order to check sample contamination. No DNA bands appeared on the agarose gels for these control samples. DNA quality and concentration were evaluated on a 1% agarose gel in comparison with standard lambda DNA. The DNA samples were stored at –20 °C.

AFLP procedure

The AFLP analysis was carried out following the procedure described by Vos *et al.* (1995) and Ajmone-Marsan *et al.* (1997). Genomic DNA was digested with *Taq*I at 65 °C for 1 h and then with *Eco*RI at 37 °C for 1 h. Ligation was carried out at 37 °C for 4 h. Pre-amplification started with an initial 2-min extension step at 72 °C for the complete adapter ligation (Riberon *et al.* 2004). All PCR amplifications were performed using a GeneAmp® PCR System 9700 (Perkin Elmer Corp., Norwalk, CT, USA). PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel at 90 W for 2.5 h using the SequiGen 38 × 50 cm gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA). AFLP patterns were then visualized by silver staining (Merril *et al.* 1981, Bassam *et al.* 1991). In order to reduce the effect of erroneous genotypes on the analysis of molecular data sets through quality control (Bonin *et al.* 2004, Pompanon *et al.* 2005), 25 samples were randomly chosen for each of 16 candidate primer combinations to be amplified for three times following the above protocol. Among the 16 candidate primer com-

binations, 7 primer combinations showed stable fingerprintings among 3 replicates. We selected these 7 primer combinations with consistent DNA fingerprinting for further analysis of all the 101 samples of Hong Kong Newts.

Data analysis

The AFLP markers were manually scored as present (1) or absent (0) in each newt sample and each primer combination. The binary matrix of polymorphic bands was analyzed using the program POPGENE ver. 1.32 (Yeh *et al.* 1997) to calculate the observed number of alleles (A_o), effective number of alleles (A_e), Nei's (1973) gene diversity (H), Shannon's information index (I), total genetic diversity (H_{tot}), genetic diversity within populations (H_S), the coefficient of genetic differentiation among populations [$G_{ST} = (H_{tot} - H_S)/H_{tot}$], and gene flow [$N_m = 0.25(1 - G_{ST})/G_{ST}$] (McDermott & Mcdonald 1993). We carried out an analysis of molecular variance (AMOVA) to partition total genetic variability into the within- and between-population components and calculated Nei's genetic distances between populations, using program GENALEX 6.1 (Peakall & Smouse 2006). AMOVA used the Φ_{PT} to measure the relative genetic variance among the three populations (TSS, PNS, and TTT) relative to the total genetic variance and used the permutation of 1000 times to test for the significance of Φ_{PT} ($p < 0.05$; Peakall & Smouse 2006).

Results

Genetic variation

The seven selected primer combinations produced a total of 476 clear fragments/markers for the entire 101 samples. Fragment sizes ranged from approximately 80 to 500 bp. The number of markers varied from 45 (E-GAG/T-GAA) to 78 (E-AAG/T-ATC or E-AAG/T-AAA) and averaged 68 markers per primer combination (Table 1). Of the total 476 scored markers, 102 (21.42%) were polymorphic. Correspondingly,

at the species level, the observed number of alleles was 2.0, the effective number of alleles was 1.24, Nei's gene diversity was 0.16, and Shannon's information index was 0.27 for *P. hongkongensis* (Table 2).

Within-population genetic variation

The percentages of polymorphic loci (P) in individual populations varied from 5.9% to 17.4% and averaged 12.4%. The observed number of alleles (A_o) ranged from 1.28 to 1.92 averaging 1.62, and the effective number of alleles (A_e) ranged from 1.09 to 1.25 and averaged 1.24. Nei's gene diversity (H) averaged 0.16 and ranged from 0.06 to 0.17, whereas Shannon's information index was 0.27 and ranged from 0.1 to 0.29. The mainland PNS population had the highest level of genetic diversity whereas the mainland TSS population had the lowest variability (Table 2).

Population genetic structure

The total genetic diversity (H_{tot}) was 0.16 ± 0.03 (mean ± SD), and within-population genetic diversity (H_S) was 0.12 ± 0.01. Moreover, the coefficient of genetic differentiation (G_{ST}) was 0.26, indicating that about 26% of the total genetic variability could be explained by genetic differences between populations, whereas 74% was explained by genetic differences among individuals. Between-population variation was significant (AMOVA: $p = 0.001$) and explained 32% of total genetic variability, whereas within-population variation explained 68%. The genetic distance between the two mainland populations (PNS and TSS) was twice as long as the distance between either PNS or TSS and the Hong Kong Island population (TTT). The average level of gene flow (N_m) was estimated to be 1.45 effective migrants per generation among the three *P. hongkongensis* populations. Gene flow between the

Table 1. The number of bands generated by seven primer combinations of amplified fragment length polymorphism (AFLP) markers.

Primer combination	Marker size	Number of markers	Number of polymorphic markers	Percentage of polymorphic markers
E-GAG/T-GAA	100–400 bp	45	9	20
E-AAG/T-ATC	115–410 bp	78	10	13
E-AAT/T-GAA	84–350 bp	60	9	15
E-AAG/T-ATT	100–480 bp	75	20	27
E-GAA/T-ATT	100–500 bp	72	29	40
E-AAG/T-AAA	95–400 bp	78	13	17
E-AAG/T-CTC	80–420 bp	68	12	18
Total		476	102	

Table 2. Genetic variability parameters of the Hong Kong newt populations at three streams (Pak Ngau Shek Stream, Tai Shing Stream, and Tai Tam Tuk Stream) based on amplified fragment length polymorphism (AFLP) markers. A_o = observed number of alleles; A_e = effective number of alleles; H = Nei's gene diversity; I = Shannon's information index.

Population	Sample size	Percentage of polymorphic loci	A_o (mean ± SD)	A_e (mean ± SD)	H (mean ± SD)	I (mean ± SD)
Pak Ngau Shek	34	17.44	1.92 ± 0.27	1.25 ± 0.27	0.17 ± 0.14	0.29 ± 0.19
Tai Tam Tuk	37	13.87	1.65 ± 0.48	1.18 ± 0.27	0.12 ± 0.15	0.20 ± 0.21
Tai Shing	30	5.88	1.28 ± 0.45	1.09 ± 0.20	0.06 ± 0.12	0.10 ± 0.18
Mean		12.40	1.62	1.24	0.16	0.27
Total	101	21.43	1.99 ± 0.09	1.24 ± 0.31	0.16 ± 0.16	0.27 ± 0.21

PNS and TSS populations was 3.87, 1.6 between the PNS and TTT populations, and 1.26 between the TSS and TTT populations.

Discussion

This study was the first to examine the effect of landscape barriers on the spatial population genetic variation of *P. hongkongensis*, indeed of any Asian salamander. The current genetic distance between the two mainland populations (PNS and TSS) was twice that between either mainland population and newts on Hong Kong island (TTT), which suggests that — contrary to our expectations — the isolation effect of the highest mountain ridge in Hong Kong was significantly stronger than the effects of a salt-water barrier, which was created when sea levels rose 6000 years ago around Hong Kong, and isolated newt populations on the island (Dudgeon & Corlett, 2004). Evidently, isolation by the mountain ridge may exceed those brought about by 6000 years of separation by an impenetrable marine barrier. Similar isolating effects of mountain ridges on gene flow have been shown in other amphibian species (Lougheed *et al.* 1999, Tallmon *et al.* 2000). The TSS and PNS streams were located on the southern and northern slope of Tai Mo Shan, and although distance between these two newt populations was shorter than that between either of the mainland populations and that on Hong Kong Island (Fig. 1), gene flow (N_m) between the two mainland newt populations was less than half of that between either mainland population and the Hong Kong Island population. Genetic differences between the two newt populations (PNS and TSS) in the New Territories were greater than that between a newt population (PNS or TSS) in the New Territories and the Hong Kong Island newt population

(TTT) (Table 3). Since it is unlikely for migration to have occurred across a marine barrier during the past 6000 years, the Hong Kong newt populations between the mainland and the Hong Kong Island must have remained isolated. This implies one of two things: either genetic divergence between the two mainland populations has proceeded more rapidly than between island and mainland populations or, alternatively, that divergence between the two mainland populations had occurred prior to the isolation of island from mainland populations.

The mainland PNS population, which was at the most lowland site, had higher genetic diversity than the other two populations (PNS > TTT > TSS) and this difference appeared to be negatively related to a low-high elevation gradient. We speculate that the genetic diversity among the Hong Kong newt populations might be negatively correlated with elevation, because effective population sizes at high elevation sites are smaller than those at low elevation (author's pers. obs.); and the landscape barrier effect on gene flow can be influential for high elevation populations. Funk *et al.* (2005) found that mountain ridges and elevational differences (from 733–2652 m a.s.l.) were associated with increased genetic differentiation among sites for the Columbia spotted frog (*Rana luteiventris*). Since frogs in low-elevation ponds had high genetic variation within populations, it was considered that large low-elevation populations are important sources of immigrants and genetic variation for small populations at high elevations. Because topographic relief significantly limits gene flow, extinction of low-elevation populations may have the unexpected impact of reducing the persistence of mountain populations (Funk *et al.* 2005). Such altitudinal gradients can shape the spatial distribution of genetic variation of a species. Giordano *et al.* (2007) showed a significant negative correlation between genetic diversity and altitude in the long-toed salamander (*Ambystoma macrodactulym*), which parallels the pattern seen in *P. hongkongensis*. They considered high-altitude uplands to be landscape barriers that restricted gene flow among high-altitude populations relative to low-altitude populations, thus reducing genetic variability among high-altitude populations.

Table 3. Nei's genetic distance between the Hong Kong newt populations at Pak Ngau Shek Stream, Tai Shing Stream, and Tai Tam Tuk Stream.

	Pak Ngau Shek	Tai Tam Tuk
Tai Tam Tuk	0.044	–
Tai Shing	0.085	0.08

The population-specific proportion of polymorphic loci in *P. hongkongensis* appears relatively low (5.9%–17.4%) in comparison with other animals, such as some warblers with a 25%–45% AFLP-marker polymorphism range (Bensch *et al*. 2002) and pigs (*Sus scrofa*) with a 26.4%–59.8% range (Kim *et al*. 2002). Riberon *et al*. (2004) postulated that genetic polymorphism of amphibians is low in general, but this is certainly not the case for all amphibians. For instance, the proportion of polymorphic AFLP loci was 54.8% in the microhylid frog *Gastrophryne carolinensis*, which ranges widely in the south of the United States, although population genetic differentiation was low (Makowsky *et al*. 2009). Curtis and Taylor (2004) also showed that the percentage of polymorphic AFLP markers exceeded 50% in coastal giant salamanders (*Dicamptodon tenebrosus*), a threatened but widely distributed species in the Pacific Northwest. Our data suggest that genetic polymorphism may be relatively low in endemic salamanders, especially those with restricted distribution ranges, which may be related to a low evolutionary rate in the salamander genome (Wake *et al*. 1983).

Although genetic polymorphism appeared low, *P. hongkongensis* populations exhibited considerable genetic differentiation with a coefficient of differentiation (G_{ST}) of 0.26, exceeding the value of 0.25 that suggests a high degree of inter-population genetic differentiation (Freeland 2005). AFLP analysis is a powerful tool to assess intra-population genetic diversity and population genetic structure of threatened species (Holderegger & Wagner 2008), however, few studies have used AFLP markers to evaluate intra- and inter-population genetic variation of salamanders (Makowsky *et al*. 2009). Riberon *et al*. (2004) used AFLP to reveal genetic variation between and within populations in two subspecies of *Salamandra atra* in Europe, although, in that case, low genetic differentiation was ascribed to small sample sizes (1–5). Our results indicate that AFLP markers can certainly detect population genetic structure and differentiation with a sample size of 30–40 individuals. Milá *et al*. (2010) recently reported a study on the Pyrenean brook newt (*Calotriton asper*) with extreme dispersal limitation across four drainages in the French Pyrenean, revealing marked population genetic structuring by genome-wide AFLP but not by mtDNA. In this only other AFLP study on newts, a high level of variation in 382 AFLP loci revealed isolation by distance effect, which was consistent with long-term restriction of gene flow among populations of *C. asper*. Milá *et al*. (2010) concluded that severely underestimated population divergence by using only mtDNA sequence data can lead to erroneous inferences on the true pattern of gene flow of *C. asper*.

Our results suggest that isolation due to geographic landscape barrier or habitat fragmentation, especially in a mountainous region, can result in substantial genetic differentiation between small populations, according to findings by Gibbs (1998). Elsewhere, Curtis and Taylor (2004) showed that forest clear-cutting in British Columbia can contribute to genetic differentiation of Giant salamander populations in clear-cut and forested sites due to smaller population sizes and genetic drift in clear-cut sites.

Our findings on physical barrier effects of an upland ridge and a marine barrier on the genetic divergence of the Hong Kong newt have implications for the conservation and management of amphibians. Given the urgency in the global decline of amphibian diversity (Rovito *et al*. 2009) and the threat of climate warming on fragmented populations (Sinervo *et al*. 2010), it is crucial to identify threatened and endangered amphibian species populations with low genetic diversity, restricted gene flow and small population sizes for future protection, because the combination of these three limiting factors can cause even higher vulnerability in geographically restricted endemic amphibian species. To better understand divergence among newt populations and determine those that warrant priority for protection (Bonin *et al*. 2007), further phylogeographic investigation of the genetic linkages of *P. hongkongensis* populations using mitochondrial DNA markers is needed.

Acknowledgments

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