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Genetic differentiation among populations of *Brachytrupes portentosus* (Lichtenstein 1796) (Orthoptera: Gryllidae) in Thailand and the Lao PDR: the Mekong River as a biogeographic barrier

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Abstract

The Mekong River is known to act as a boundary between a number of terrestrial and freshwater species, including various parasites and their intermediate hosts as well as endangered mammal species. Little information is available, however, on the genetic differentiation between terrestrial invertebrates to the east and the west of this wide river. The genetic diversity among eight natural populations of *Brachytrupes portentosus* (Lichtenstein, 1796) (Orthoptera: Gryllidae) collected from Thailand and the Lao People's Democratic Republic (PDR) were analyzed by multilocus enzyme electrophoresis. The allelic profiles of 20 enzymes encoding 23 loci were analyzed. An average of 41% fixed differences was detected between the populations from Thailand and Lao PDR, which are separated by the Mekong River. The percent fixed differences ranged between 4% and 26% within the populations from Thailand and between 4% and 22% within the populations from Lao PDR. A phenogram shows that the eight populations fell into two major clusters based on the Thai and Lao sampling sites. The genetic distance between the samples within Thailand and within Lao PDR was related to the distances between sampling areas. The genetic variability between populations of this cricket indicates that genetic relationships are influenced by a natural barrier as well as by the geographical distance between these allopatric populations.

Keywords: *Brachytrupes portentosus*, genetic differentiation, multilocus enzyme electrophoresis, Mekong River, natural barrier

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Introduction

The biogeography of Southeast Asia is in part defined by the major river systems which run through the area (Meijaard & Groves, 2006). Thus, species distributions of both freshwater as well as terrestrial animals often end on the banks of the major rivers such as the Mekong or, in the case of some bird species, are confined to the deep river valleys cut by the Brahmaputra, Salween, Mekong and Yangtze (Johansson *et al.*, 2007). Both invertebrate species, such as the intermediate snail hosts of the small liver fluke *Opisthorchis viverrini sensu lato* and *O. viverrini sensu stricto* (Saijuntha *et al.*, 2007; Laoprom *et al.*, 2009), and the intermediate hosts of *Schistosoma mekongi* and *S. ovuncatum* (Attwood *et al.*, 2002, 2008) differ genetically between Thai populations to the west of the Mekong and those from Laos to the east. The Mekong also acts as a dividing line between various mammal species, such as primates (Long *et al.*, 1994; Groves, 2007; Roos *et al.*, 2008), with species richness being appreciably higher to the east (Meijaard & Groves, 2006). These findings are consistent with the riverine barrier hypothesis, which states that rivers and their flood plains act as barriers to gene flow between populations (Haffer, 1997; Jalil *et al.*, 2008). Relatively little information is available from the Mekong Subregion, however, for terrestrial invertebrates.

Brachytrupes portentosus (Lichtenstein, 1796), the large brown or big-headed field cricket, belongs to the order Orthoptera, family Gryllidae (there are several synonyms, e.g. *Tarbinskiellus portentosus* (Lichtenstein, 1796) or *Acheta portentosa* Lichtenstein, 1796). This species spends most of its time up to 30 cm underground with a single specimen per burrow. It feeds on young plants and in some areas can be gathered in thousands at sundown (DeFoliart, 2002). It is widely distributed in Southeast Asia where it is important to humans both as a food source (Bristowe, 1932; DeFoliart, 2002), as well as a pest of various agricultural crops, vegetables, lawns and ornamental plants (Chatterjee, 1965; Atim *et al.*, 1992). As a food source, this cricket has a higher economic value than other crickets in Southeast Asia, especially in Thailand, with the price of a hundred live giant crickets being about $US1.5–4.5 (50–150 Thai baht) and, after being cooked (normally by deep frying), up to $US4.5–6.0 (150–200 Thai baht) (Kittibanpacha, unpublished data). It is a valuable protein source, with 100 g dry weight providing 12.8 g protein and energy worth about 113 kcal (Viwatpanich, unpublished data). This is of particular importance as sources of animal protein in this area are limited, with freshwater fish supplying by far most of the requirements for the local population (Sverdrup-Jensen, 2002).

Previous studies have shown a relatively high genetic variation in other species of field cricket, with genetic differentiation among their populations depending mostly on geographic features, especially among geographical populations of North American field crickets of the genus *Gryllus*, Hawaiian crickets of the genus *Laupala* and the Jerusalem cricket of the genus *Stenopelmatus* endemic to southern California (Huang *et al.*, 2000; Parsons & Shaw, 2001; Gray *et al.*, 2006; Vandergast *et al.*, 2007). Moreover, within the singing Orthoptera, many closely related, reproductively isolated species are divergent in ecological and sexual traits yet exhibit little or no morphological divergence (Braswell *et al.*, 2006). Such cryptic species pose particular problems for taxonomy. Speciation events can be associated with reduced gene flow caused by physical barriers to migration, along with limited vagility and natal philopatry (Buston *et al.*, 2007). There is usually a geographic range within which individuals are more closely related to one another than to those randomly selected from the general population, leading to genetic structuring (Repaci *et al.*, 2007). The populations of many ground crickets are structured in this way, e.g. sequence divergence in mitochondrial genes between different geographical populations of field crickets, *Gryllus* spp., sampled from areas of both allopatry and sympatry. The pattern of cytochrome oxidase 1 (CO1) sequence divergence and genetic variation is consistent with allopatric or peripatric speciation in the southeastern and south-central American field cricket (Gray *et al.*, 2006). In addition, the divergence of cytochrome *b* sequence was reported among North American field cricket populations (Huang *et al.*, 2000).

Many molecular markers have been used to address the genetic diversity/variation of morphologically or geographically cryptic species. These include allozyme electrophoresis, amplified fragment length polymorphism (AFLPs), restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), mitochondrial DNA (mtDNA) sequence variation and microsatellite markers (e.g. Parsons & Shaw, 2001; Dawson *et al.*, 2003; Bretman *et al.*, 2008). Allozyme surveys are straightforward and inexpensive and are capable of detecting variation among geographical and/or morphologically distinct populations (Loxdale & Lushai, 1998).

In this study, the genetic variation and geographical relationships among large field cricket populations from Thailand and the Lao People's Democratic Republic (PDR), separated by the Mekong River (on average, about 1 km wide), were investigated by multilocus enzyme electrophoresis (MEE). Three additional ground cricket species, *Acheta confirmata* Walker, *Gryllus bimaculatus* de Geer and *Gryllus testaceus* Walker, were compared as out-groups.

Materials and methods

Sample collection

Two hundred ninety-six adult large brown crickets were collected from five areas in Thailand and three in the Lao PDR (table 1, fig. 1). The specimens were collected by digging them up in grass fields, rice paddies, forests or by buying them at local markets. All crickets came from natural habitats. The other crickets tested, *A. confirmata*, *G. bimaculatus* and *G. testaceus*, were caught in houses in the Maung district, Maha Sarakham Province of Thailand. Species were identified morphologically as described in Triplehorn & Johnson (2005). The crickets were transported back to the laboratory alive and, after killing by freezing, the hind legs were removed and immediately stored deep frozen at −80°C. Enzyme homogenates were individually prepared from the muscle of a left hind leg. Femoral muscle was manually ground with an equal volume of lysing solution (100 ml distilled water, 100 µl β-mercaptoethanol, 10 mg NADP) using a glass rod and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatants were stored in capillary tubes as 5 µl aliquots at −20°C until used.

Multilocus enzyme electrophoresis (MEE)

Multilocus enzyme electrophoresis (MEE) was performed by using cellulose acetate (Cellogel, Milan, Italy) as the

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Table 1. Geographical locations of *B. portentosus* collection from Thailand and Lao PDR.

Code	*N**	District	Province	Country	Source of collection	Latitude, longitude
MSm	35	Muang	Maha Sarakham	Thailand	Local market	16°11′3″ N, 103°17′24″ E
MSg	42	Gudrang	Maha Sarakham	Thailand	Rice paddy	16°05′38″ N, 103°00′38″ E
MSn	38	Nadun	Maha Sarakham	Thailand	Grass field	15°43′03″ N, 103°13′48″ E
UBk	40	Khemarat	Ubon Ratchathani	Thailand	Rice paddy and forest	16°02′21″ N, 105°13′15″ E
UBm	30	Muang Sam Sip	Ubon Ratchathani	Thailand	Local market	15°30′50″ N, 104°43′29″ E
SVg	40	Kaison Phomvihan	Savannakhet	Lao PDR	Local market	16°34′13″ N, 104°46′00″ E
SVs	37	Songkorn	Savannakhet	Lao PDR	Grass field and forest	16°15′28″ N, 105°17′58″ E
CSp	34	Pakse	Champasak	Lao PDR	Local market	15°06′58″ N, 105°48′08″ E

* Sample size.

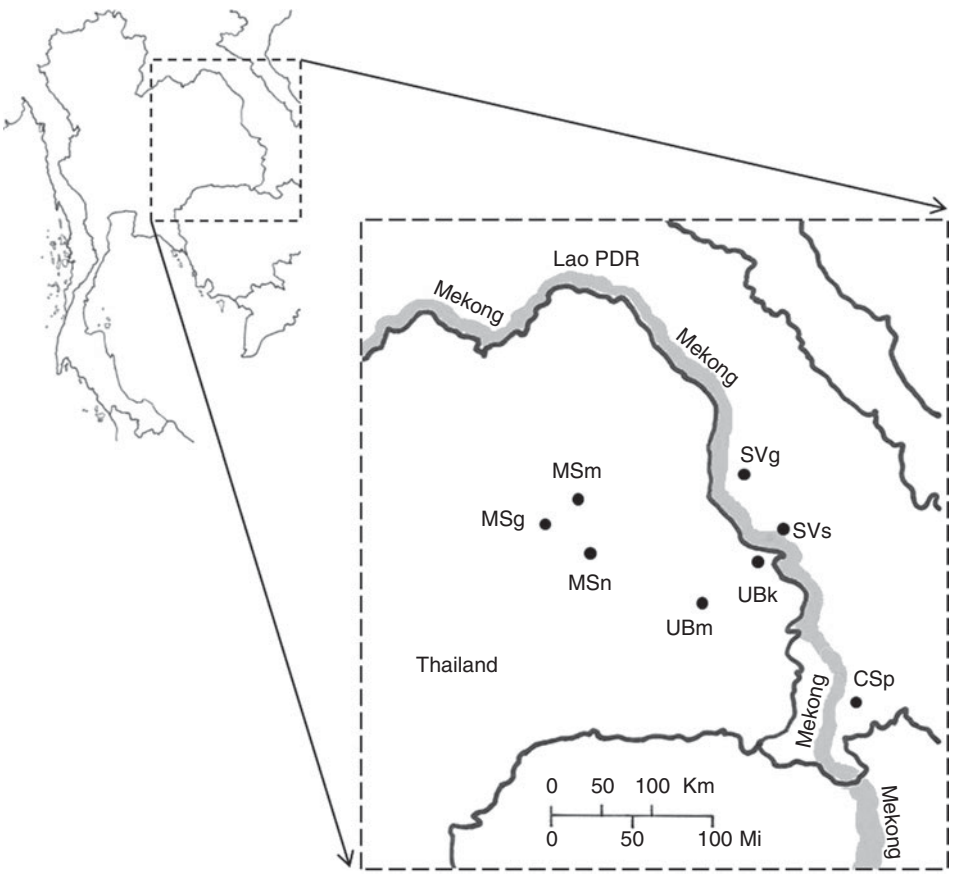

Fig. 1. Map showed the eight different geographical localities of *B. portentosus* collection. The samples codes as listed in table 1.

support medium. The cellulose acetate gel was cut into an appropriate size (15 × 30 cm), soaked in 30% methanol and kept at 4°C until used. The cellulose acetate gel was blotted dry between sheets of blotting paper and rapidly transferred to a soaking tank containing approximately 400 ml of the running buffer for at least ten minutes to equilibrate the gel with the buffer. The loaded gel was run at a constant 200 V, running time being adjusted between 120–150 min according to the mobility of each enzyme. Twenty enzymes were examined as follows (abbreviation, Enzyme Commission no.); adenylate kinase (Ak, 2.7.4.3), aldolase (Ald, 4.1.2.13), creatine kinase (Ck, 2.7.3.2), enolase (Enol, 4.2.1.11), fructose-1,6-diphosphatase (Fdp, 3.1.3.11), aspartate amino transferase (Got, 2.6.1.1), glucose-phosphate isomerase (Gpi, 5.3.1.9), alanine amino transferase (Gpt, 2.6.1.2), hexokinase (Hk, 2.7.1.1), isocitrate dehydrogenase (Idh, 1.1.1.42), malate dehydrogenase (Mdh, 1.1.1.37), malic enzyme (Me, 1.1.1.40), mannose-phosphate isomerase (Mpi, 5.3.1.8), peptidase leucine-glycine-glycine (PepB, 3.4.11.4), peptidase phenylalanine-proline (PepD, 3.4.13.9), 6-phosphogluconate dehydrogenase (6Pgd, 1.1.1.44), phosphoglycerate kinase (Pgk, 2.7.2.3), phosphoglucomutase (Pgm, 2.7.5.1), pyruvate kinase (Pk, 2.7.1.40), triose phosphate isomerase (Tpi, 5.3.1.1) and general protein (Gp).

The staining protocol described by Richardson *et al*. (1986) was followed with minor modifications. The stain ingredients commonly used were substrates in solution or dry

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Table 2. Pairwise comparison of the percentage of fixed difference (indicated in bold), F_{ST} values (indicated in italics, lower triangle) and geographical distance (in km, upper triangle) among isolates of *B. portentosus* collected from different geographical localities.

Code*	MSm	MSg	MSn	UBk	UBm	SVg	SVs	CSp
MSm	–	32	54	207	171	164	215	296
MSg	**4**/*0.753*	–	48	236	194	196	245	293
MSn	**9**/*0.787*	**4**/*0.879*	–	216	162	190	229	295
UBk	**17**/*0.830*	**17**/*0.870*	**22**/*0.879*	–	79	76	26	120
UBm	**17**/*0.852*	**22**/*0.882*	**26**/*0.889*	**13**/*0.752*	–	117	102	124
SVg	**48**/*0.947*	**52**/*0.953*	**52**/*0.952*	**39**/*0.905*	**48**/*0.912*	–	67	195
SVs	**35**/*0.937*	**39**/*0.939*	**39**/*0.943*	**22**/*0.899*	**35**/*0.903*	**4**/*0.518*	–	140
CSp	**43**/*0.966*	**43**/*0.969*	**43**/*0.971*	**30**/*0.938*	**43**/*0.935*	**22**/*0.861*	**17**/*0.857*	–

* Code of eight geographical samples listed in table 1. F_{ST} values compare between all localities were significant different with *P*-value <0.001.

components, which were weighed prior to preparation of the final stain, staining buffer, stock solution and linking enzymes. The stain solution was thoroughly mixed and spread evenly on the staining plate to the width of the gel by using a disposable plastic bulb-pipette. The gel was removed from the electrophoresis chamber inverted so that the porous, loading surface was in contact with the stain mixture over the surface of the gel. The stain was evenly spread over the gel within five seconds of application. The gel was then left on the stain for 30–60 s with occasional agitation every 10–15 s. The gel was blotted to remove excess stain and quickly transferred, porous side down, onto the plastic sheet. This was wrapped around the gel so that no creases were present to obscure enzyme bands. Wrapping the gel in this manner facilitates handling, whilst allowing the enzyme reaction(s) to be monitored safety. Gels were incubated at 37°C to increase the rate of the enzyme reaction and checked frequently for the appearance of band activity. Highly active enzymes appeared within seconds of stain application, whereas weaker enzymes took up to an hour to stain.

Data analysis

Alleles at each locus were designated by letters of the alphabet, starting with the allele encoding the most anodally migrating allozymes. To examine the genetic differences and relationships between different geographical populations, percent fixed difference (i.e. where a sample did not share any alleles in common with another sample at a particular locus) was calculated and a phenogram constructed based on UPGMA (unweighted pair group method of analysis: Sneath & Sokal, 1978) using the GWbasic program to measure the fixed differences between different populations. GENEPOP, version 3.3 (Raymond & Rousset, 1995) was used to calculate allele and genotype frequencies for each locus and genetic differentiation among populations (F_{ST}) based on Weir & Cockerham (1984) estimation. The correlation between two matrices of genetic distance (% fixed differences) and geographic distance among *B. portentosus* populations was performed using a Mantel test (Mantel, 1967) based on a two-tailed test (Pearson) at 95% CI in XLSTAT. The correlation between three matrices of genetic distance vs. geographic distance and river barrier (Mekong River) among *B. portentosus* populations was performed using a two-tailed partial Mantel test (Quemere *et al.*, 2010) with 95% CI using ZT software (Bonnet & Van de Peer, 2002).

Results

Eight geographical samples of *B. portentosus* showed a relatively high genetic diversity. Allele frequencies at all 23 loci tested in each sample are shown in Appendix 1. Allelic variability within a population of *B. portentosus* was observed at *Enol*, *Gpt-1*, *Gpt-2*, *Me*, *Mpi*, *PepB*, *6Pgd* and *Tpi* (see Appendix 1). Two populations of *B. portentosus* from Ubon Ratchathani Province showed allelic variability at five loci (i.e. *Enol*, *Gpt-1*, *Gpt-2*, *Mpi* and *Me*), whereas the three populations from Laos showed allelic variability at six loci, three as in Thailand (i.e. *Gpt-1*, *Gpt-2* and *Mpi*) and three additional ones (i.e. *PepB*, *6Pgd* and *Tpi*) (Appendix 1). However, there was highly significant differentiation in the pairwise F_{ST} values between eight different geographical populations of *B. portentosus*. Pairwise F_{ST} values between the populations from Thailand and the populations from Laos varied from 0.899 to 0.971. Pairwise F_{ST} values between five populations from Thailand varied from 0.752 to 0.889, whereas between three populations from Laos varied from 0.518 to 0.861 (Appendix 1).

Of the 23 enzyme loci, fixed differences between different geographical populations of *B. portentosus* were observed at 12 loci, i.e. *Ak*, *Ald*, *Ck*, *Gp*, *Hk*, *Idh*, *Mdh*, *Me*, *PepD*, *Pgk*, *Pgm* and *Pk* (Appendix 1). Fixed differences were also observed between the populations from Thailand and Lao PDR at eight loci, i.e. *Ak*, *Ald*, *Ck*, *Gp*, *Hk*, *Idh*, *Me* and *Pgk* (22–52% fixed difference) (Appendix 1, table 2). A total of 4–26% fixed differences were observed when the allelic patterns among five different geographical populations from Thailand were compared. Fixed differences were observed between the two populations from Ubon Ratchathani Province (UBk and UBm) and the three populations from Maha Sarakham Province (MSm, MSg and MSn) (17–26% fixed difference) at five loci, namely *Ck*, *Gp*, *Mdh*, *PepD* and *Pgm* (Appendix 1, table 2). Moreover, comparison among three populations from Maha Sarakham Province revealed that there were fixed difference between the MSn population vs. the MSm and MSg populations at the *Ald* locus and the MSm population vs. the MSg and MSn populations at *Mdh* locus. Similarly, it was found that fixed differences occurred between the UBk and the UBm populations from Ubon Ratchathani Province at three loci, i.e. *Ck*, *Gp* and *PepD*, with 13% fixed differences (Appendix 1, table 2). Comparison among the three populations from Lao PDR showed that fixed differences were observed at three loci (*PepD*, *Pgm* and *Pk*) between the two populations from Savannakhet Province (SVg and SVs) and one population from Champasak Province (CSp) at 17–22% fixed differences.

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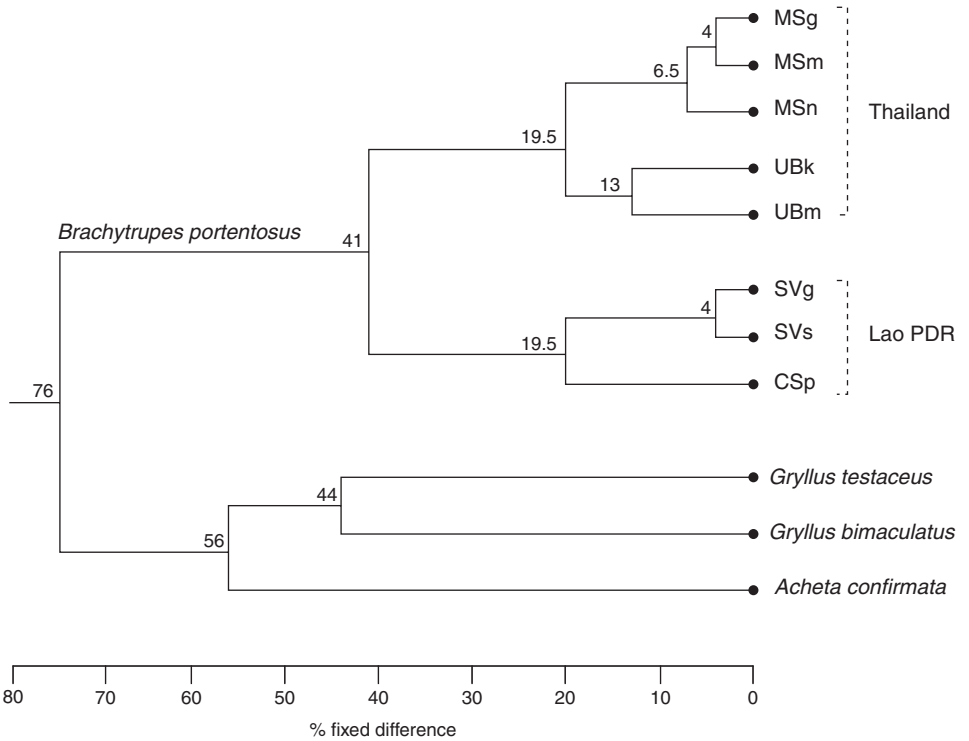

Fig. 2. Phenogram depicts % fixed difference (indicated in each branch) between eight different geographical populations of the field cricket, *B. portentosus*, from Thailand and Lao PDR and three other species of ground crickets.

In contrast, 4% fixed differences were observed between the SVg and SVs populations, which were differed at *Mdh* locus (Appendix 1, table 2).

Two distinct clusters of *B. portentosus* samples are shown in a phenogram generated from the allelic profile of 23 loci examined (fig. 2). The first cluster contained the five samples from Thailand, which is seen to be genetically different from the cluster comprising the three samples from Laos at 41% fixed differences (fig. 2). The three samples from Maha Sarakham Province (MSg, MSm and MSn) were aligned as a cluster with 4–6.5% fixed differences. The population from Ubon Ratchathani Province, UBk, was clustered with UBm at 13% fixed differences. Moreover, the three samples from Maha Sarakham Province appeared to be genetically different from the two populations from Ubon Ratchathani at 19.5% fixed difference. The cluster of the samples from Laos showed that two populations, SVg and SVs, from Savannakhet Province were clustered at 4% fixed differences. The CSp population from Champasak Province was genetically different from SVg and SVs with 19.5% fixed differences. In addition, *B. portentosus* was genetically distinct from the other three species of ground cricket tested (i.e. >76% fixed differences). A Mantel test (r = 0.515, *P*-value = 0.0061; table 3) supports the notion that the null hypothesis, indicating that the matrices of % fixed difference and Euclidean geographical distance are not correlated, be rejected. A partial Mantel test between 'geographic distance' and 'genetic distance' after controlling for 'river barrier' revealed that r = 0.415 (*P* = 0.0275) (table 3). Another partial Mantel test between 'river barrier' and 'genetic distance' after controlling for 'geographic distance' gave r = 0.486 (*P* = 0.0007) (table 3). These results suggest that the river is even a more important factor in limiting gene-flow than the geographical distance. Our results also showed that genotypes at all loci deviated from Hardy-Weinberg expectations (HWE) with a highly significant heterozygote deficiency ($P < 0.001$) except for *Mpi* ($P = 0.117$) and *PepB* ($P = 0.289$) (Appendix 1).

Table 3. Correlation test between genetic distance vs. geographical distance and river barrier.

Correlation	Controlling factor	r	*P*-value	Test
Genetic distance vs. geographic distance	No	0.515	0.0061	Mantel test
Genetic distance vs. geographic distance	River barrier	0.415	0.0275	Partial Mantel test
Genetic distance vs. river barrier	Geographic distance	0.486	0.0007	Partial Mantel test

Discussion

As with other species belonging to the order Orthoptera (e.g. grasshoppers: Gill, 2008; Ademolu *et al.*, 2009; mole crickets: e.g. Nevo *et al.*, 2000; and many field or ground crickets: e.g. Braswell *et al.*, 2006), MEE has proven to be an extremely use-efficient tool for examining the genetic variation and relationships between different geographical populations

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of *B. portentosus* in Thailand and Lao PDR. In our study, over 23 informative enzyme loci markers were generated with up to 12 loci (52%) being polymorphic among *B. portentosus* populations. The grouping of *B. portentosus* samples based on the UPGMA phenogram was in line with the geographic area and distances as recorded, implying that genetic relationships of *B. portentosus* samples, would depend on geographic features in Thailand and Lao PDR. Moreover, the genetic distance was also concordant with the Euclidean geographical distance between populations compared within Thailand or within Lao PDR, i.e. the samples which were collected between 32–79 km from one another showed 4–13% fixed differences and F_{ST} values of 0.518–0.879, whereas those separated by between 102–236 km showed 17–26% fixed differences and F_{ST} values of 0.830–0.971. Other studies have shown that this is not always the case; thus although genetic difference of cytochrome *b* sequences of *G. bimaculatus* De Geer showed a significant correlation with spatial distance between South African and European samples, there was no significant correlation between different geographical populations from within South Africa (Ferreira, 2006).

The observed deficit of heterozygotes as compared with Hardy-Weinberg expectations could be the result of a variety of factors, such as the Wahlund effect (i.e. due to the mixing of apparently contiguous population samples with different allele frequencies which are in reality separated by geographic barriers), natural selection, phenotypic assortative mating or inbreeding (Karlsson & Mork, 1999; Holsinger & Weir, 2009). Which of these acts in the case of *B. portentosus* cannot be determined from the data available, although the very high F_{ST} levels indicate concomitantly very limited gene flow between the populations sampled (Holsinger & Weir, 2009), suggesting that the strong Wahlund effect, selection or inbreeding may be possible. The use of burrows by these crickets may be an indication of limited dispersal, which would be in line with both of these possibilities. Here, more information on the behavior and ecology of *B. portentosus* is required.

The genetic difference observed between the population samples from Thailand and Lao PDR (average 40% fixed difference) can probably be attributed to the major natural barrier, the Mekong River. The distance between UBk from Thailand and SVs from Laos is only 26 km, but there were 22% fixed differences between populations on either side of the river. Moreover, the correlation between genetic distance was more highly significantly correlated with river barrier than geographic distance. This finding provides strong evidence that the Mekong River obstructs gene flow of this cricket between Thailand and Lao PDR, as is the case for other organisms. The liver fluke, *Opisthorchis viverrini*, which uses *Bithynia* snails as first intermediate host and cyprinid fish as a second intermediate host, shows very low levels of gene flow between the samples from Thailand and Lao PDR (Saijuntha *et al.*, 2007). Gene flow between populations of freshwater fish species, such as cyprinid spp. and catfish, is also interrupted across the Mekong River (So *et al.*, 2006; Ngamsiri *et al.*, 2007; Hurwood *et al.*, 2008). This conforms to the riverine barrier hypothesis, which was first suggested by Alfred Russell Wallace to describe the pattern of species distribution in the Amazon area of South America (Wallace, 1853; Haffer, 1997). Over the last few decades, tests of the hypothesis have usually involved taxonomic groups from this area (Gascon *et al.*, 1998, 2000; Aleixo, 2004; Noonan & Wray, 2006), although some support has also come from Africa (Anthony *et al.*, 2007) and islands of Southeast Asia (Jalil *et al.*, 2008) for gorillas and orang-utans, respectively.

Nevertheless, the two closest areas in Thailand and the Lao PDR, i.e. UBk and SVs, showed the lowest genetic difference when compared with the other samples from these countries. This is possibly because there was some gene flow between these populations despite the obstruction presented by the Mekong, potentially caused by gene flow between the UBk and SVs populations due to human transport. This human-assisted movement can occur if crickets are brought from the Songkorn district in Laos for sale at local markets in the Khemarat district of Thailand. While all our analyses show substantial genetic differentiation among the *B. portentosus* samples, a comprehensive assessment of population genetics structure, allele/genotype frequency and gene flow will require larger sample sizes and also more sophisticated approaches, i.e. DNA markers such as microsatellites or mtDNA markers with higher genetic resolving power. A low-frequency of some alleles at some loci tested may have remained undetected in this study due to the comparatively lower resolving power of allozyme markers vs. microsatellites. Moreover, the variation in morphological and biological traits, e.g. flatwing, cercal filiform hairs, body size, genitalia, tegmen length, mesosternum, metasternum, male calling song and seminal protein, which have been reported in other field crickets (Parsons & Shaw, 2001; Dangles *et al.*, 2005; Braswell *et al.*, 2006; Tinghitella, 2008; Cordero *et al.*, 2009), indicates that further work to explore biological differences between geographical populations of *B. portentosus* is required. One last possibility is certainly that cryptic species may be responsible for the fixed allelic differences observed, but again this requires elucidation using other biological methods such as cross mating experiments.

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Appendix 1

Allele frequency of 23 loci of eight geographical samples of *B. portentosus*, including *Acheta confirmata* Walker (*Ac*), *Gryllus bimaculatus* de Geer (*Gb*) and *Gryllus testaceus* Walker (*Gt*). All loci deviated from HWE with highly significant heterozygote deficiency at $P<0.001$, except *Mpi* ($P=0.117$) and *PepB* ($P=0.289$).

Enzyme loci	Allele	*B. portentosus* samples*								*Ac* (3)	*Gb* (2)	*Gt* (2)
		Thailand					Lao PDR					
		MSm (35)	MSg (42)	MSn (38)	UBk (40)	UBm (30)	SVg (40)	SVs (37)	CSp (34)			
Ak	*a*	1.00	1.00	1.00	1.00	1.00				1.00		
	b						1.00	1.00	1.00			
	c										1.00	1.00
Ald	*a*						1.00	1.00		–**	–	–
	b	1.00	1.00		1.00	1.00				–	–	–
	c			1.00						–	–	–
	d								1.00	–	–	–
Ck	*a*					1.00				–	–	–
	b	1.00	1.00	1.00						–	–	–
	c				1.00					–	–	–
	d						1.00	1.00	1.00	–	–	–
Enol	*a*	1.00	1.00	1.00	0.89	0.92	1.00	1.00	1.00	1.00	1.00	1.00
	b				0.11	0.08						
Fdp-1	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–
Fdp-2	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			
	b									1.00		
	c										1.00	1.00
Gp	*a*									1.00	1.00	1.00
	b				1.00		1.00	1.00	1.00			
	c					1.00						
	d	1.00	1.00	1.00								
Got-1	*a*									1.00		
	b										1.00	1.00
	c	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			
Got-2	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–
Gpt-1	*a*					0.10	0.07	0.11				
	b	1.00	1.00	1.00	1.00	0.90	0.93	0.89	1.00	1.00	1.00	1.00
Gpt-2	*a*							0.12	0.09	–	–	–
	b	1.00	1.00	1.00	0.88	0.90	1.00	0.88	0.91	–	–	–
	c				0.12	0.10				–	–	–
Hk	*a*									1.00	1.00	1.00
	b						1.00	1.00	1.00			
	c	1.00	1.00	1.00	1.00	1.00						
Idh	*a*	1.00	1.00	1.00	1.00	1.00					1.00	
	b						1.00	1.00	1.00			
	c											1.00
	d									1.00		
Mdh	*a*									1.00	1.00	1.00
	b							1.00				
	c		1.00	1.00								
	d	1.00			1.00	1.00	1.00		1.00			
Me	*a*				0.09	0.20	1.00	1.00	1.00			
	b	1.00	1.00	1.00	0.91	0.80						
	c										1.00	
	d									1.00		
	e											1.00

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Appendix 1 (*Cont.*)

Enzyme loci	Allele	*B. portentosus* samples*								*Ac* (3)	*Gb* (2)	*Gt* (2)
		Thailand					Lao PDR					
		MSm (35)	MSg (42)	MSn (38)	UBk (40)	UBm (30)	SVg (40)	SVs (37)	CSp (34)			
Mpi	*a*	0.81	0.80	0.88	0.84	0.83	0.81	0.82	0.81	0.90	1.00	
	b	0.12	0.12	0.05	0.11	0.10	0.13	0.10	0.12			1.00
	c						0.06	0.08	0.07	0.10		
	d	0.07	0.08	0.07	0.05	0.07						
PepB	*a*										1.00	
	b									1.00		
	c											1.00
	d							0.11	0.09			
	e	1.00	1.00	1.00	1.00	1.00	1.00	0.89	0.91			
PepD	*a*					1.00					1.00	
	b	1.00	1.00	1.00	1.00		1.00	1.00				
	c								1.00			1.00
	d									1.00		
6Pgd	*a*									1.00		
	b										1.00	1.00
	c	1.00	1.00	1.00	1.00	1.00	0.88	0.86	1.00			
	d						0.12	0.14				
Pgk	*a*									1.00		
	b										1.00	
	c	1.00	1.00	1.00	1.00	1.00						1.00
	d						1.00	1.00	1.00			
Pgm	*a*									1.00		
	b										1.00	1.00
	c				1.00	1.00	1.00	1.00				
	d	1.00	1.00	1.00								
	e								1.00			
Pk	*a*									1.00	1.00	1.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00				
	c								1.00			
Tpi	*a*						0.96	1.00	1.00			
	b	1.00	1.00	1.00	1.00	1.00	0.04			1.00	1.00	1.00

* Sample codes as listed in table 1 and sample size indicated in parenthesis.
** Enzyme band could not be stained.

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