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Allozyme data reveal genetic diversity and isolation by distance in sympatric *Glyphidrilus* Horst, 1889 (Oligochaeta: Almidae) of the Lower Mekong River Basin

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ABSTRACT

The genus *Glyphidrilus*, comprised of semi-aquatic freshwater earthworms that live in an ecotone between terrestrial and freshwater ecosystems, are widely distributed along riverbanks and rice paddy systems. Two *Glyphidrilus* species (*Glyphidrilus vangviengensis* and *Glyphidrilus mekongensis*) are endemic in the Lower Mekong River Basin and are sympatric from Northern Thailand to Southern Laos. However, species delimitation among the Mekong *Glyphidrilus* remains unclear because the key morphological traits in semi-aquatic earthworms are highly polymorphic. This study assessed the distinction between *G. vangviengensis* and *G. mekongensis* using allozyme electrophoresis. A total of 752 individuals collected from 33 localities were screened for 10 putative loci from seven enzymatic systems, revealing that *G. vangviengensis* and *G. mekongensis* are two distinct species, according to their different allelic patterns and high genetic distance. A low genetic differentiation within each species was indicated by the pairwise Nei's D and F_{ST} analyses, and the absence of population structure was detected by AMOVA and Bayesian structure analyses. However, a significant isolation by distance, but not vicariance, was observed, which is probably due to the river current causing translocation downstream and so gene flow between adjacent localities. The genetic diversity of the Mekong *Glyphidrilus* was relatively high and comparable to other earthworm taxa, and several localities showed deviation from Hardy–Weinberg equilibrium. An additional cryptic species from Ban Hat Khamphi, Loei, Thailand, was inferred.

1. Introduction

Semi-aquatic freshwater earthworms of the genus *Glyphidrilus* Horst, 1889 live in an ecotone between terrestrial and freshwater ecosystems. They are widely distributed along the banks of rivers, streams and canals, and are even found in ponds and rice paddy fields. The key morphological differences between semi-aquatic and terrestrial earthworms are that the

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former have a square-shaped cross-section of their posterior body and unusual expanded epidermises in the clitellar region, known as 'wings' or 'alae' (Brinkhurst and Jamieson, 1971; Chanabun et al., 2013). In Thailand, *Glyphidrilus* is widely distributed along the major river systems, including the Chao Phraya and the Mekong. The latter river system is an important international border between Laos and Thailand and serves as the main economic lifeline for the local people of both nations. *Glyphidrilus* was first reported in 2011 in the Lower Mekong River Basin and subsequently described as *Glyphidrilus vangviengensis* Panha and Chanabun, 2011 in the Song River, one of the Mekong tributaries in Vangvieng, Laos (Chanabun et al., 2011). In a later investigation (Chanabun et al., 2012), the *Glyphidrilus* species inhabiting the Mekong River bank was described as *Glyphidrilus mekongensis* Panha and Chanabun, 2012 based on specimens exclusively from its type locality of Khong Chiam, Ubon Ratchathani, Thailand. However, our intensive investigation found that *G. vangviengensis* and *G. mekongensis* coexist sympatrically along the middle section of the Mekong River from Northern Thailand to Southern Laos (pers. obs.).

G. vangviengensis and *G. mekongensis* range from 104 to 224 mm in length and live in the sandy mud topsoil on the river shore, as well as in underwater sediment, to a depth of about 10–20 cm. Morphologically, *G. vangviengensis* has a shorter clitellum (starting in segment 19 or 20 and ending in 35, 36 or 37) and shorter wings (starting in 24 or 25 and ending in 31 or 32) than *G. mekongensis* (clitellum starting in 19 and ending in 37 or 38; wings starting in 24 and ending in ½32, 33, 34 or ½35). Moreover, *G. mekongensis* has only one pair of genital markings on segment 23, unlike *G. vangviengensis*. Female, male and spermathecal pores are not visible in either species (Chanabun et al., 2011, 2012). However, the external morphology is highly variable in all semi-aquatic and aquatic taxa (Brinkhurst and Jamieson, 1971; Chanabun et al., 2013), so it is still unclear as to whether *G. vangviengensis* and *G. mekongensis* may be regarded as distinct species.

Earthworms typically have limited gene flow between populations because of their low dispersal rate (James, 2004; King et al., 2008; Siqueira et al., 2013) and their affinity with a specific type of soil (Novo et al., 2010, 2012; Viktorov, 1997). This has resulted in a high level of genetic differentiation among earthworm populations, as reported in several species (Dupont et al., 2011; Fernández et al., 2013; Novo et al., 2009; Siqueira et al., 2013). However, the semi-aquatic earthworm species in the Mekong River Basin might be dispersed downstream by river currents, resulting in a somewhat unidirectional enhanced gene flow, which indeed has been reported previously in some earthworms (Terhivuo and Saura, 2006).

At present, little is known about the biology and ecology of the semi-aquatic earthworms of the Mekong River Basin (see Jouquet et al., 2008). Regarding the ambiguity of species delimitation using key morphological characters, the aims of this study were to: (1) determine whether *G. vangviengensis* and *G. mekongensis* along the Lower Mekong River are reproductively isolated taxa that may be regarded as distinct species under the biological species concept; and (2) assess the extent to which their variable morphology allows their specific separation.

2. Material and methods

2.1. Sample collection

A total of 735 *Glyphidrilus* individuals were collected from 32 localities along the banks of the Mekong River and its tributaries in Thailand and Laos during Dec 2013–Apr 2014, spanning an approximate distance of 1580 km. The sample sizes ranged from one to 43 individuals per locality. In addition, 17 individuals of a different *Glyphidrilus* morphospecies were collected from an adjacent paddy field and included for comparison (Fig. 1). Localities, geographical coordinates, and sample sizes are given in Appendix A (Table S1). Earthworms were sampled by digging up the topsoil and hand sorting, then rinsed in running water to remove soil particles, snapped frozen in liquid nitrogen and stored at −20 °C until used for analysis. All specimens were identified to either species or morphospecies level under a stereomicroscope based on the guidelines of Chanabun et al. (2013).

2.2. Allozyme electrophoresis

Allozyme electrophoresis was used to determine whether *G. vangviengensis* and *G. mekongensis* are reproductively isolated taxa that may be regarded as distinct species under the biological species concept (Henry, 1999). The whole body part of each earthworm, from the anterior to clitellum, was cut and homogenized. The resulting crude protein extract from each homogenate was subjected to horizontal starch gel electrophoresis using a citrate-aminopropylmorpholine (pH 6.0) buffer system (Clayton and Tretiak, 1972) and screened for 10 putative allozyme loci: aspartate aminotransferase (*Aat-1,2*; E.C. 2.6.1.1); esterase (*Est-1,2*; E.C. 3.1.1-); glucose-6-phosphate isomerase (*Gpi*; E.C. 5.3.1.9); malate dehydrogenase (*Mdh*; E.C. 1.1.1.37); isocitrate dehydrogenase (*Idh-1,2*; E.C. 1.1.1.42); mannose-6-phosphate isomerase (*Mpi*; E.C. 5.3.1.8); and phosphoglucomutase (*Pgm*; E.C. 2.7.5.1). Electrophoresis and enzyme staining procedures followed Murphy et al. (1996). The remaining body parts were labeled, registered as voucher specimens and deposited at the Chulalongkorn University Museum of Zoology (CUMZ), Bangkok, Thailand.

2.3. Data analysis

Localities with less than five individuals of a given (morpho)species were not included in subsequent calculations (except in the STRUCTURE analysis). This resulted in a total of 731 individuals used in the analysis (*G. vangviengensis* = 253; *G.*

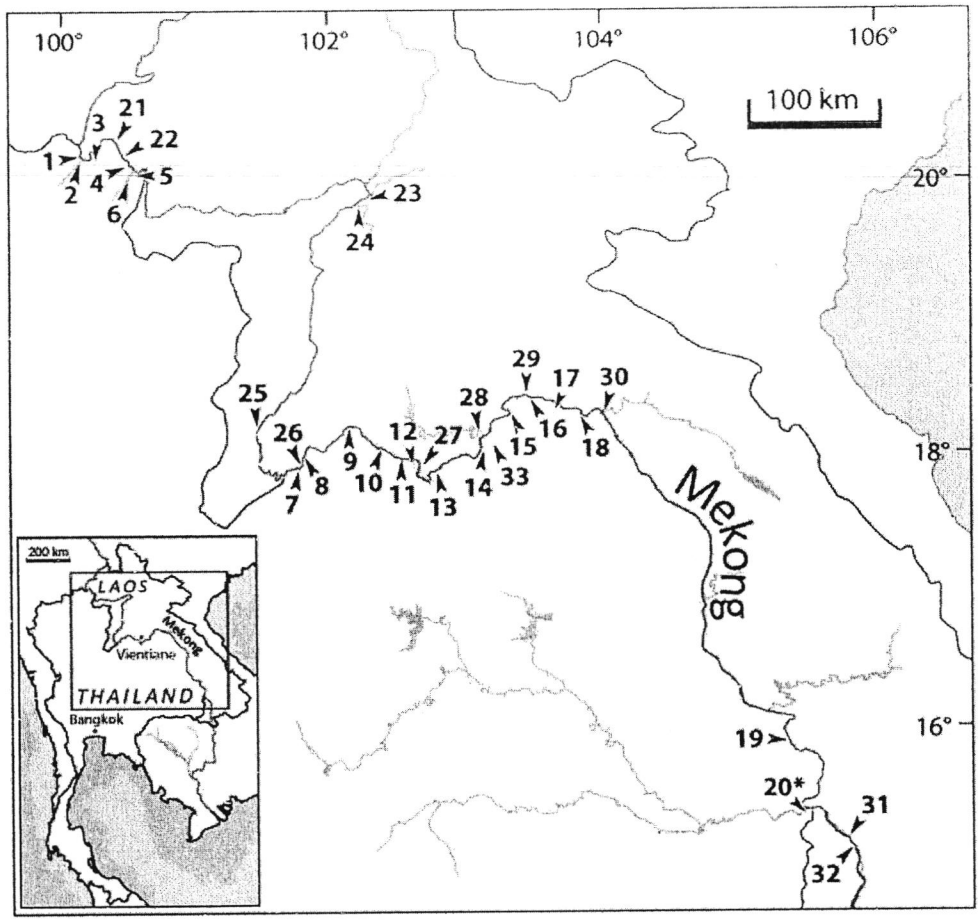

Fig. 1. Map of the Mekong River in Thailand and Laos, showing the 33 *Glyphidrilus* sampling localities in this study. Locality numbers correspond to those in Table S1 (Appendix A). An asterisk designates the type locality of *G. mekongensis*.

mekongensis = 461; *Glyphidrilus* sp. = 17). Genotype frequencies at polymorphic loci were tested for deviations from Hardy–Weinberg equilibrium (HWE) conditions by performing permutation tests (10,000 iterations) in GENEPOP v.4.2.2 (Rousset, 2008). In addition, the program BIOSYS-1 (Swofford and Selander, 1981) was used to estimate the genetic diversity within each population by calculating the mean number of alleles per locus (A), the mean observed heterozygosity (H_{obs}) and the mean expected heterozygosity (H_{exp}). The mean allelic richness (Ar) was calculated for all loci in all populations of both species with FSTAT v.2.9.3.2 (Goudet, 1995).

To estimate the heterogeneity among localities, estimators of F-statistics of each locus and pairwise F_{ST} values between populations were calculated according to Weir and Cockerham (1984), using GENEPOP v.4.2.2. F_{IS} values were determined for a significant heterozygote excess or deficient in each population and locus. The significance of pairwise F_{ST} values was tested in FSTAT v.2.9.3.2 using 10,000 permutations. The mean F_{IS} and F_{ST} values from each species were also tested for significant differences using FSTAT v.2.9.3.2 with 10,000 permutations. The sequential Bonferroni correction was applied to adjust for multiple test comparisons.

Nei's (1978) unbiased genetic distance (D) was calculated for all pairwise comparisons of populations using BIOSYS-1. An UPGMA dendrogram was constructed using PHYLIP v.3.695 (Felsenstein, 2005), and bootstrap support of each node was calculated with 1000 replications.

In order to establish the population structure and assign individuals to population clusters, STRUCTURE v.2.3.4 (Pritchard et al., 2000) was used to infer the most likely number of genetic clusters (K) with the admixture model. The analyses were run for 10 repetitions of the K value from one to 10, with 200,000 burn-ins and a 500,000 simulation length. The results from STRUCTURE were subsequently analyzed by STRUCTURE HARVESTER Web v.0.6.94 (Earl and von Holdt, 2012) to calculate ΔK based on the Evanno method, which correctly shows the number of population clusters (Evanno et al., 2005).

An analysis of molecular variance (AMOVA) was performed to test whether the genetic differentiation within each *Glyphidrilus* species is related to their geographic region. The partitioning of the genetic variation was examined: (i) within populations; (ii) among populations within groups; and (iii) among different groups using ARLEQUIN v.3.1 (Excoffier et al., 2005).

To examine the association between the genetic difference and geographic distance, isolation by distance (IBD) of the populations in each species was tested by a Mantel test (Mantel, 1967), using the correlation between the natural logarithms

of the geographic distances (measurements between localities along the course of the Mekong River on the map) and pairwise values of $F_{ST}/(1-F_{ST})$ between all localities. The genetic differentiation and geographic distance matrices were permutated 10,000 times in the ISOLDE program in GENEPOP v.4.2.2. This program was also used to compute the regression line describing the relationship between $F_{ST}/(1-F_{ST})$ and the natural logarithms of the geographic distances. The IBD analysis was performed on all localities of: (i) *G. vangviengensis* and (ii) *G. mekongensis*. In order to identify the effect of vicariance on IBD testing (Kuchta and Tan, 2005; Meirmans, 2012), the tests were performed with some clusters of localities: (iii) the northern populations of *G. vangviengensis* (Loc. 1–3, 5–6, 21–22); and (iv) the large central populations of *G. mekongensis* (Loc. 7–18, 26, 30).

3. Results

Morphological examination of the 735 individuals classified them as 266 *G. vangviengensis* individuals from 22 localities and 469 *G. mekongensis* individuals from 25 localities, and the two species occurred sympatrically at 15 of these localities (Fig. 2B).

Idh-2 was fixed for the same allele in all populations, while *G. vangviengensis* and *G. mekongensis* were fixed for different alleles at *Aat-2*. Moreover, the *Est-1* allele 'a' was nearly fixed in *G. vangviengensis*, whereas it was absent in *G. mekongensis* apart from at Loc. 11 and Loc. 15, where it occurred at low frequencies. Overall, there was a difference in the allele frequencies of each species between loci (Tables S2 and S3; Appendix A). Interestingly, two different fixed allelic patterns were observed at Loc. 9 (Ban Hat Khamphi, Loei, Thailand). A subpopulation here with different allelic frequencies from *G. mekongensis* in the same locality could be distinctively characterized by possession of three unique alleles at the *Gpi*, *Idh-1* and *Mpi* loci (alleles 'd', 'd' and 'a', respectively). Moreover, this subpopulation possessed alleles 'c' at the *Gpi* and 'b' at the *Pgm* loci that were only shared with the population from Khong Chiam, Ubon Ratchathani, Thailand (Loc. 20). Therefore, *G. vangviengensis*, *G. mekongensis*, the individuals from Loc. 9 that were distinct from *G. mekongensis* (*Glyphidrilus* sp. 1), and *Glyphidrilus* sp. 2 from the adjacent paddy field were analyzed separately in subsequent calculations. Allele frequencies at polymorphic loci are given in Appendix A (Tables S2 and S3).

The genetic diversity of all *Glyphidrilus* species in this study is given in Table 1. Heterozygote excess was mostly observed in some populations from Northern Thailand and Laos in both species, as well as in *Glyphidrilus* sp. 2. Heterozygote deficiency was observed in the lower section of the Mekong in this study (e.g. Loc. 20) (Table 1). Two out of eight loci (*Mdh* and *Pgm*) showed a heterozygote excess, while three (*Gpi*, *Idh-1* and *Est-1*) were deficient in heterozygotes in *G. vangviengensis*. In contrast, only two loci (*Gpi* and *Est-2*) were heterozygote deficient in *G. mekongensis* (Table 2). The F_{IS} and F_{ST} values for *G. vangviengensis* and *G. mekongensis* did not differ significantly (Table 2).

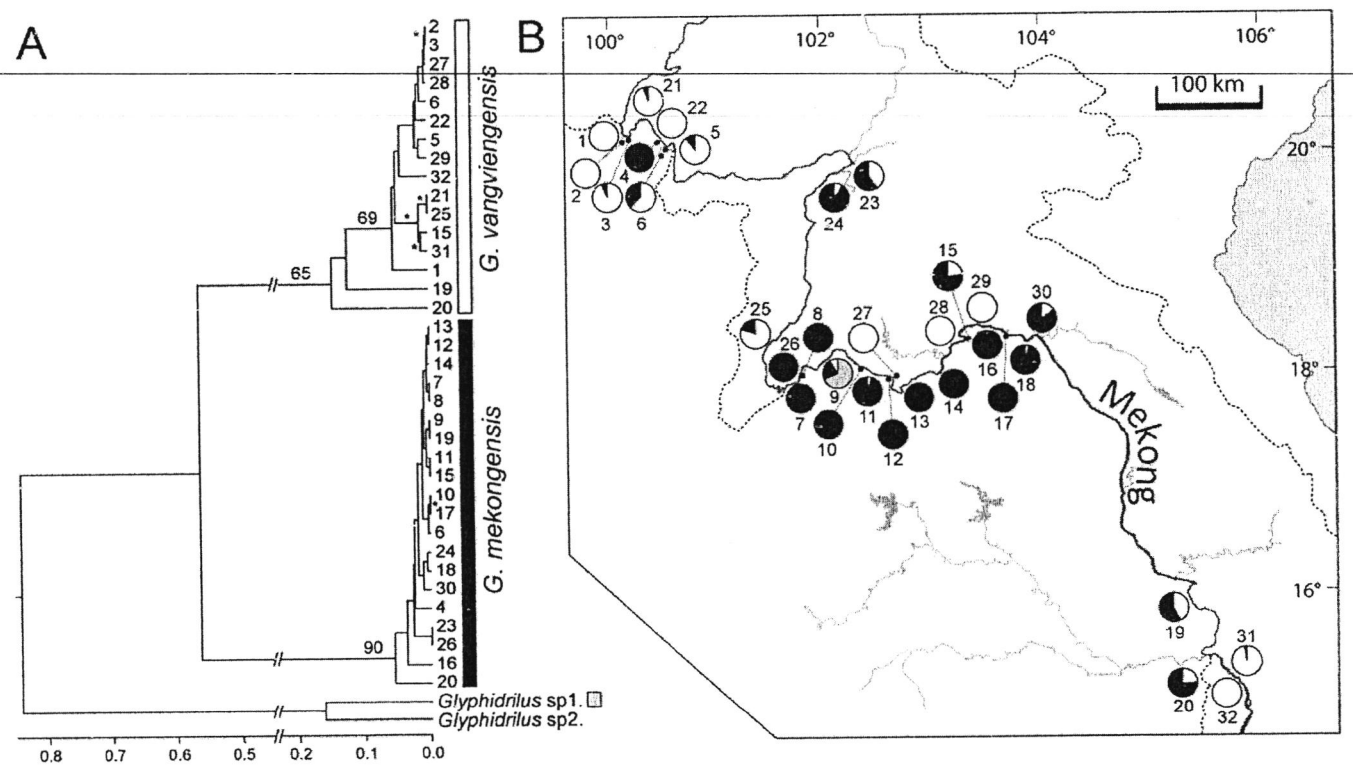

Fig. 2. A) UPGMA dendrogram for all *Glyphidrilus* populations based on Nei's (1978) unbiased genetic distance. Bootstrap values for the major branches are shown on the tree, with asterisks indicating bootstrap values above 50%. B) Pie charts display the relative frequencies of each sympatric *Glyphidrilus* species sampled from a given locality. Locality numbers correspond to those in Fig. 1 and Table S1 (Appendix A).

Table 1
Genetic diversity of *Glyphidrilus vangviengensis*, *G. mekongensis*, *Glyphidrilus* sp. 1 & *Glyphidrilus* sp. 2.

Species	Geographic region	Locality[a]	*N*	*A*	H_{obs}[b]	H_{exp}[b]	*Ar*	F_{IS}
G. vangviengensis	Northern Thailand	1	8	1.7	0.338 (0.109)	0.272 (0.076)	1.60	−0.260
		2	26	1.8	0.328 (0.116)	0.239 (0.077)	1.60	−0.386[c]
		3	28	1.7	0.269 (0.108)	0.225 (0.076)	1.56	−0.197
		5	16	1.8	0.260 (0.085)	0.217 (0.060)	1.57	−0.163
		6	15	1.7	0.360 (0.099)	0.287 (0.069)	1.64	−0.266
	Northeastern Thailand	15	8	1.7	0.140 (0.060)	0.167 (0.056)	1.54	0.218
		19	11	2.1	0.247 (0.083)	0.388 (0.072)	1.96	0.388[d]
		20	10	2.3	0.095 (0.029)	0.389 (0.090)	2.07	0.747[d]
	Northern Laos	21	16	1.8	0.183 (0.053)	0.200 (0.060)	1.59	0.088
		22	28	1.6	0.368 (0.138)	0.229 (0.078)	1.49	−0.628[c]
		25	8	1.6	0.211 (0.069)	0.204 (0.060)	1.54	−0.065
	Central Laos	27	8	1.8	0.354 (0.120)	0.283 (0.087)	1.67	−0.352
		28	9	1.6	0.232 (0.110)	0.197 (0.082)	1.49	−0.238
		29	8	1.7	0.182 (0.087)	0.199 (0.085)	1.61	0.081
	Southern Laos	31	38	1.5	0.030 (0.014)	0.053 (0.024)	1.19	0.444[d]
		32	16	2.1	0.172 (0.057)	0.221 (0.072)	1.61	0.210
	Mean			1.78 (0.21)	0.236 (0.100)	0.236 (0.080)	1.61 (0.19)	−0.024 (0.036)
G. mekongensis	Northern Thailand	4	28	1.5	0.296 (0.151)	0.161 (0.082)	1.36	−0.873[c]
		6	9	1.7	0.322 (0.142)	0.205 (0.085)	1.53	−0.628[c]
	Northeastern Thailand	7	35	1.6	0.170 (0.086)	0.181 (0.078)	1.46	0.079
		8	35	1.6	0.193 (0.085)	0.192 (0.085)	1.52	−0.007
		9	8	1.7	0.196 (0.095)	0.239 (0.090)	1.64	0.186
		10	27	1.6	0.207 (0.098)	0.185 (0.087)	1.50	−0.130
		11	23	2.0	0.229 (0.103)	0.231 (0.089)	1.71	0.024
		12	28	1.7	0.195 (0.090)	0.178 (0.082)	1.50	−0.095
		13	28	1.6	0.190 (0.096)	0.182 (0.085)	1.51	−0.035
		14	28	1.6	0.169 (0.082)	0.178 (0.084)	1.48	0.051
		15	26	1.9	0.213 (0.095)	0.230 (0.089)	1.66	0.086
		16	27	1.7	0.205 (0.082)	0.219 (0.081)	1.60	0.069
		17	28	1.6	0.157 (0.066)	0.189 (0.083)	1.49	0.173
		18	23	1.7	0.151 (0.090)	0.151 (0.081)	1.44	0.037
		19	15	1.9	0.230 (0.097)	0.233 (0.083)	1.64	0.004
		20	31	2.2	0.120 (0.046)	0.283 (0.070)	1.80	0.578[d]
	Northern Laos	23	6	1.6	0.177 (0.088)	0.225 (0.094)	1.56	0.210
		24	10	1.7	0.152 (0.073)	0.179 (0.079)	1.52	0.165
	Central Laos	26	5	1.7	0.165 (0.078)	0.237 (0.099)	1.68	0.309
		30	17	1.7	0.130 (0.067)	0.168 (0.075)	1.51	0.220
	Mean			1.72 (0.17)	0.193 (0.050)	0.202 (0.033)	1.56 (0.11)	0.021 (0.309)
Glyphidrilus sp. 1		9	24	1.6	0.253 (0.124)	0.171 (0.079)	1.42	−0.461
Glyphidrilus sp. 2		33	17	1.6	0.262 (0.129)	0.187 (0.088)	1.48	−0.452[c]

A, mean number of alleles per locus; *Ar*, mean allelic richness; H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity.
[a] Locality numbers correspond to those in Fig. 1 and Table S1 (Appendix A).
[b] Standard errors are indicated in parentheses.
[c,d] Significant (*p*-value adjusted after sequential Bonferroni correction) heterozygote [c]excess or [d]deficient.

Table 2
Summary of *F*-statistics for all eight polymorphic loci in *G. vangviengensis* and *G. mekongensis*.

Locus	*G. vangviengensis*		*G. mekongensis*	
	F_{IS}	F_{ST}	F_{IS}	F_{ST}
Mdh	−0.458[a]	0.156	−0.011	0.012
Gpi	0.451[b]	0.134	0.771[b]	0.124
Idh-1	0.122[b]	0.096	−0.036	0.097
Est-1	0.509[b]	0.484	−0.108	0.033
Est-2	0.037	0.163	0.634[b]	0.147
Pgm	−0.465[a]	0.192	0.770	0.377
Aat-1	0.105	0.068	0.155	0.026
Mpi	−0.020	0.126	−0.045	0.075
Mean	**−0.098**	**0.162**	**0.037**	**0.080**

[a,b] Significant (*p*-value adjusted after sequential Bonferroni correction) heterozygote [a]excess or [b]deficient.

Table 3
Comparison of mean pairwise Nei's (1978) unbiased genetic distance (below diagonal) and F_{ST} values (above diagonal) among pooled populations of *G. vangviengensis*, *G. mekongensis*, *Glyphidrilus* sp. 1 and *Glyphidrilus* sp. 2 across all loci. All F_{ST} values were significant after sequential Bonferroni correction.

Species	*G. vangviengensis*	*G. mekongensis*	*Glyphidrilus* sp. 1	*Glyphidrilus* sp. 2
G. vangviengensis	–	0.621	0.658	0.647
G. mekongensis	0.563	–	0.728	0.765
Glyphidrilus sp. 1	0.684	0.887	–	0.361
Glyphidrilus sp. 2	0.573	1.144	0.162	–

Table 4
Estimates of the genetic cluster number (K) and ΔK from the STRUCTURE and STRUCTURE HARVESTER analyses of 10 allozyme loci for $K = 1$–10 clusters. The most probable value ($K = 2$) is given in bold.

K	Ln(Pr) ± SD	ΔK
1	−9827.59 ± 0.06	–
2	−6276.01 ± 0.44	**6996.28**
3	−5826.20 ± 484.83	0.54
4	−5636.99 ± 333.18	0.26
5	−5360.82 ± 50.45	2.62
6	−5216.65 ± 56.11	3.24
7	−5254.06 ± 33.90	2.27
8	−5368.40 ± 99.56	1.15
9	−5368.03 ± 26.62	0.06
10	−5366.00 ± 35.80	–

Pairwise Nei's D and F_{ST} values between populations ranged from 0 to 1.271 (mean ± S.D. of 0.359 ± 0.319) and −0.057 to 0.845 (0.403 ± 0.277), respectively. Within each species, the mean pairwise Nei's D and F_{ST} values were lower (*G. vangviengensis*: $D = 0.061 \pm 0.055$; $F_{ST} = 0.156 \pm 0.109$; *G. mekongensis*: $D = 0.022 \pm 0.019$; $F_{ST} = 0.077 \pm 0.055$). The mean Nei's D and pairwise F_{ST} values of each species pair are compared in Table 3, and the complete matrices of pairwise Nei's D and F_{ST} are given in Appendix A (Tables S4 and S5).

The UPGMA dendrogram constructed from Nei's unbiased genetic distances showed that the different populations of *G. vangviengensis* and *G. mekongensis* resolved as two distinct clusters with a high genetic distance (0.56) between them. In addition, *Glyphidrilus* sp. 1 from Loc. 9 has clearly diverged from *G. vangviengensis* and *G. mekongensis* by a very high genetic distance (0.84), and is more closely related to *Glyphidrilus* sp. 2 (Fig. 2A). The STRUCTURE and STRUCTURE HARVESTER analyses revealed the highest ΔK values occurred for $K = 2$ (Table 4), supporting that *G. vangviengensis* and *G. mekongensis* belong to different genetic clusters, but this analysis failed to distinguish *Glyphidrilus* sp. 1 and sp. 2 from *G. vangviengensis* (Fig. S1A; Appendix A). However, the Bayesian structure analysis of $K = 3$ assigned *Glyphidrilus* sp. 1 and sp. 2 together to a different genetic cluster (Fig. S1B; Appendix A).

AMOVA revealed that neither species showed any significant genetic structure based on the defined geographic group, with the genetic variance being partitioned to the greatest extent within populations (*G. vangviengensis* = 83.27%, *G. mekongensis* = 89.58%: $p < 0.001$; Table S6; Appendix A).

A significant IBD pattern was detected for both *G. vangviengensis* and *G. mekongensis* populations, and also among the large central populations of *G. mekongensis* ($p < 0.05$; Fig. 3). By contrast, the Mantel test failed to detect an IBD among the northern populations of *G. vangviengensis* ($p > 0.05$; Fig. 3C).

4. Discussion

Two *Glyphidrilus* species endemic to the Lower Mekong River Basin (*G. vangviengensis* and *G. mekongensis*) were confirmed to be different biological species despite their apparent coexistence in 15 localities in Thailand and Laos (Fig. 2B). The coexistence of both species does not itself support sympatric speciation, but rather it is probably due to the current of the Mekong River acting as a water-mediated dispersal mode that facilitates translocation of separate (upstream) allopatric species occurring in the same area. Indeed, species delimitation was established using allozyme electrophoresis data for the closely related terrestrial earthworm species *Eisenia fetida* and *Eisenia andrei* (Henry, 1999). The high pairwise Nei's D obtained among different *Glyphidrilus* species were comparable with the genetic distances previously reported in other earthworm taxa (Table 5). This strongly suggests an absence of gene flow among the Mekong *Glyphidrilus*, and supports the notion that they are reproductively isolated biological species. Thus, based on the initial identification of the specimens used in this study by following Chanabun et al. (2013), the position of clitellum, wings and genital markings appear to be valid markers in discriminating between these semi-aquatic earthworms.

Within each species, the river current is also responsible for gene flow between localities along the Mekong River, as water dispersal has been observed in other earthworm species (Terhivuo and Saura, 2006). The low level of genetic differentiation

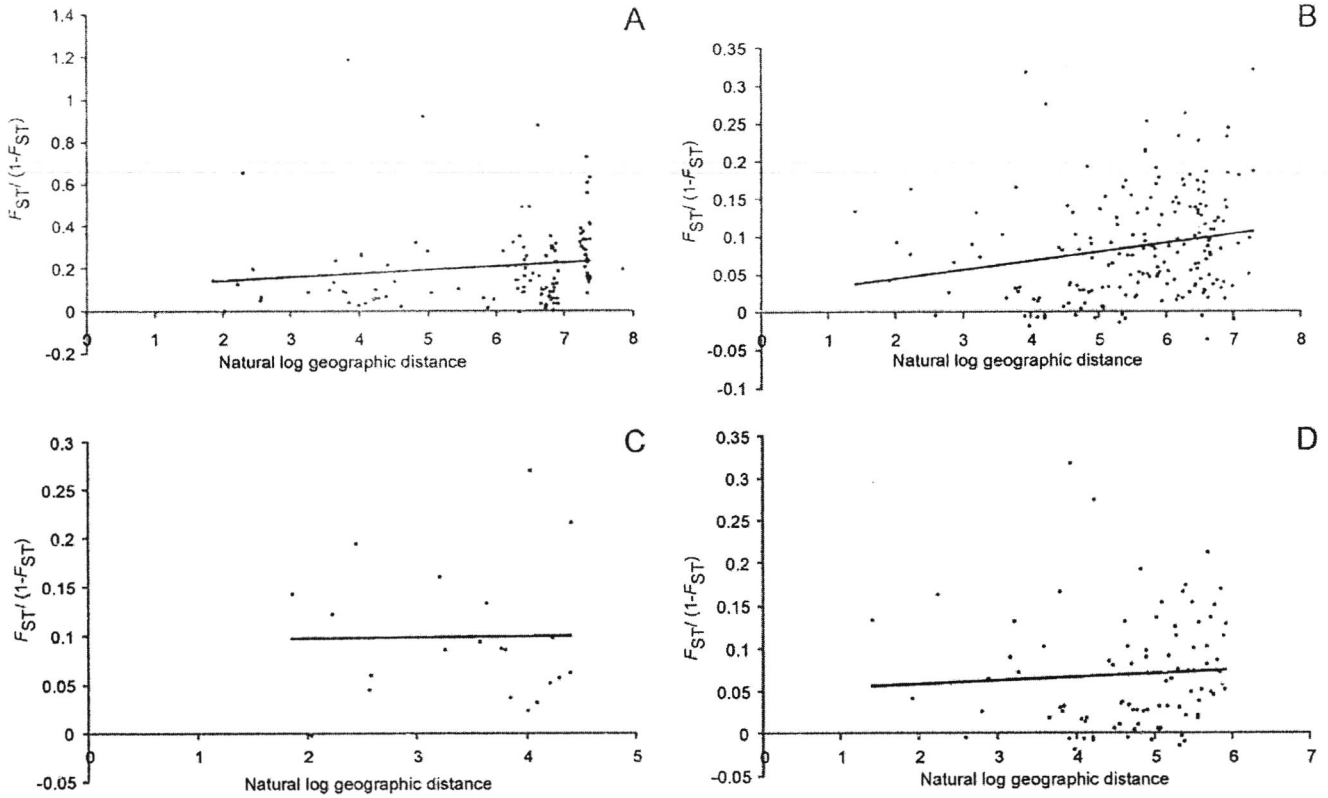

Fig. 3. Isolation by distance in: A) all *Glyphidrilus vangviengensis* populations; B) all *G. mekongensis* populations; C) northern *G. vangviengensis* populations; and D) central *G. mekongensis* populations.

found, as reflected in the estimates of Nei's *D* and pairwise F_{ST} values, is similar to that reported in other earthworms (e.g. *Aliolobophora tuberculata* and *Metaphire peguana*) that was ascribed as being due to a large population size and a high level of gene flow across different geographic regions associated with human activities (Prasankok et al., 2013; Stilie et al., 1980). Water-mediated gene flow also results in genetic similarity among nearby localities. However, this may be limited by distance resulting in IBD over longer ranges, which was detected in both *G. vangviengensis* and *G. mekongensis*. That the patterns truly reflect IBD, and not the effect of vicariance, was supported by performing the Mantel tests separately in some localities (Kuchta and Tan, 2005; Meirmans, 2012), and also by the absence of population structure observed in the UPGMA tree or detected by AMOVA. In contrast, the IBD pattern found in other earthworms was probably due to the underlying population genetic structure instead (Novo et al., 2009, 2010; Siqueira et al., 2013).

The genetic diversity in *G. vangviengensis* and *G. mekongensis* was relatively high, and well within the ranges reported for other earthworm taxa (McElroy and Diehl, 2001; Peles et al., 2003; Prasankok et al., 2013; Simonsen and Klok, 2010), suggesting a large and long-term stable effective population size (Frankham, 1996). Specifically, with respect to the level of heterozygosity, further analyses revealed deviation from HWE in several populations of both *G. vangviengensis* and *G.*

Table 5
Intra- and interspecific Nei's (1978) unbiased genetic distance reported in this study and other earthworm taxa.

Taxa	Interspecific distance		Intraspecific distance		No. of loci used	Reference
	Range	Mean ± S.D.	Range	Mean ± S.D.		
Hormogaster redii & *H. pretiosa*	1.1–3.1	1.8	*H. redii*:0.118–0.440 *H. pretiosa*:0.171–0.9	N/A	26	Sbordoni et al., 1992
Eisenia fetida & *E. andrei*	0.465–0.571	0.513 ± 0.047	N/A	*E. fetida*: 0.005 *E. andrei*: 0.031	8	McElroy and Diehl, 2001
Metaphire peguana & *M. bahli*	0.432–0.520	0.470 ± 0.026	*M. peguana*:0.002–0.170	*M. peguana*: 0.048 ± 0.040	18	Prasankok et al., 2013
Glyphidrilus vangviengensis & *G. mekongensis*	0.227–0.811	0.563 ± 0.122	*G. vangviengensis*: 0.000–0.206 *G. mekongensis*: 0.000–0.097	*G. vangviengensis*: 0.061 ± 0.055 *G. mekongensis*: 0.022 ± 0.019	10	This study
Glyphidrilus sp. 1 & *G. mekongensis*	0.788–1.068	0.887 ± 0.076				

mekongensis. Heterozygote deficiency could be attributed to inbreeding or to the Wahlund effect (Dupont et al., 2011). Even though self-fertilization has been reported in some earthworms (Cosín et al., 2011), and *Glyphidrilus* itself is hermaphroditic (Chanabun et al., 2013), inbreeding is unlikely to have been responsible for the heterozygote deficiency observed in this study due to the incongruent results among all the loci (Prasankok et al., 2013; Velavan et al., 2009). A more plausible explanation of the heterozygote deficient is the Wahlund effect, possibly as the result of recent mixing by river currents or seasonal migration (Chanabun et al., 2013). In contrast, a heterozygote excess has been reported from some loci in a number of earthworms and was purported to correlate with heterosis (McElroy and Diehl, 2001, 2004) or selection for heavy metal resistance genotype (Peles et al., 2003; Simonsen and Klok, 2010; Simonsen and Scott-Fordsmand, 2004). Furthermore, polyploidy could occur in some earthworms (Garbar et al., 2009; Shen et al., 2011; Vlasenko et al., 2011), which is caused by a form of parthenogenesis (Cosín et al., 2011). This breeding system could lead to the maintenance of heterozygosity (Cosín et al., 2011) and possibly explain the excess of heterozygotes observed in this study. However, the mechanisms underlying this phenomenon are unclear, and further analysis is needed to explore the actual causes.

Two sympatric species of *Glyphidrilus* (*G. vangviengensis* and *G. mekongensis*) now constitute the Mekong semi-aquatic earthworm fauna, and an additional cryptic species (*Glyphidrilus* sp. 1) was also inferred from allozyme result. In addition, the genetic distance of *G. vangviengensis* between Loc. 19 and 20, and the other populations was similar to the divergence between *Glyphidrilus* sp. 1 and sp. 2 (Fig. 2A), suggesting more cryptic species could yet be found. Further analysis using finer molecular markers will verify the species delimitation, reveal cryptic diversity of the Mekong *Glyphidrilus*, and elucidate the genetic differentiation of the respective species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2015.05.003.

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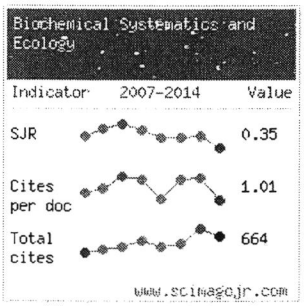

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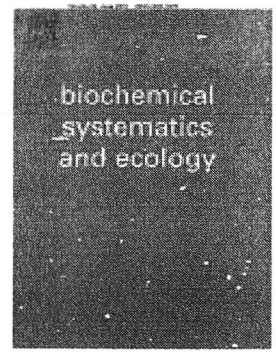

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