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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

Parin Jirapatrasilp [a, b], Pongpun Prasankok [c], Ratmanee Chanabun [d], Somsak Panha [b, *]

[a] Biological Sciences Program, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Animal Systematics Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[c] School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[d] Program in Animal Science, Faculty of Agriculture Technology, Sakon Nakhon Rajabhat University, Sakon Nakhon 47000, Thailand

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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

* Corresponding author. Tel./fax: +66 22185273.
E-mail address: somsak.pan@chula.ac.th (S. Panha).

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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.*