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Molecular phylogenetics and sequence analysis of two cave-dwelling *Dugesia* species from Southeast Asia (Platyhelminthes: Tricladida: Dugesiidae)

Tsung Fei Khang[1*], Siew Hwa Tan[2], Somsak Panha[3] & Zulqarnain Mohamed[2]

Abstract. The speciose freshwater planarian genus *Dugesia* Girard, 1850 has been extensively studied for more than a century, with over 80 species known to date. However, the absence of molecular characterisation of *Dugesia* species from Southeast Asia has created gaps in the understanding of global *Dugesia* systematics. We report hitherto unavailable DNA sequence data from cytochrome oxidase I (COI), internal transcribed spacer 1 (ITS-1) and 28S rRNA sequences of two *Dugesia* species from Southeast Asia: *Dugesia batuensis* Ball, 1970 from the Batu Caves in Malaysia, and *Dugesia deharvengi* Kawakatsu & Mitchell, 1989 from Tham Nen Noi in northeastern Thailand. Sequence analyses revealed a novel 25bp duplication in the ITS-1 sequence of *D. deharvengi*. Phylogenetic analysis of homologous sequences from other *Dugesia* species found in GenBank showed that *D. batuensis* does not share a most recent common ancestor with *D. deharvengi*, but rather with the geographically distant *Dugesia ryukyuensis* Kawakatsu, 1976. The basal position of *D. deharvengi* in the clade consisting of *Dugesia* species from the Oriental-Australasia region supports a previous hypothesis that *Dugesia notogaea* Sluys & Kawakatsu, 1998 from Australia was the result of speciation from ancestral forms in Southeast Asia.

Key words. *Dugesia*, phylogenetics, Batu Caves, Tham Nen Noi

INTRODUCTION

The freshwater flatworm genus *Dugesia* Girard, 1850 (Platyhelminthes: Tricladida: Dugesiidae) comprises over 80 species, making it one of the most speciose genera in the family Dugesiidae (Sluys et al., 2013). Its species are found throughout Europe, the Middle East, Africa, Asia and the Australasian region (Sluys et al., 1998), in varied habitats such as lakes, streams and caves. Since they rely on connected freshwater rivers and bodies for dispersion in nature, their biogeographical distribution has been suggested to be potentially useful for historical biogeography studies (Ball & Fernando, 1969; Solà et al., 2013). More recently, platyhelminths have become increasingly important as model organisms for regeneration biology (e.g., Sánchez Alvarado et al., 2002; Umesono & Agata, 2009; Nishimura et al., 2012), neurobiology (Inoue et al., 2015) and the study of mechanisms for the evolution of regressive phenotypes such as eye loss (Mannini et al., 2004).

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The importance of molecular evidence in *Dugesia* systematics cannot be overemphasised, since morphological characters are insufficient to produce a well-resolved phylogeny (Sluys et al., 1998). Although molecular phylogenies of *Dugesia* species (e.g. Lázaro et al., 2009; Sluys et al., 2013) spanning major continents are available, they lack adequate taxon coverage from critical geographical regions in Southeast Asia. While at least 20 *Dugesia* species from Southeast Asia are known (Kawakatsu & Mitchell, 2004), currently no molecular sequence data from these species are available in GenBank. In addition, many of the species found in Southeast Asia were morphologically re-examined more than 40 years ago, and lack basic karyological information. Some examples include *D. hymanae* (Sivickis, 1928) from the Philippines (see Sivickis, 1928; Kawakatsu, 1972a); *D. burmaensis* (Kaburaki, 1918) and *D. annadalei* (Kaburaki, 1918) from Myanmar (see Kaburaki, 1918); *D. borneana* Kawakatsu, 1972 from Borneo Island, Malaysia (see Kawakatsu, 1972b); and *D. indonesiana* Kawakatsu, 1973 from Indonesia (see Kawakatsu, 1973). Variation in chromosomal number was a key factor in the decision to recognise *D. ryukyuensis* Kawakatsu 1976 as distinct from *D. japonica* Ichikawa & Kawakatsu, 1964. An initial intention to distinguish the two species on the basis of penial anatomy was considered weakly justified owing to the presence of transitional forms in different populations (Kawakatsu et al., 1976).

In Malaysia, *D. batuensis* Ball, 1970 is known to inhabit shallow pools and streams in the Dark Cave of Batu Caves, near Kuala Lumpur. The limestones of Batu Caves originate in the Silurian (Cocks et al., 2005). Freshwater planarians

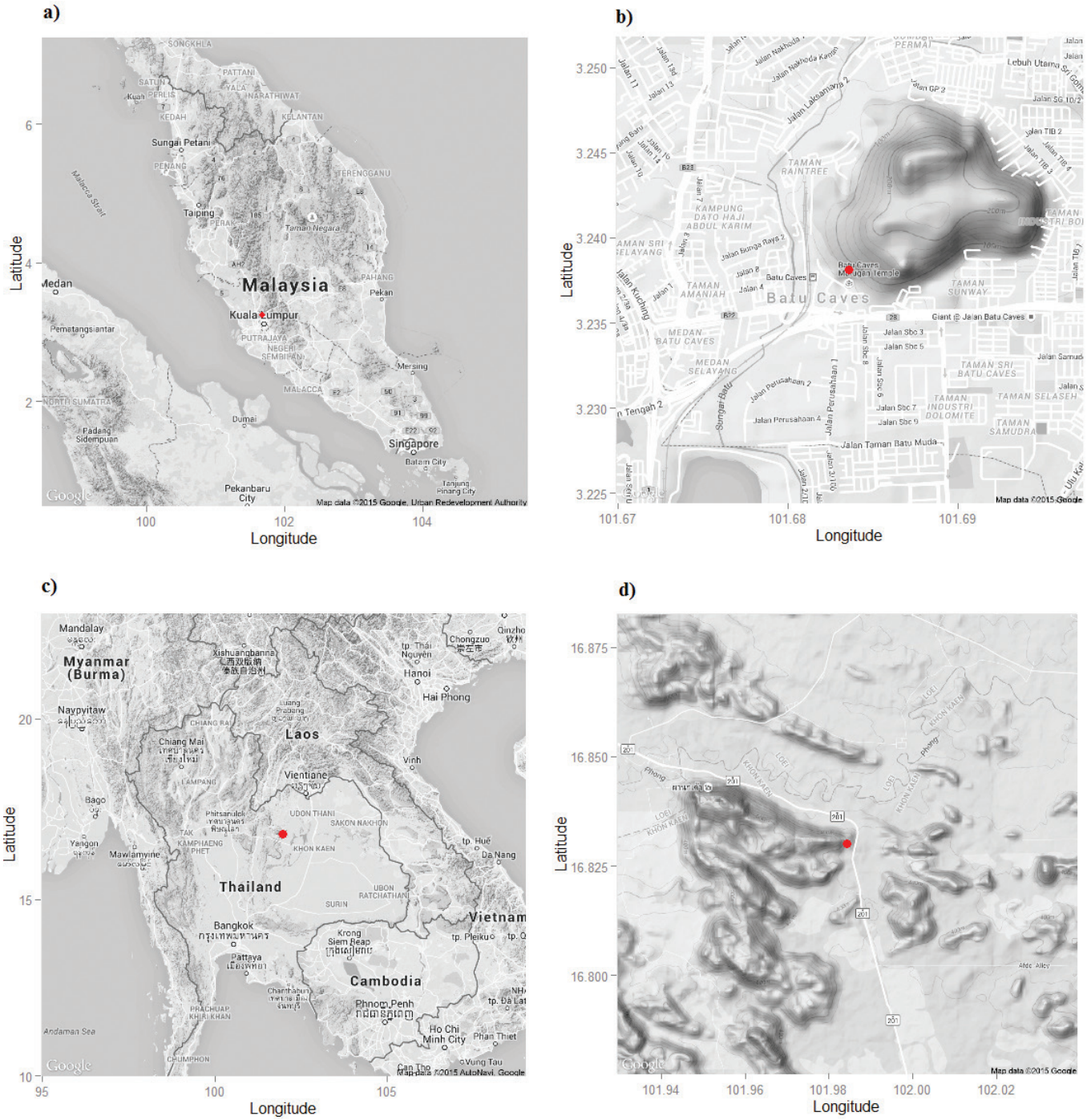

Fig. 1. *Dugesia* collection sites in Malaysia and Thailand. a, Dark Cave at Batu Caves, Peninsular Malaysia; b, Topological details of the area around Batu Caves; c, Tham Nen Noi, Thailand; d, Topological details of the area around Tham Nen Noi. Maps were created using the ggmap R package (Kahle & Wickham, 2013). Image source: Google Maps.

in the Dark Cave of Batu Caves were first recorded by Heynes-Wood & Dover (1929), and then by McClure et al. (1967). *Dugesia batuensis* does not show regressive phenotypes common to troglobitic organisms, such as lack of eyes and pigmentation (Kawakatsu & Mitchell, 2004). Ball (1970) provided its taxonomic description, and karyological information was added some 20 years later by Kawakatsu et al. (1989). In Thailand, an extensive speleological expedition during the late 1980s (Deharveng & Delnatte, 1988) led to the discovery of the first triglobitic triclad in Tham [=Cave] Kubio (sic: Tham Nen Noi [Louis Deharveng, pers. comm., 2012]) at Chum Phae District in Khon Kaen Province, northeastern Thailand. This species, which lacks eyes and pigmentation, was subsequently described by Kawakatsu & Mitchell (1989) and named *Dugesia deharvengi* after Dr. Louis Deharveng who collected it.

The present study aims to elucidate the molecular sequence data from *D. batuensis* and *D. deharvengi*, and to provide an updated phylogenetic analysis of global *Dugesia* species in the light of these new data.

MATERIAL AND METHODS

Specimen collection. *Dugesia* species were assigned based on type locality and karyological evidence (for *D. batuensis*). Living *D. batuensis* individuals were collected from the type locality—the Dark Cave, at Batu Caves (N3°14′E101°41′; Fig. 1A, B) in the state of Selangor, Malaysia on 7 December 2010, and immediately transferred to a laboratory at the University of Malaya. Individuals of *Dugesia deharvengi* were collected from the type locality—Tham Nen Noi (N16°50′E101°59′; Fig. 1C, D), in Chum Phae District of Khon Kaen Province, Thailand on 1 May 2012. They were fixed immediately in absolute ethanol and samples were sent to the University of Malaya for molecular sequencing work.

Karyological analysis. We modified the karyological analysis protocol of Sluys & de Jong (1984) for *Dugesia* species to validate the chromosome number in *D. batuensis.* First, we induced tissue regeneration in four individuals by cutting each of them into half. After that, the tissues from regenerating blastemas at the wounded site were excised using a sterile scalpel blade after three days (two individuals) and five days (two individuals). The blastemas were treated in 0.3% colchicine for about four hours, followed by washing with distilled water. They were then fixed using a 3:1 alcohol-acetic acid solution for at least 30 minutes, and stained with lacto-aceto-orcein for about 30 minutes. Having properly stained the tissues, we then transferred them into a drop of stain on a microscope slide. The slide was sandwiched with tissue papers, gently squashed, sealed using paraffin-balsam, and then checked under the microscope. Slides with reasonably clear metaphase chromosome spreads were photographed under a Leica DM2500 microscope with Leica QWin Plus software (Leica, Wetzlar, Germany).

DNA extraction, PCR amplification and sequencing. Tissues from living *D. batuensis* (n=9) that had been starved for one week, and ethanol-preserved *D. deharvengi* specimens (n=4) were used for DNA extraction using the QIAamp® DNA Mini Tissue Kit (Qiagen, Germany), according to the manufacturer's protocols.

Primers specific to three molecular markers (Table 1): the nuclear ITS-1 marker (Lázaro et al., 2009), the nuclear 28S rRNA marker (Álvarez-Presas et al., 2008), and the mitochondrial COI marker (Lázaro et al., 2009) were used in polymerase chain reaction (PCR) for amplifying these markers. The reaction mixtures were prepared in 50 µl reactions containing 100 ng template DNA, 1 unit of *Taq* polymerase, 1× PCR reaction buffer, 2 mM $MgCl_2$ (EuRex, Poland), 200 mM of each dNTP (Fermentas, USA) and 1 mM of each forward and reverse primers. PCR was performed in an Arktik thermal cycler (Thermo Scientific, USA), and the thermal cycling programme consisted of an initial denaturation step of 94°C of 5 min, followed by 35 cycles of denaturation at 94°C for 1 minute, an annealing step for 1 minute and an extension step at 72°C for 1 minute. A final elongation step was carried out at 72°C for 5 minutes. The annealing temperatures used were according to those described in Álvarez-Presas et al. (2008) for the 28S rRNA marker, and Lázaro et al. (2009) for the ITS-1 and COI markers.

The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Germany) following the manufacturer's protocol. Sequencing was performed using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. All samples were sequenced for both forward and reverse DNA strands. Electrophoresis and detection of the sequencing reaction products were carried out in the capillary electrophoresis system ABI PRISM 3730xl capillary DNA Sequencer with a capillary length of 80 cm.

Bioinformatic and phylogenetic analyses. For within-species sequence analysis, we performed multiple sequence alignment of COI, ITS-1 and 28S rRNA markers for *D. batuensis* and *D. deharvengi* samples using MAFFT (Online Version 7.245 at http://mafft.cbrc.jp/alignment/software/; Katoh et al., 2002; Katoh & Standley, 2013). For ITS-1, we searched for the presence of genomic signatures such as duplicated sequences in both species using Dotlet (http://myhits.isb-sib.ch/cgi-bin/dotlet), a dot matrix plot program (Junier & Pagni, 2000). Such signatures were phylogenetically useful for distinguishing molecular synapomorphies in two *Dugesia* species, viz. *Dugesia aethiopica* Stocchino, Corso, Manconi & Pala, 2002 from Ethiopia, and *Dugesia sicula* Lepori, 1948 from the Mediterranean region (Baguñà et al., 1999).

To obtain sequences for molecular phylogenetic inference in the current study, we used BLASTN (Altschul et al., 1990) to search for 28S rRNA, ITS-1 and COI sequences in the GenBank database that are homologous to corresponding query sequences from *D. batuensis*. The search set filter for organism was restricted to "*Dugesia* (taxid:6160)" and "*Schmidtea* (taxid:55270)", and program selection was optimised for "Somewhat similar sequences". All default algorithm parameters were kept except for "max target sequences", which was set to 250. Generally, retrieved sequences that satisfied the criteria of having at least 70% query coverage and E-value less than 10^{-10} were collected, and then filtered for species redundancy and annotation uncertainty (e.g. lack of species name, species name given but with "nomen nudum" warning). However, for taxa-poor markers such as 28S rRNA, we found it necessary to lower the query coverage criterion to 50%. We excluded 28S rRNA data for *D. subtentaculata* (DQ665970) due to concerns that it may be a paralogous sequence. For outgroup rooting of the phylogenetic tree, we used sequences of *Schmidtea mediterranea* Benazzi, Baguñà, Ballester, Puccinelli & Del Papa, 1975. Table 2 shows the GenBank accession numbers of all sequences used in this study.

For phylogenetic analysis, we adopted a supermatrix approach (De Queiroz & Gatesy, 2007). Multiple sequence alignment was done using MAFFT (Online Version 7.245). For both ITS-1 and 28S rRNA, we used the following alignment parameters: Q-INS-i iterative refinement method, 1PAM/

Table 1. PCR primer details for COI, ITS-1 and 28S rRNA markers. Abbreviations: F for forward primer; R for reverse primer.

Marker	Primer sequence 5′to 3′	Annealing temperature (°C)
COI		
COI (F)	CCTGGGTTTGGTATTGTTTCACA	45
COI (R)	CCAGTTAAACCACCTATAGTAAA	45
ITS-1		
ITS 9F	GTAGGTGAACCTGCGGAAGG	45
ITSR	TGCGTTCAAATTGTCAATGATC	45
28S rRNA		
28S 1F	TATCAGTAAGCGGAGGAAAAG	52
28S 3R	CCTTGGGTCCGTGTTTCAAGAC	52
28S 2F	CTGAGTCCGATAGCAAACAAG	49
28S 4R	CCAGCTATCCTGAGGG	49
28S 3F	GTCTTGAAACATGGACCAAGG	53
28S 6R	GGAACCCCTTCTCCACTTCAGT	53

Table 2. GenBank accession numbers of DNA sequence data of *Dugesia* species used in the study. Sequences contributed from the present study are marked with an asterisk. Retrieval dates: 10 August 2015 (1); 11 August 2015 (2); 16 August 2015 (3). Sequence data sources indicated by superscripts: a) Álvarez-Presas et al. (2008); b) Lázaro et al. (2009); c) Carranza et al. (1999a); d) Solà et al. (2013); e) Baguñà et al. (2001); f) Sluys et al. (2013); g) Lázaro et al. (2011); h) Carranza et al. (1999b); i) Álvarez-Presas & Riutort (2014); j) Chen et al. (2015).

Region	Sub-region	Species	Markers		
			COI[1]	ITS-1[2]	28S rRNA[3]
Oriental	East Asia	*D. japonica*	DQ666034[a]	FJ646904[b]	DQ665966[a]
	East Asia	*D. ryukyuensis*	FJ646946[b]	FJ646909[b]	DQ665968[b]
	East Asia	*D. sinensis*	KP401592[j]		
	India	*D. bengalensis*		FJ646897[b]	
	Southeast Asia	*D. batuensis*	KF907818*	KF907815*	KF907821*
	Southeast Asia	*D. deharvengi*	KF907820*	KF907817*	KF907824*
Australia		*D. notogaea*	FJ646945[b]	FJ646908[b]	KJ599720[i]
Ethiopia		*D. aethiopica*	FJ646952[b]	FJ646911[b]	
Western Palearctic	Western Europe	*D. gonocephala*	DQ666033[a]	FJ646901[b]	DQ665965[a]
	Mediterranean Basin	*D. subtentaculata*	DQ666036[a]	U84369[c]	
		D. sicula	FJ646962[b]	FJ646925[b]	DQ665969[a]
		D. cretica	KC006974[d]	KC007050[d]	
		D. ariadnae	KC006972[d]	KC007048[d]	
		D. malickyi	KC006989[d]	KC007066[d]	
		D. arcadia	KC006971[d]	KC007044[d]	
		D. elegans	KC006984[d]	KC007062[d]	
		D. sagitta	KC007000[d]	KC007074[d]	
		D. aenigma	KC006963[d]	KC007038[d]	
		D. damoae	KC006979[d]	KC007056[d]	
		D. hepta	FJ646943[b]	FJ646902[b]	
		D. benazzii	FJ646935[b]	FJ646890[b]	
		D. etrusca	AF178310[e]	FJ646898[b]	
		D. ilvana	FJ646944[b]	FJ646903[b]	
		D. liguriensis		FJ646907[b]	
		D. naiadis	KF308758[f]		
		D. effusa	KF308779[f]		
		D. improvisa	KF308771[f]		
		D. parasagitta	KF308738[f]		
Outgroup		*Schmidtea mediterranea*	JF837062[g]	AF047854[h]	DQ665992[a]

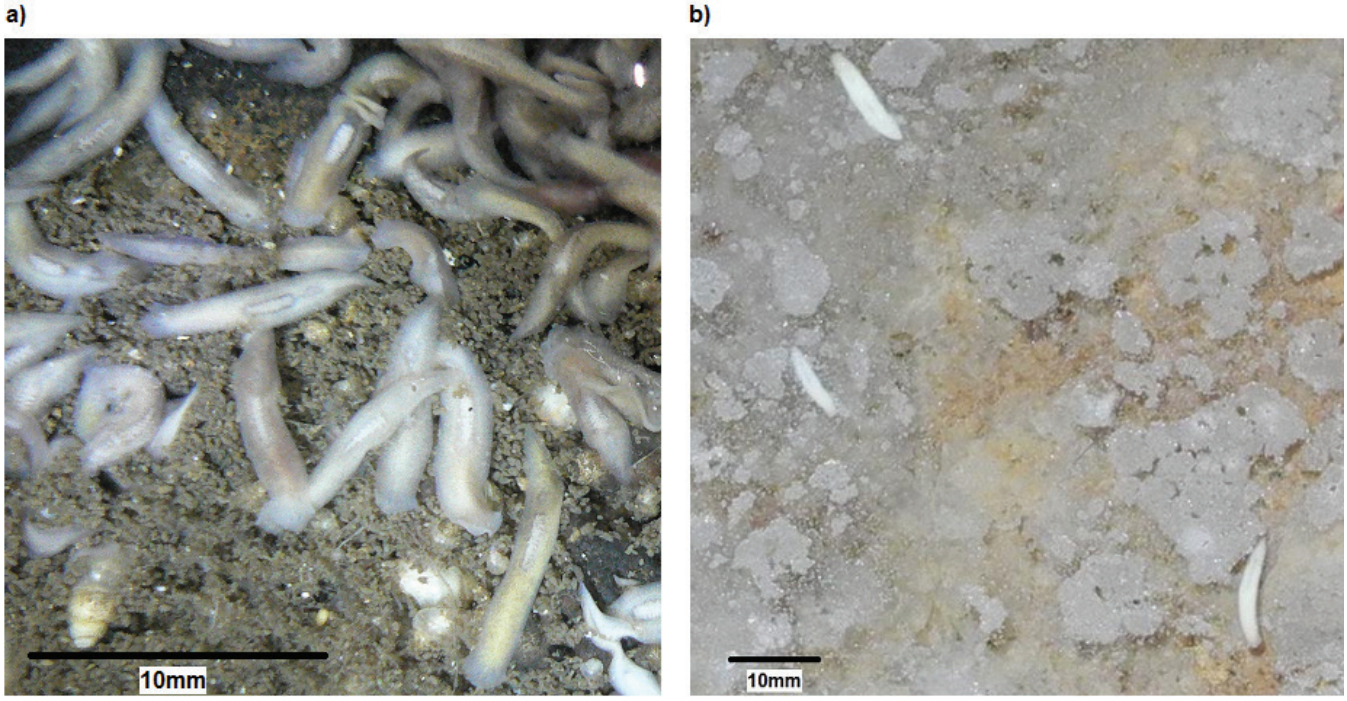

Fig. 2. *Dugesia* species from Malaysia and Thailand. a, Living *D. batuensis* in a shallow stream in the Dark Cave, Batu Caves, Malaysia; b, Living *D. deharvengi* in a freshwater pool inside Tham Nen Noi, Thailand.

Fig. 3. *Dugesia batuensis*. a, A living specimen with visible eyes and clearly pigmented body; b, Karyogram (2n=14).

ê=2 nucleotide scoring matrix and gap penalty of 1.53. For aligning the protein-coding COI sequences, we used the TranslatorX pipeline (http://translatorx.co.uk; Abascal et al., 2010). Nucleotide sequences were translated to amino acid sequence using the Echinoderm and Flatworm Mitochondrial Code (Translation Table 9), followed by MAFFT alignment (FFT-NS-2 progressive alignment method; BLOSUM62 amino acid scoring matrix; gap opening penalty of 1.53), and then back-translated to nucleotide sequences. We disabled the Gblocks (Talavera & Castresana, 2007) option, because automated removal of gap columns and variable regions has been reported to worsen the accuracy of the inferred phylogeny (Dessimoz & Gil, 2010; Tan et al., 2015). The multiple sequence alignments (doi: 10.6084/

Table 3. Characteristics of multiple sequence alignment (MSA) of COI, ITS-1 and 28S rRNA sequences. Abbreviations for DNA substitution models and their site properties: Kimura's three-parameter model with unequal base frequencies (TPM3u); Hasegawa-Kishino-Yano (HKY) model; gamma-distributed rate variation among sites (G).

Sequence	Number of species	MSA length	Percentage of constant sites (%)	Model selection	Estimated G parameter
COI	27	252	50	HKY + G	0.25
ITS-1	24	665	50	TPM3u + G	0.75
28S rRNA	8	1683	82	TPM3u + G	0.10

m9.figshare.3839547) were then concatenated in the order of COI—ITS-1—28SrRNA using an in-house R script (doi:10.6084/m9.figshare.3839538; Version 3.2.1; R Core Team, 2015).

To construct phylogenetic trees, we used IQ-TREE (Nguyen et al., 2015), a state-of-the-art pipeline for fast construction of maximum likelihood (ML) trees (Felsenstein, 1981) that incorporates a model selection (Posada & Crandall, 1998) procedure based on the robust Bayesian Information Criterion (Luo et al., 2010). Sequence evolution (partition models with edge-linked option) in the concatenated multiple sequence alignments was modelled using nucleotide substitution models. Bootstrap support (10,000 replicates) was computed using the ultrafast bootstrap option (Minh et al., 2013) in IQ-TREE. The resulting ML tree was visualised and annotated using MEGA 6 (Version 6.06; Tamura et al., 2013).

RESULTS

General observations and karyological analysis. *Dugesia batuensis* and *D. deharvengi* (Fig. 2A, 2B) are shown in their natural cave environment. We were able to keep some *D. batuensis* individuals alive in distilled water in the laboratory for up to three months. Fig. 3A shows photograph of a living specimen of *D. batuensis*. Karyological results of 21 mitotic cells examined confirm the 2n=14 diploid chromosome number of *D. batuensis* (Fig. 3B) previously reported by Ball (1970).

Bioinformatic analysis. Sequence length of 28S rRNA (1,605 bp) and COI (289 bp) fragments amplified were the same for both species. The ITS-1 sequence in *D. deharvengi* was 47 bp longer (708 bp) compared to *D. batuensis* (661 bp). No intraspecific sequence variation was detected in all three markers for *D. deharvengi* (three individuals for 28S rRNA; four individuals for ITS-1 and COI). However, low levels of intraspecific sequence variation in *D. batuensis* samples were detected. For 28S rRNA (four individuals), polymorphic sites at positions 6 (A/T) and 53 (A/C) yielded three haplotypes: AA (one individual), TC (one individual) and TA (two individuals). For COI (nine individuals), a polymorphic site at position 208 (T/C) was detected in a single sequence. For ITS-1, all sites were conserved with the exception of position 255, where an A/R base ambiguity (nine individuals; two As and seven Rs) was noted.

The dot matrix plot of *D. deharvengi* ITS-1 sequence (Fig. 4A) revealed a novel 25 bp repeat sequence: ATACTTAAAAATGGGCGTAT(A/G)CAAT, which is located at positions 224–248 and 277–301. A 30 bp repeat sequence: ATGCATATTTAATAAAAGGTGTATGCATGA, which was shared by both *D. sicula* and *D. aethiopica* (Fig. 4B), was also located similarly at positions 216–245 and 271–300. No novel repeat sequence in ITS-1 was detected in *D. batuensis*.

Phylogenetic analysis. The resulting concatenated multiple sequence alignment consisted of 29 taxa and 2,600 columns with 366 informative sites. The percentage of missing data was 52%. Table 3 summarises the number of species, the length of multiple sequence alignment, the percentage of constant sites, the optimal substitution model and associated model parameter for each of the three genetic markers.

Figure 5 shows the ML tree inferred from concatenated COI, ITS-1 and 28SrRNA sequence data. Several features are noteworthy. Firstly, the tree suggests the presence of three major clades with strong bootstrap support that are associated with broad geographical locations of the species: the Mediterranean-Ethiopian clade (Clade I), the Western Palearctic clade (Clade II), and the Oriental-Australasia clade (Clade III). These clades agree with results from previous phylogenetic analyses (Lázaro et al., 2009; Solà et al., 2013). Specifically in Clade III, *D. batuensis* clusters with the geographically distant *D. ryukyuensis* (100% bootstrap support), and not with the geographically closer *D. deharvengi*. In addition, *D. bengalensis*, which is found in West Bengal in India, clusters with *D. notogaea* from Australia, also with very strong bootstrap support (100%). The other two *Dugesia* species from East Asia appeared to be phylogenetically closer to Clade III (*D. japonica*) and Clade II (*D. sinensis*) respectively than to each other.

DISCUSSION

The present results provide new molecular evidence to support the hypothesis that *D. ryukyuensis* shares a most recent common ancestor with *D. batuensis* rather than *D. japonica*, despite the latter being geographically closer. In fact, the observation that *D. deharvengi* is basal to Clade III suggests that *D. ryukyuensis* is phylogenetically closer to *D. deharvengi* than to *D. japonica*, while simultaneously supporting the hypothesis proposed by Sluys et al. (1998) that

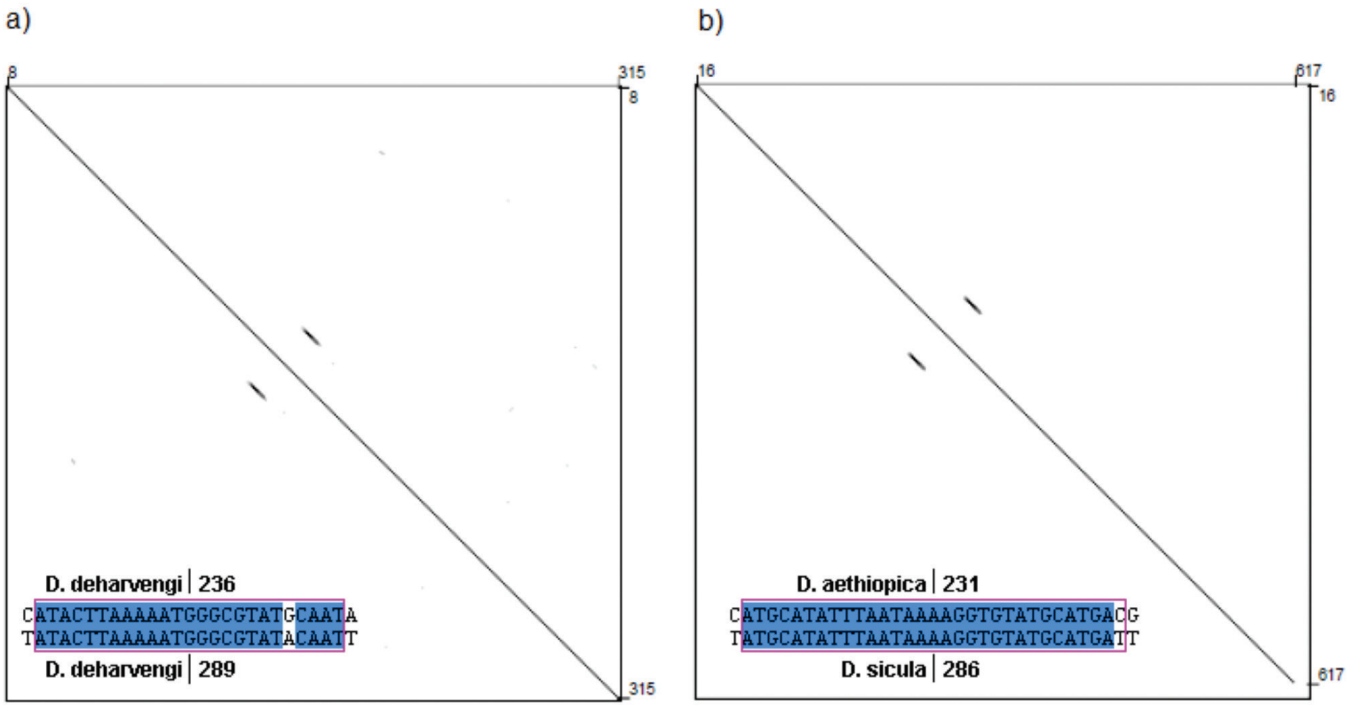

Fig. 4. Dot matrix plots for ITS-1 sequences of *Dugesia* species. a, *D. deharvengi* sequence (708 bp) showing a 25 bp repeat sequence: ATACTTAAAAATGGGCGTAT(A/G)CAAT at positions 224–248, and 277–301; b, *D. aethiopica* sequence (616 bp) against *D. sicula*. Note the presence of a 30 bp repeat sequence ATGCATATTTAATAAAAGGTGTATGCATGA at positions 216–245 and 271–300.

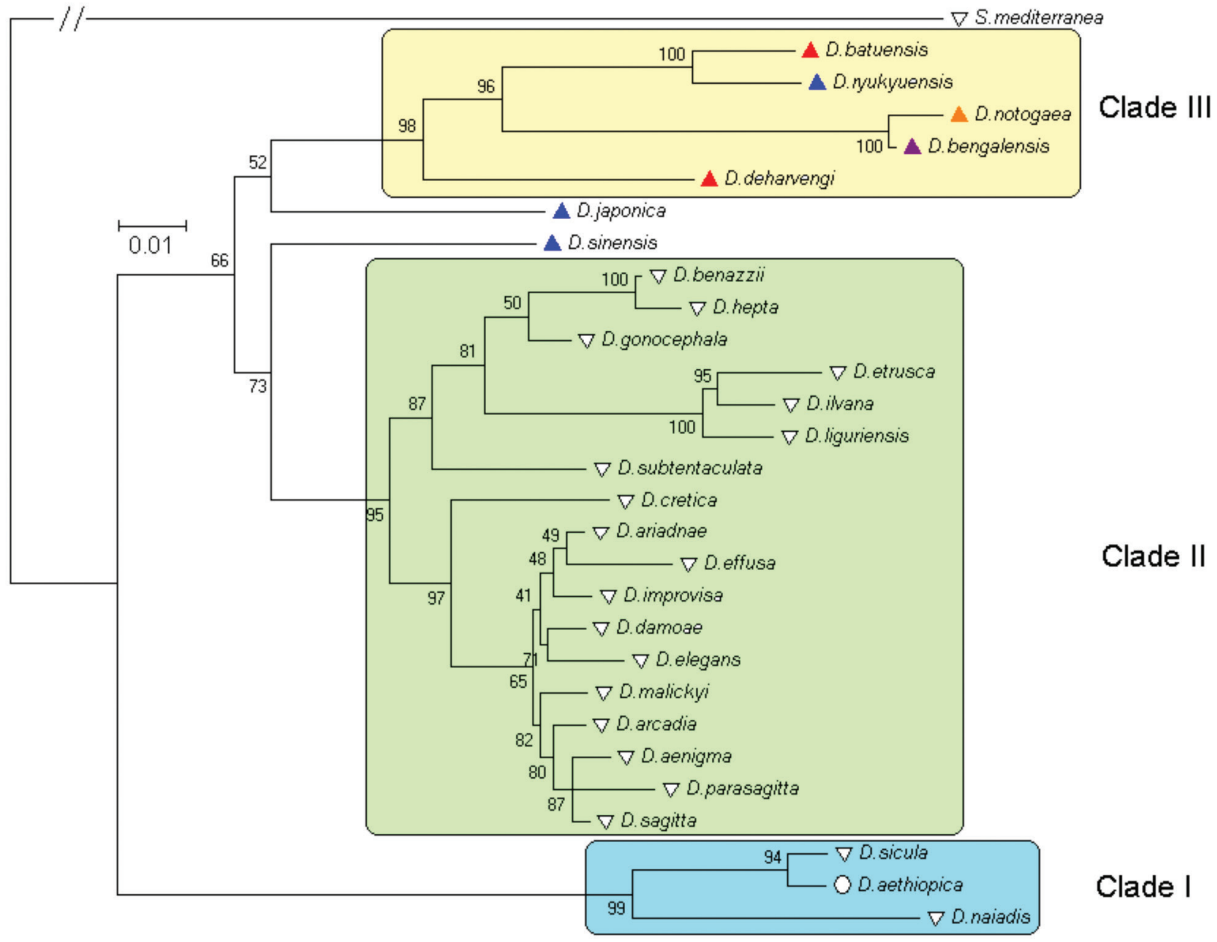

Fig. 5. Maximum likelihood tree inferred from concatenated COI, ITS-1 and 28SrRNA sequence data. Species from the Western Palearctic region are marked by inverted triangles, those from Ethiopia by circles, and those from the Oriental-Australasia region by coloured triangles, with colours indicating geographical location (red: Southeast Asia; blue: East Asia; purple: India; orange: Australia). The three main clades in the tree with strong bootstrap support at the base are highlighted in blue (Clade I), green (Clade II), and beige (Clade III). Branch length for *S. mediterranea* was 0.37 nucleotide substitutions per nucleotide site.

D. notogaea in Australia arose as an outcome of speciation from ancestral forms in Southeast Asia. Thus, expansion of taxon coverage of *Dugesia* species from the Southeast Asian region could be invaluable for further strengthening this hypothesis. In terms of molecular species delimitation, the 25-bp repeat discovered in ITS-1 for *D. deharvengi* is a novel molecular apomorphy for this species, which is the second one reported after those in *D. sicula* and *D. aethiopica*.

Taxonomically, *D. ryukyuensis* was elevated to the rank of species from *D. japonica* based on morphological and karyological (Kawakatsu et al., 1976; Kawakatsu et al., 1989; Kawakatsu et al., 1995) evidence. *Dugesia japonica* has a wide distribution encompassing mainland China, Taiwan, the Korean peninsula and the Japanese islands, whereas *D. ryukyuensis* has only been recorded from the Ryukyu Islands in southwest Japan. It seemed natural to attribute the divergence of these two species to vicariance. However, analysis of the correlation between the anatomical characters of the penis papilla and the karyotype prompted Kawakatsu et al. (1995) to suggest a closer affinity between *D. ryukyuensis* and *D. batuensis*, which have geographically separated distributional ranges. Their conclusion was based on two lines of argument: i) both species share an asymmetrical penis papilla with a well-developed valve surrounding its basal part; ii) both have the same chromosome number (2n=14 against 2n=16 of *D. japonica*) with similar chromosome morphology. Indeed, a maximum parsimony phylogenetic analysis of 68 *Dugesia* species using 15 morphological characters (Sluys et al., 1998) clustered both species together as well.

At present, it remains unclear whether the ancestral species of the Oriental-Australasia clade originated in the Far East, or possibly diverged from an ancestor in a broad region covering Ethiopia and the Middle East, since sequence data are to date not available from species distributed in the regions across Middle East and India (e.g. *D. iranica* De Vries, 1988 from Iran, and *D. lindbergi* De Beauchamp, 1959 from Pakistan; *D. tamilensis* Kawakatsu, 1980 and *D. andamanensis* (Kaburaki, 1925) from India).

We are still a long way from achieving confident inference of the sequence of paleogeographical events consistent with the known biogeographical distribution of extant *Dugesia* species and their updated molecular phylogeny in Southeast Asia, since this region has a complex palaeogeography (Hall, 2009). The present Southeast Asian land mass is an outcome of multiple allochthonous continental terranes of Gondwanaland origin joining together sequentially at different geological periods, a process likely to have been completed by the end of the Triassic (Metcalfe, 1988). Interestingly, the type localities of Batu Caves and Tham Nen Noi for *D. batuensis* and *D. deharvengi*, respectively, are located in different terranes–the former in the Sibumasu terrane and the latter in the Indochina terrane. The sequence of suturing of these two terranes to the South China terrane may be important for explaining the phylogeny of Southeast Asian *Dugesia* species, but whether the Indochina terrane sutured to the South China terrane first before the Sibumasu terrane remains an open question (Metcalfe, 1988). However, biogeographical analyses need not always be explained in the context of a particular paleogeographical scenario, since paradoxical results can also be an indication of problems in existing paleogeographical hypotheses (Sluys, 1995).

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