Zootaxa 5277 (3): 585–599
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5277.3.11
http://zoobank.org/urn:lsid:zoobank.org:pub:97763772-418C-4C74-9EB4-A4A01D823C85

Rediscovery of *Bipalium admarginatum* de Beauchamp, 1933 (Platyhelminthes, Tricladida, Geoplanidae) in Malaysia, with molecular characterisation including the mitogenome

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Abstract

We present here the first observation of *Bipalium admarginatum* de Beauchamp, 1933 since its original description 90 years ago. Three specimens were found on Perhentian Kecil Island, off Terengganu State, Malaysia and photographed in the field, and two were collected. This report thus includes the first colour photographs published for this species, from a locality close to the type-locality, Tioman Island (which is ca. 200 km south of the locality in this study, on the east coast of Peninsula Malaysia). We describe the external morphology and colour pattern of the species, which correspond well to the original description, itself based only on two preserved specimens. We performed an in-depth molecular characterisation of the species, including its complete mitochondrial genome, the *18S* sequence and elongation 1-alpha (*EF1-α*) sequence. In addition, *EF1-α* sequences were also retrieved for 5 additional geoplanid species. No *tRNA-Thr* could be detected in the mitogenome of *B. admarginatum*, a lack already reported in several species of geoplanids, but we found a 13 bp sequence that contains the anticodon loop and seems to be conserved among geoplanids and might thus possibly represent a non-canonical undetected tRNA. We discuss the difficulties encountered in trying to reconstruct the cluster of nuclear ribosomal genes, a problem already mentioned for other Triclads. Three phylogenies, based respectively on all mitochondrial proteins, *18S*, and *EF1-α*, were computed; the position of *B. admarginatum* within the Bipaliinae was confirmed in each tree, as sister-group to various bipaliine species according to the sequences available for each tree. In the mitochondrial proteins tree, which had high support, *B. admarginatum* was sister to *Bipalium kewense* and *Diversibipalium multilineatum*.

Key words: Land flatworms, *18S*, *EF1-α*, mitochondrial genome, phylogeny

Penemuan semula *Bipalium admarginatum* de Beauchamp, 1933 (Platyhelminthes, Tricladida, Geoplanidae) di Malaysia dengan percirian molekular yang merangkumi mitogenome.

Kajian ini membentangkan pemerhatian pertama *Bipalium admarginatum* de Beauchamp, 1933 sejak penerbitan asal dari 90 tahun lalu. Tiga spesimen telah dirakam di Pulau Perhentian Kecil, Terengganu, Malaysia, dan dari tiga spesimen ini, dua telah dikumpul untuk kajian selanjutnya. Penemuan ini memaparkan gambar berwarna pertama yang diterbitkan untuk spesies ini yang asalnya telah dijumpai di Pulau Tioman, lokaliti 'type' *B. admarginatum* di Malaysia. Morfologi luaran dan corak warna spesies telah dihuraikan dan pemerhatian ini adalah sepadan dengan spesies dari penerbitan asal, berdasarkan dua spesimen yang telah dikumpul dalam kajian ini. Kami turut menjalankan pencirian molekul bagi spesies, termasuk genom mitokondria lengkap, jujukan *18S* dan pemanjangan '1

Accepted by E. Solà: 11 Apr. 2023; published: 3 May 2023

alpha'. Selain itu, jujukan *EF1-α* juga telah dikumpulkan untuk 5 spesies geoplanid tambahan. Tiada *tRNA-Thr* yang dikesan dalam mitogenome *B. admarginatum* yang sebelum ini telah dilaporkan dalam penerbitan beberapa spesies geoplanid. Kami mendapati jujukan 13 bp mengandungi gelung anti-kodon yang nampaknya dipelihara di kalangan geoplanid dan mungkin mewakili tRNA bukan kanonik yang tidak dapat dikesan. Kami turut membincangkan kesukaran yang dihadapi dalam percubaan membina semula kelompok gen ribosom nuklear dan ini adalah satu masalah yang telah diperhatikan di kalangan 'Triclad' lain. Tiga filogeni, yang masing-masing berdasarkan semua protein mitokondria, *18S*, dan *EF1-α*, telah dihasilkan. Kedudukan *B. admarginatum* dalam Famili Bipaliinae telah disahkan dalam setiap pokok filogeni, sebagai kumpulan saudara kepada spesies bipaliine berlainan mengikut jujukan terdahulu untuk setiap pokok filogenetik. Dalam pokok filogeni protein mitokondria yang mempunyai sokongan yang kukuh, *B. admarginatum* menunjukkan hubungan filogenetik yang dekat dengan *B. kewense* dan *Diversibipalium multilineatum*.

Introduction

Land flatworms (family Geoplanidae) include about 300 species, mainly found in tropical areas, and are famous for their ability to conquer new habitats worldwide and to become invasive alien species (Carbayo *et al.*, 2016; Justine *et al.*, 2015, 2018, 2020b, 2021, 2022b; Sluys, 2016; Winsor, 1983). The Bipaliinae or hammerhead flatworms are among the giants in this family, with species reaching almost one metre in length (Kawakatsu *et al.*, 1982); their area of origin is South-East Asia and Madagascar. A recent study modelling potential distribution (Fourcade *et al.*, 2022) found that the five more common bipaliines, already found on various continents, could still invade more regions of the world. According to scenarios of future climate change, two species (*Bipalium kewense* Moseley, 1878 and *Bipalium vagum* Jones & Sterrer, 2005) that already have the largest observed global range are predicted to further increase their potential distribution (Fourcade *et al.*, 2022).

This study is not about an invasive species found far away from its area of origin, but instead it is about a rarely recorded species, *Bipalium admarginatum* de Beauchamp, 1933, which we collected in the area where it was first described, islands off the East coast of Peninsular Malaysia. We subjected our specimens to molecular techniques that had been, until now, mostly used on invasive species. The complete mitogenome was sequenced and compared with existing data (Gastineau *et al.*, 2019; Justine *et al.*, 2022a). The phylogenetic position of *B. admarginatum* was studied with 3 different datasets. Special attention was paid to the search for a missing mitochondrial tRNA. Finally, an attempt to reconstruct the two variants of the cluster of nuclear ribosomal RNA genes was undertaken.

This paper provides information about a rarely mentioned species and molecular data which we hope will be useful for comparison with other geoplanids.

Material and methods

Origin of the specimens

Observations, photographs, and collections were performed by one of us (GV). Three specimens were found on the soil on the edge of primary jungle on the east coast of Perhentian Kecil Island, off Terengganu State in Peninsular Malaysia. The first one, found on 26 August 2019, was photographed in natural light but not collected. Two specimens were found (5°54'22.2"N 102°43'55.7"E) on 28 September 2019; they were photographed live (both in natural light and under artificial light) and then fixed in gin (ca. 40% ethanol), in the absence of a more scientific fixative. After several days, the specimens were shipped to Kuala Lumpur for examination, and the gin was replaced by 100% ethanol. Specimens were then shipped to Paris and deposited in the collection of the Muséum National d'Histoire Naturelle under registration numbers MNHN JL354 and MNHN JL355. Photographs of preserved specimens were taken, and the posterior part of the body of specimen MNHN JL354, about 1 centimetre in length, was taken for molecular studies.

Morphometric data

Morphometric data of living and preserved specimens were calculated from scaled photographs. Colour names and codes were taken from the RAL colour chart at https://www.ralcolorchart.com/.

Sequencing and assembly

A piece of specimen MNHN JL354 of *B. admarginatum* was sent to the Beijing Genomics Institute (BGI) in Shenzhen (China). DNA was extracted by BGI in accordance with their internal protocol. A total of ca. 80M clean 150 bp paired-end reads were obtained from a DNBSEQ platform and assembled using SPAdes 3.15.5 (Bankevich *et al.*, 2012) with a k-mer parameter of 125. The sequences of interest were found among the contig file resulting from assembly by using blastn command-line (Boratyn *et al.*, 2012) with databases made of the full mitogenome, partial *18S* and partial elongation factor 1-alpha (*EF1-α*) genes of *Bipalium adventitium* Hyman, 1943 (GenBank accession numbers MZ561467, MZ520993 and KJ599681, respectively). Annotation of the mitogenome was carried out using MITOS (Bernt *et al.*, 2013). The position of tRNA was checked using Arwen v1.2 (Laslett & Canbäck, 2008). The OGDRAW web portal was used to draw the map of the mitogenome (Lohse *et al.*, 2013). The LOGO figure of the tRNA-Thr alignment was obtained using WebLogo3 online (Crooks *et al.*, 2004).

Phylogeny

Three different maximum likelihood phylogenies were generated. The first two were obtained by appending the new data on *B. marginatum* to datasets already used in previous papers (Gastineau *et al.*, 2022; Justine *et al.*, 2022a), consisting of concatenated alignments of the mitochondrial proteins (21 sequences) and alignment of the partial *18S* gene (15 sequences). The third one was obtained from alignment of the partial *EF1-α* gene nucleotidic sequences obtained in the course of this study with references coming from previous works (Almeida *et al.*, 2021; Álvarez-Presas & Riutort, 2014; Carbayo *et al.*, 2013) for a total of 279 sequences. The percentage identity between *EF1-α* sequences was calculated after alignment by Clustal Omega (Sievers *et al.*, 2011). All other alignments were done using MAFFT 7 (Katoh & Standley, 2013) with the -auto option. For the multigene phylogeny, amino-acid sequences were aligned separately, trimmed and then concatenated using Phyutility 2.7.1 (Smith & Dunn, 2008). All trimming was performed with trimAl (Capella-Gutiérrez *et al.*, 2009) and the -automated1 option. Evolutionary models were selected based on results returned by ModelTest-NG v0.1.7 (Darriba *et al.*, 2019), with the concatenated sequence of mitochondrial proteins being considered a single amino-acid sequence. Maximum likelihood (ML) phylogenies were obtained using IQ-TREE 2.2.0 (Minh *et al.*, 2020) after 1 000 bootstrap replications. In the case of the amino-acid dataset, a Bayesian inference (BI) phylogeny was also performed using MrBayes v3.2.7 (Ronquist *et al.*, 2012) on files formatted with ALTER (Glez-Peña *et al.*, 2010). The model of evolution was also obtained from ModelTest-NG v0.1.7. The Bayesian inference phylogeny was stopped after 100000 generations, with sampling and print frequencies of 100 and diagnostic frequency of 1000. GenBank accession numbers of all sequences used in the phylogenies are indicated in Figures 6–8.

Results

Description of the specimens (Figures 1-3)

An elongate living specimen (MNHN JL355) measured approximately 103 mm long, and 2.9 mm maximum wide over the pharyngeal region (**Figure 2B**). The fan-shaped headplate measured 2.1 mm in maximum width, less than the maximum body width, and had a length to width ratio of 1:1.8, with very slightly recurved small lappets. The transverse body shape is broadly convex. Eyes are visible as fine black spots, mainly around the lateral quarters of the headplate, and continuing posteriorly just above the marginal black stripes.

The preserved specimen (MNHN JL355) measured 77.7 mm long, and 4.1 mm maximum wide over the pharynx, with the mouth 25.0 mm from the anterior end, and gonopore 5.9 mm behind the mouth (**Figure 3B**). The narrow creeping sole is estimated to be 20% of the body width and is slightly raised above the convex surface.

Since colours appear slightly different for specimens photographed in natural light and under artificial light, we show photographs taken under both conditions (**Figures 1-2**). The colour and pattern of the living specimen (**Figure 1**) are as follows: the anterior two-thirds of the headplate, reaching in a curve from lappet to lappet, is Black-grey (RAL 7021) in colour, with the posteriad headplate lighter Telegrey 4 (RAL 7047). Behind the headplate are three transverse bands, Jet Black (RAL 9005) in colour. The margins of these bands are irregular, and the length of each band is slightly greater than the one before it. The black bands are separated by two transverse bands coloured Traffic White (RAL 9016). The dorsal ground colour of the 85% of the remaining body is slightly darker than Telegrey 4. Three dorsal longitudinal stripes that terminate just short of the rounded posterior tip are present. They comprise a broad, gently tapering mid-dorsal Jet Black stripe extending from just behind the hindmost black band, and widest over the pharyngeal region, and paired narrow Jet Black marginal stripes of even thickness that extend posteriorly from the last black transverse band. In the living specimen, a fine faint grey, discontinuous longitudinal stripe, slightly darker than the ground colour, is present between the median and marginal stripes (**Figure 2B**), but not visible in the preserved specimen.

The ventral ground colour is Telegrey 4, with whitish creeping sole delineated on either side by a fine Black-grey longitudinal stripe. In the anterior ventral surface of the preserved specimen (JL355), the two white bands, and the first two transverse black bands, though less strongly pigmented, wrap around the body as far as the ventral zone.

The form of the dorsal transverse bands and longitudinal stripes appeared constant in the other specimens examined (JL354), as well as a specimen from Tioman Island Mersing, Jahor, Malaysia (iNaturalist 21 February 2021; https://www.inaturalist.org/observations/69891718 accessed January 2023). The ventrad extension of the transverse bands was observed in both preserved specimens JL354 and JL355. Morphometric data for the preserved specimens are provided in **Table 1**. Neither of these specimens were examined histologically.

TABLE 1. The three specimens found and their measurements. Percentages are of total length

Specimen	**Living**	**Preserved**						
	Length	**Length**	**Width**	**Creeping sole: body width**	**Distance of the mouth from the anterior end**		**Distance of the gonopore from the mouth**	
Unit	mm	mm	mm	%	mm	%	mm	%
Not collected	ca. 45	-	-		-	-	-	-
MNHN JL354	ca. 131	58.0	3.6	25	15.9	27.4	-	-
MNHN JL355	ca. 103	77.7	4.1	20	25.0.	32.2	5.9	6.9
de Beauchamp, 1933	-	85	3	25	30	35.3	6	7.1

FIGURE 1. *Bipalium admarginatum*, live specimens photographed in the field under natural light. A, specimen not collected, photographed 16-08-2019. B, specimen MNHN JL354 (a part of this specimen was used for the molecular analysis). Unscaled. Photos by George Verdon.

FIGURE 2. *Bipalium admarginatum*, live specimens photographed under artificial light. A, specimen MNHN JL354; B, Specimen MNHN JL355. Photos by George Verdon.

FIGURE 3. *Bipalium admarginatum*, preserved specimens. A, specimen MNHN JL354, undamaged; B-D, specimen MNHN JL355; B, undamaged; C, partially showing ventral surface; D, damaged, showing ventral surface and position of mouth and gonopore. A, B, photographs taken in 2019 vs. C, D, photographs taken in 2023, note that artefactual yellow colour seen in the earlier photographs has vanished. Photos by Jean-Lou Justine.

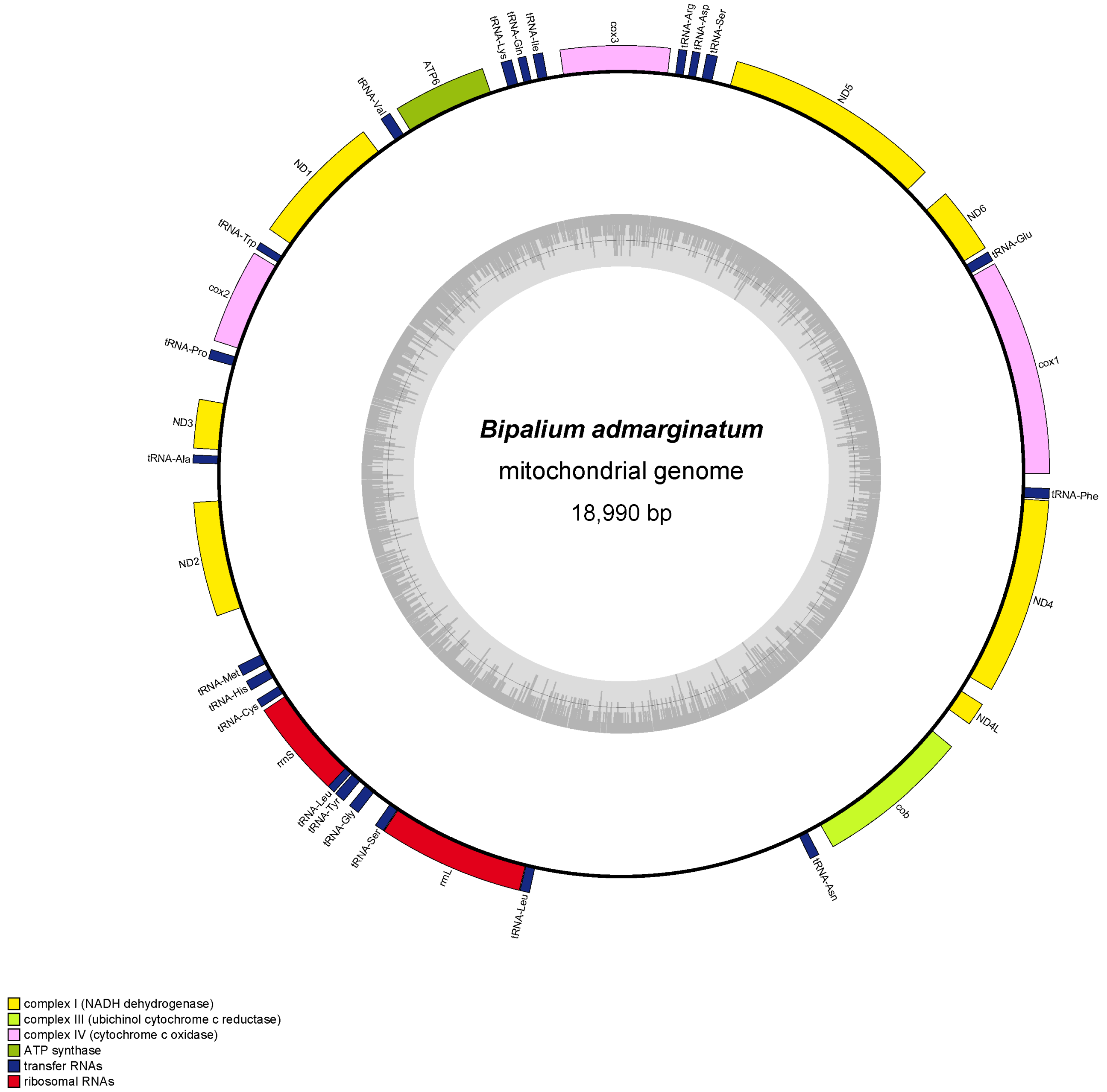

FIGURE 4. *Bipalium admarginatum*, map of mitochondrial genome. The mitogenome codes for 12 conserved protein-coding genes, 21 tRNA and 2 rRNA.

Mitogenome

Although the specimen was originally processed with a somewhat unusual protocol, including fixation in a non-scientific ethanol solution (gin), sequencing allowed to recover the mitogenome with a high coverage.

A 19 115 bp long contig with a coverage of 178.61 × and redundant endings was retrieved from the assembly. After trimming and circularisation, the mitogenome (GenBank OQ308795) is 18 990 bp long **(Figure 4).** It codes for 12 conserved protein-coding genes, 21 tRNA and 2 rRNA. It is for now the largest known mitogenome for a Bipaliinae (Table 2). This extra length results from intergenic sequences, especially a large region located between *rrnL* and *cob*. This region is 2 288 bp long and contains only 2 tRNA. A comparison of the size of this region among Bipaliinae is presented in **Table 2**. In a more general way, it should be noted that the mitogenome is not compact, with intergenic lengths parsed among it.

TABLE 2. Mitogenomes of Bipaliinae and various characteristics

Species	GenBank	Size	Length of the *rrnL-cob* intergenic region and presence of tRNA
Bipalium admarginatum	OQ308795	18 990 bp	2288 bp (*tRNA-Leu, tRNA-Asn*)
Bipalium kewense	MK455837	15 666 bp	934 bp (*tRNA-Leu, tRNA-Thr, tRNA-Asn*)
Bipalium vagum	MZ561468	17 149 bp	1552 bp (*tRNA-Leu, tRNA-Thr, tRNA-Asn*)
Bipalium adventitium	MZ561467	15 494 bp	741 bp (no tRNA detected)
Diversibipalium multilineatum	MZ561469	15 660 bp	Impossible to circularize (*tRNA-Leu*, *tRNA-Thr*, *tRNA-Asn*)
Diversibipalium mayottensis	MZ561470	15 989 bp	1479 bp (*tRNA-Leu, tRNA-Thr, tRNA-Asn*)
Humbertium covidum JL351	MZ561472	15 540 bp	691 bp (*tRNA-Leu, tRNA-Asn*)
Humbertium covidum JL090	MZ561471	15 524 bp	672 (*tRNA-Leu, tRNA-Asn*)

The case of the missing *tRNA-Thr*

No *tRNA-Thr* could be detected in the mitogenome of *B. admarginatum*. This lack has already been noticed in the Bipaliinae *Humbertium covidum* Justine, Gastineau, Gros, Gey, Ruzzier, Charles & Winsor, 2022 (Justine *et al.*, 2022a), but also among three species of Rhynchodeminae, namely *Parakontikia ventrolineata* (Dendy, 1892) Winsor, 1991 (Gastineau & Justine, 2020), *Platydemus manokwari* de Beauchamp, 1963 (Gastineau *et al.*, 2020) and *Australopacifica atrata* (Steel, 1897) (Gastineau *et al.*, 2022).

We tried to align the *tRNA-Thr* genes from mitogenomes of Geoplanidae where they were detected, including the Geoplaninae *Obama nungara* Carbayo, Álvarez-Presas, Jones & Riutort, 2016 (Solà *et al.*, 2015) and *Amaga expatria* Jones & Sterrer, 2005 (Justine *et al.*, 2020a), with the complete mitogenomic sequences of *B. admarginatum* and *H. covidum* JL090 and JL351. The *tRNA-Thr* genes aligned in an area that corresponds to their assessed position in other species, between *cob* and *rrnl*. The alignment showed the presence of a nearly perfectly conserved 13 bp sequence that contains the anticodon loop as it can be seen on the LOGOS alignment provided as **Figure 5**. Only *Diversibipalium mayottensis* Justine, Gastineau, Gros, Gey, Ruzzier, Charles & Winsor, 2022 and *Bipalium vagum* exhibited each a single polymorphism in this sequence. The 5' and 3' ends of the 72 bp sequence derived from *B. admarginatum* were completely conserved for 4 bp in 5' and 6 bp in 3' with *Bipalium kewense* Moseley, 1878. Attempting to manually model a functional tRNA for *B. admarginatum* based on this sequence remained non-conclusive (figure not shown), leading to a tRNA that would have the following characteristics: an acceptor stem with a single mismatch out of 7 pairs, but one mismatch plus one unpaired base in the anticodon arm; complete lack of a D-arm; the putative TΨC-arm would have had at maximum 3 well matched pairs of nucleotides and 21 unpaired bases.

Trying to reconstruct the cluster of nuclear ribosomal RNA genes

The cluster of rRNA did not come as a single sequence of high coverage. Instead, several contigs returned a positive blastn result. These contigs varied in both size and coverage. **Table 3** summarizes the names of the contigs that were used in our attempt to reconstruct the rRNA, with their sizes and coverages.

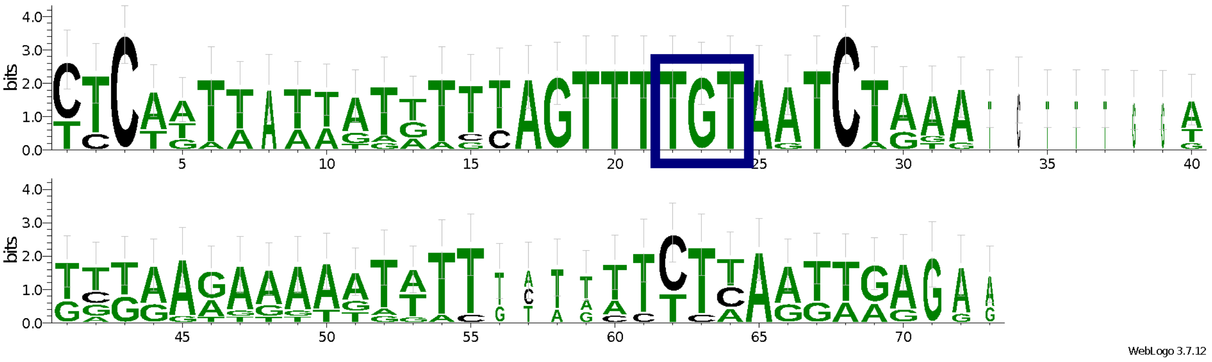

FIGURE 5. Sequence motifs obtained by aligning with WebLogo3 the *tRNA-Thr* genes annotated in the mitogenomes of *Diversibipalium multilineatum*, *Bipalium kewense*, *Diversibipalium mayottensis*, *Bipalium vagum*, *Obama nungara* and *Amaga expatria* with the suspected corresponding sequences of *Humbertium covidum* and *Bipalium admarginatum*. The height of each nucleotide indicates the conservation at that position. The blue box shows the conserved sequence of the anticodon.

TABLE 3. Contigs used to reconstruct the nuclear ribosomal RNA genes

Name of the contig	Size (in bp)	Coverage (in ×)
NODE_62	5230	629.757884
NODE_127	3569	99.339431
NODE_190	2948	98.593340
NODE_203	2863	65.301315
NODE_245	2559	859.470008
NODE_339	2141	1147.058532
NODE_1121	1182	1437.595080
NODE_4793	762	1499.594976
NODE_84672	313	1800.345745

Some of these contigs were overlapping with others by a number of 125 bp, corresponding to the k-mer used for assembly. Two of these highly covered contigs, NODE_4793 and NODE_84672, were found to merge with both high and low coverages contigs. This situation was understood as the presence of two types of rRNA, first evidenced among Dugesiidae (Carranza *et al.*, 1996, 1999) and later among other Tricladida (Breugelmans *et al.*, 2012). We attempted to reconstruct the complete clusters, which we will therefore refer to as 'high' and 'low', by merging overlapping contigs with respect to their coverages (except for the aforementioned NODE_4793 and NODE_84672). **Table 4** provides the names and order of the contigs used in the attempt to reconstruct both clusters.

TABLE 4. Contigs used in the attempt to reconstruct both clusters

Type	Contigs (5' to 3', starting with *18S*)
High	NODE_62-NODE_4793-NODE_1121-NODE_339-NODE_84672-NODE_245
Low	NODE_203-NODE_4793-NODE_190-NODE_84672-NODE_127

For the *18S* phylogeny computed in this paper, only contig NODE_4793 was used, as it is common to both putative types and also corresponds to the portion of the *18S* gene previously used in Justine *et al*. (2022). Only this fragment has been deposited in GenBank (accession number: OQ308840). The sequences of the two putative clusters can be downloaded from Zenodo, as indicated in the Data availability statement.

EF1-α gene

It was possible to retrieve the complete *EF1-α* gene of *B. admarginatum* (OQ326523). The intronless gene is 1 392 bp long. This complete coding sequence was later used to datamine the previous sequencing results from Gastineau *et al*. (2019) and Justine *et al*. (2022a). It was possible to retrieve the same complete coding sequence for *Bipalium kewense* JL184A (OQ326519), *Humbertium covidum* JL090 (OQ326520), *Diversibipalium multilineatum* JL177 (OQ326521) and *Bipalium vagum* JL307 (OQ326522). All genes were of identical lengths. **Table 5** lists the species, the GenBank accession number of their *EF1-α* gene obtained in the course of this study. and the percentage of identity between them as calculated after alignment by Clustal Omega.

TABLE 5. Species of Bipaliinae and the percentage identity of *EF1-α* gene between them

Name	*B. vagum*	*H. covidum*	*B. admarginatum*	*B. kewense*	*D. multilineatum*	GenBank
B. vagum	100.00	90.23	91.02	91.09	91.24	OQ326522
H. covidum		100.00	91.88	91.95	92.60	OQ326520
B. admarginatum			100.00	94.32	94.47	OQ326523
B. kewense				100.00	97.92	OQ326519
D. multilineatum					100.00	OQ326521

Molecular phylogenies

The best models of evolution suggested by ModelTest-NG were mtZOA+I+G4 and CPREV+I+G4 for respectively the ML and BI phylogenies based on the mitochondrial proteins, TVM+I+G4 for *18S* and GTR+I+G4 for *EF1-α*. The two first phylogenies were congruent with previously published works (Justine *et al.*, 2022). The mitochondrial amino acid inferred phylogeny (**Figure 6**) placed *B. admarginatum* as sister species to a clade containing *B. kewense* and *Diversibipalium multilineatum* Makino & Shirasawa, 1983, with strong support at the nodes and identical topology for ML and BI. In the main clade of the Geoplanidae, the lowest support was found at the node separating Bipaliinae and Rhynchodeminae, with only 66% support (ML). In the BI phylogeny, all posterior probability were equal to 1. The *18S*-inferred phylogeny (**Figure 7**) associated *B. admarginatum* with *Novibipalium venosum* (Kaburaki, 1922), but with weak support at the node. For the *EF1-α* inferred tree, only the subtree containing the Bipaliinae is shown (**Figure 8**). The tree associated *B. admarginatum* with *Bipalium adventitium*, although with low support at the node. The tree displayed *B. mayottensis* at some distance from the rest of the Bipaliinae in a similar way to the mitochondrial protein inferred phylogeny.

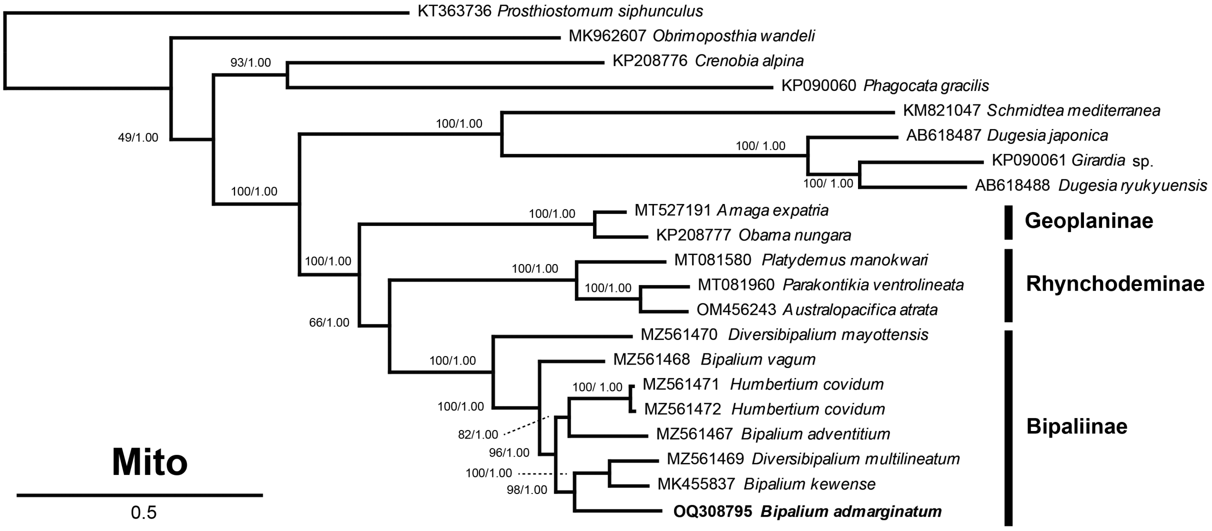

FIGURE 6. Maximum likelihood phylogenetic tree obtained from concatenated amino-acid sequences of the mitochondrial proteins of *Bipalium admarginatum* and other flatworms. The tree represents both Maximum likelihood and Bayesian inference phylogenies, performed using mtZOA+I+G4 and CPREV+I+G4 models of evolution, respectively. The tree with the best likelihood is shown, and bootstrap values are indicated. The BI tree had an identical topology, posterior probabilities are indicated on the right as decimal values. Subfamilies of Geoplanidae are indicated on the right. Based on the matrix and method used in Gastineau *et al.* (2022) with addition of the new sequence of *B. admarginatum*.

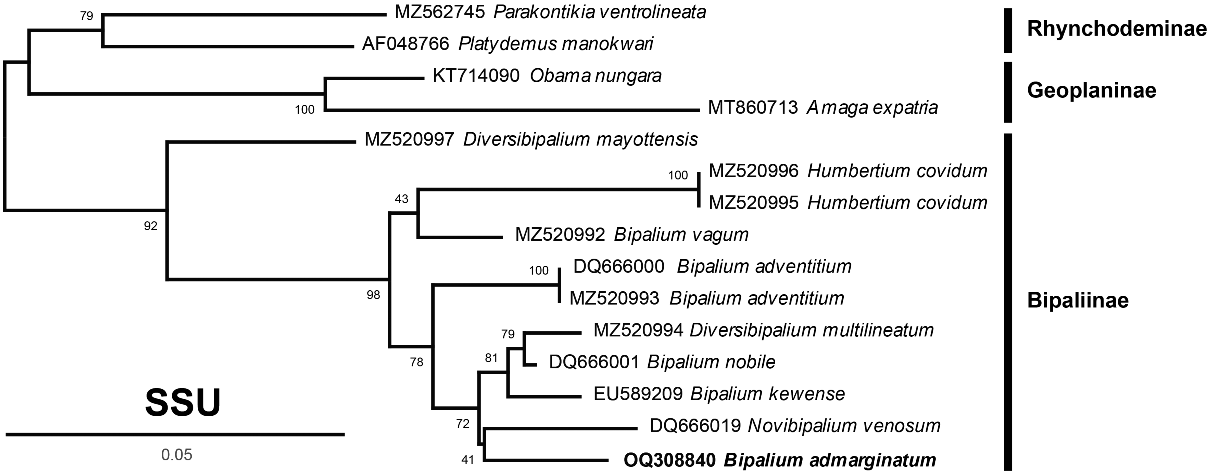

FIGURE 7. *18S* phylogenetic tree of bipaliine geoplanids. Maximum likelihood phylogenetic tree based on 15 partial *18S* genes, using the TVM+I+G4 model of evolution. The tree with the best likelihood is shown, and ML bootstrap support values are indicated. The subfamilies within the Geoplanidae (Rhynchodeminae, Geoplaninae and Bipaliinae) are indicated. Based on the matrix and method in Justine *et al.* (2022) with the addition of the new sequence of *Bipalium admarginatum*.

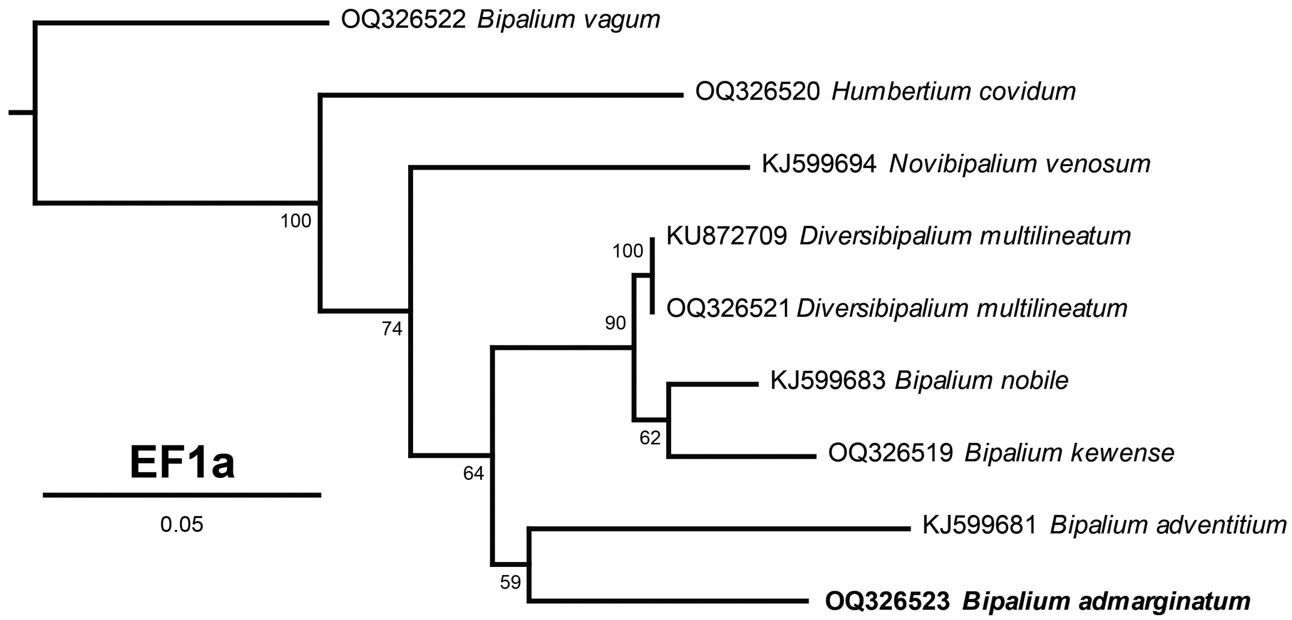

FIGURE 8. Phylogenetic tree of geoplanids based on nine sequences of elongation factor 1-alpha (*EF1-α)*, including five new; only the part of the tree containing the Bipaliinae is shown. Maximum likelihood phylogenetic tree based on 9 sequences, using the GTR+I+G4 model of evolution. All OQ sequences are our own and new.

Discussion

Identification and rediscovery of *Bipalium admarginatum*

Bipalium admarginatum was described by de Beauchamp (1933) from two specimens collected in Sedagong, altitude 1 000 feet, on Tioman Island, in 1927. The illustration associated with the description includes a schematic diagram of the eyes on the head and a longitudinal section of the histology of the copulatory apparatus, and a half-tone drawing of the dorsal side of what is approximately the anterior half of a specimen.

The locality where we found our specimens (Perhentian Kecil Island) is ca. 200 km north of the type-locality (Tioman Island) and both localities are on the east coast of Peninsular Malaysia. Interestingly, each record was on an island. There is a single observation of this species in iNaturalist, in Tioman Island, the type-locality, on 12 May 2018 (iNaturalist observation #69891718). The present paper is the first scientific record of the species since its original description in 1933, 90 years ago.

We present here the first coloured photograph of a living specimen of *Bipalium admarginatum*. The ground colours of our specimens differ significantly from the light brown of the preserved specimens from Tioman Island upon which the original description (De Beauchamp, 1933) was based. The brown colour of these specimens was probably artefactual alcohol browning. Otherwise, the band, stripe, and eye pattern of the specimens examined in this study agree closely with the original description. Our preserved specimens are an artefactual yellowish colour, and are not as long as the two examined by de Beauchamp (85 mm and 90 mm), but the relative positions of the mouth and gonopore of our specimen (JL355) align well with those in the original description (**Table 1**). The extension of the transverse bands to the ventral zone, observed in both preserved specimens of *B. admarginatum*, has also been observed in another transversely and longitudinally striped species, *Diversibipalium boehmigi* (Müller, 1902) recorded from Mount Matang, Sarawak, Malaysia (Müller, 1902).

Molecular characteristics

The fact that we found a conserved pattern of the anticodon loop of the *tRNA-Thr* among *B. admarginatum* and *H. covidum* is intriguing at least, keeping in mind that all dedicated software failed until now to detect it among these mitogenomes. We are not ruling out the possibility that this tRNA indeed exists with a heavily modified structure as it can sometimes be found among Metazoa (Krahn *et al.*, 2020). Armless mitochondrial tRNA have, for example, been found *in silico* among Nematoda (Jühling *et al.*, 2012, 2018) and their presence assessed by biochemical proofs (Wende *et al.*, 2014). However, in the absence of similar proofs, we refrain from including this *tRNA-Thr* in our annotations, which does not preclude looking for the conserved anticodon loop among other mitogenomes of Geoplanidae among which this tRNA was not detected.

In a comparable way, we have chosen to restrict our use of the nuclear rRNA to a portion of the *18S* gene that seems to be common to the two types, and only submitted this fragment to GenBank. Although our 'hand-made' reconstruction of these two clusters is plausible, we are concerned that we have possibly reached the boundaries of what can be obtained by short-read sequencing technologies. Perhaps 'long-read' technologies such as those that sequence native DNA will prove fruitful in the near future. We are nonetheless convinced of the importance of this topic for its evolutive significance, the molecular and biochemical implications it induces, but also because of the putative bias it can induce in phylogeny and molecular taxonomy.

Conclusion

This study is probably the first of a complete mitogenome for a geoplanid which has *not* been recorded as an invasive species. We expect to continue such studies on various members of the geoplanids, which will provide additional data to understand the evolution of land flatworms.

This study is also pioneering with regard to the determination of *EF1-α* sequences; it will be necessary to confirm on other species whether these sequences are interesting in terms of understanding the phylogeny of the Geoplanidae.

Regarding conservation issues, we note that this species has only been found on islands off the east coast of Peninsular Malaysia, which are probably more unspoiled habitats than the coast. It would be interesting to look for this species, and others, on the mainland.

We also note that excellent sequencing results were obtained for specimens originally preserved in the field with a very non-scientific product (gin), pending the correct fixation of the specimens in the laboratory in 100% ethanol. This is encouraging for future research. In the absence of the proper DNA fixative, those who unexpectedly happen upon interesting species could temporarily preserve the specimens in a similar way to that described here, until the flatworms can reach a laboratory. We already have molecular results (unpublished) on a flatworm which was fixed in locally produced moonshine. We also note that a recent paper (Kawahara *et al.*, 2023) reported successful DNA barcoding of most specimens of moth larvae in mezcal, which is, as gin, a beverage containing ethanol.

Data availability

All the sequences obtained in the course of this study can be downloaded from Zenodo following this link: https://doi.org/10.5281/zenodo.7573203.

Acknowledgements

We thank Dr Tan Koh Siang from the Raffles Museum who kindly checked whether any colour illustration of *Bipalium admarginatum* was available and provided documentation. We also thank Liv Grant for assistance in the field; this included fending off monkeys during flatworm collection.

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