



The terrestrial flatworm *Microplana scharffi* (Geoplanidae, Microplaninae): mitochondrial genome, phylogenetic proximity to the Bipaliinae and genes related to regeneration

ROMAIN GASTINEAU^{1*}, ARCHIE K. MURCHIE², DELPHINE GEY³, LEIGH WINSOR⁴ & JEAN-LOU JUSTINE⁵

¹Institute of Marine and Environmental Sciences, University of Szczecin, Szczecin, Poland

✉ romain.gastineau@usz.edu.pl; <https://orcid.org/0000-0001-8661-5118>

²Sustainable Agri-Food Sciences Division, Agri-Food and Biosciences Institute, Belfast, BT9 5PX, Northern Ireland

✉ archie.murchie@afbini.gov.uk; <https://orcid.org/0000-0003-0941-3832>

³Département Adaptations du Vivant (AVIV), Molécules de Communication et Adaptation des Microorganismes (MCAM, UMR 7245 CNRS), Muséum National d'Histoire Naturelle, CNRS, CP 52, 57 rue Cuvier, 75231 Paris Cedex 05, France

✉ delphine.gey@mnhn.fr; <https://orcid.org/0000-0002-5656-1793>

⁴College of Science and Engineering, James Cook University of North Queensland, Townsville, Queensland, Australia

✉ leigh.winsor@jcu.edu.au; <https://orcid.org/0000-0002-6679-470X>

⁵ISYEB, Institut de Systématique, Évolution, Biodiversité (UMR7205 CNRS, EPHE, MNHN, UPMC, Université des Antilles), Muséum National d'Histoire Naturelle, CP 51, 55 rue Buffon, 75231 Paris Cedex 05, France

✉ jean-lou.justine@mnhn.fr; <https://orcid.org/0000-0002-7155-4540>

*Corresponding author

Abstract

A genome skimming approach of sequencing was undertaken on a subfamily of terrestrial flatworms that had been neglected in genomic studies until now, namely the Microplaninae as represented here by *Microplana scharffi*. A single run of short-read sequencing enabled retrieval of the complete mitogenome, the two paralogous versions of the *18S* gene, the elongation factor gene *EF1 α* , plus two genes involved in the regeneration process, namely those coding for β -CATENIN-1 and adenomatous polyposis coli (APC). The 15,297 bp mitogenome lacks a functional *tRNA-Ala* and has a mandatory alternative TTG start codon in its *cox1* gene. The multiprotein phylogeny, inferred from mitogenome proteins, positions *M. scharffi* as sister-group to the Bipaliinae with maximum support, although the organisation of the mitogenomes shows features previously never observed among Bipaliinae, such as the conserved 32 bp overlap between *ND4* and *ND4L*. Similarly to what has been observed in recent publications on other species of Geoplanidae, the two types of *18S* genes display strongly different coverages and are only 90.57% identical. Additionally, alien DNA was identified in the pool of contigs in the form of the complete mitochondrial genome of *Lumbricus rubellus*, confirming previous observations on the feeding habits of *M. scharffi*.

Key words: Mitogenome, endemic terrestrial flatworm, multiprotein phylogeny

Introduction

Microplana scharffi (Graff, 1899) is a species of the subfamily Microplaninae, family Geoplanidae. The species has mostly been observed in the UK where it is considered native but has been recorded in other countries of Europe such as Belgium, Bulgaria, Ireland, Italy, Madeira Island, and Turkey (Sluys *et al.*, 2016), and, according to iNaturalist, also in Russia, Spain and the North America (https://www.inaturalist.org/observations?taxon_id=484652). Geoplanidae, also known as land flatworms or land planarians, have received increasing attention during the last few decades as several species became invasive in Europe and North America. The taxa currently under the spotlight represent subfamilies originating from South America, Asia or Oceania: for example, the Geoplaninae, exemplified by *Obama nungara* Carbayo, Álvarez-Presas, Jones & Riutort, 2016 (Carbayo *et al.* 2016; Fourcade 2021; Justine *et al.* 2020a, 2022b; Lago-Barcia *et al.* 2019; Negrete *et al.* 2020), the Bipaliinae such as *Bipalium kewense* Moseley, 1878

(Justine *et al.* 2018; Fourcade *et al.* 2022), and several species of Rhynchodeminae, among which the most infamous might be *Arthurdendyus triangulatus* (Dendy, 1894) and *Platydemus manokwari* de Beauchamp, 1963 (Justine *et al.* 2015, 2021; Jones 1999; Murchie & Gordon 2013).

These organisms are of concern because of their apparent lack of predators, coupled with their own predation on soil invertebrates (Blackshaw & Stewart 1992; Boag 2020; Christensen & Mather 1995; Fraser & Boag 1998; Haria 1995; Haria *et al.* 1998; Murchie & Gordon 2013; Roy *et al.* 2022). The large size and unusual appearance, such as a hammer-shaped head, of some of these flatworms has attracted the attention of the public as well as that of the scientific community.

In contrast to the aforementioned species and families, Microplaninae like *M. scharffi* are not damagingly invasive; rather, they are considered endemic in Europe, although some species have been found on the African (Jones 1998), American (Ogren & Kawakatsu 1998; Murchie & Justine 2021) and Asian continents (Kawakatsu & Ogren 1998). Possibly for this reason, they might have attracted less attention from scientists and the public when compared to exotic taxa, although all the studies conducted on them highlight their noteworthy, unsuspected species richness (Álvarez-Presas *et al.* 2022; Jones *et al.* 2008; Jones & McDonald 2021; Mateos *et al.* 2017; Vila-Farré *et al.* 2011). The phylogenetic position of Microplaninae has also been re-evaluated. Early works such as Jones *et al.* (2008) tended to associate them with Geoplaninae, but simply on the basis of a single gene molecular phylogeny and with weak support at the nodes of the phylogenetic tree. Then, using a three genes dataset based on partial sequences of the mitochondrial *cox1* gene and the nuclear rRNA 18S and 28S genes, Álvarez-Presas *et al.* (2008) suggested that Microplaninae are linked with a freshwater species of the genus *Spathula* Nurse, 1950, and cluster with Rhynchodeminae. Later, Álvarez-Presas & Riutort (2014) performed additional analyses by appending the partial Elongation Factor 1 α gene (*EF1 α*) to this dataset, which resulted in Microplaninae clustering with Bipaliinae.

In recent years, efforts have been made to document Geoplanidae with complete mitochondrial genomes (Solà *et al.* 2015; Gastineau & Justine 2020; Gastineau *et al.* 2019; 2020; 2022; Justine *et al.* 2020b, 2022a; Soo *et al.* 2023; Gastineau *et al.* 2024), leading to strongly supported molecular phylogenies. However, the sampling of species of these phylogenies is of course less extensive than the sampling for earlier molecular studies that were not based upon complete mitochondrial genomes. Among the missing taxa, no Microplaninae had yet been investigated.

A specimen of *M. scharffi* was obtained and sequenced. Its complete mitochondrial genome was obtained and the results were included in a multiprotein phylogeny. As far as the depth and quality of the sequencing allowed, data were also investigated for the presence of prey DNA. With respect to previous works such as Álvarez-Presas & Riutort (2014), the *EF1 α* gene was sought. Regeneration has been observed in Geoplanidae, including a member of *Microplana* (McDonald & Jones, 2013). In the wake of the recent studies on the regeneration capacities of planarian flatworms (Vila-Farré *et al.* 2023), we tried to datamine the sequencing results for the genes coding for the β -CATENIN-1 protein and the adenomatous polyposis coli protein (APC), two genes involved in the regeneration process. As first evidenced by Carranza *et al.* (1996; 1998), Geoplanidae display duplicated, diverging versions of their nuclear ribosomal RNA clusters, a peculiarity they share with Dugesiidae. Following our newly published work on the paralogous nuclear rRNA clusters of species of Bipaliinae and Rhynchodeminae (Soo *et al.* 2023; Gastineau *et al.* 2024), we also tried to obtain the two different versions of the rRNA genes for *M. scharffi*.

Material and Methods

Origin of the specimens

A specimen of *Microplana scharffi* (Figure 1) was collected in Northern Ireland (AFBI code MS01) and identified using external morphology by Mr Stewart Rosell. The specimen was stored in 100% ethanol, sent to the Muséum National d'Histoire Naturelle (Paris) and registered as MNHN JL471. A part of the specimen was used for sequencing. An additional three specimens of *M. scharffi* were collected in France and processed for *cox1* sequencing according to the routine methods detailed in Justine *et al.* (2015). MNHN registration numbers, GenBank registration numbers, locality, dates and collectors are: MNHN JL84, PP765165, Villeneuve-de-la-Raho, Pyrénées Orientales, June 2013, Coll. Gérard Peaucellier; MNHN JL86, PP765166, Pleumeur-Bodou, Côtes-d'Armor, November 2013, Coll. Daniel Réaudin; and MNHN JL156, PP765167, Cagnes-sur-Mer, Alpes Maritimes, May 2014, Coll. Pierre Gros.



FIGURE 1. *Microplana scharffi*, live picture of specimen MNHN JL471. Photo by Archie K. Murchie. Grid under the worm: cm and mm.

Sequencing, assembly, data-mining and annotation

For next-generation sequencing, a 1 cm long piece was cut and sent to the Beijing Genomics Institute, China where DNA extraction and sequencing on a DNBSEQ platform were performed. An amount of ca. 100M clean 150 bp paired-end reads were obtained from sequencing and assembled with SPAdes 3.15.5 (Bankevich *et al.* 2012) using kmer of 85 and 125. Contigs of interest were extracted from the contigs files using standalone blastn or blastx queries (Camacho *et al.* 2009) with published sequences as references. For mitochondrial genome, rRNA clusters and *EF1 α* gene, references were the mitogenome of *B. kewense* (NC_045216), the type II *18S* of *M. scharffi* (AF050435) and the partial *EF1 α* gene of *M. scharffi* (KU872695), respectively. For the β -CATENIN-1 gene and APC genes, references were the protein sequences of *Dugesia japonica* Ichikawa & Kawakatsu 1964 (AWD06771) and *Cura pinguis* (Weiss, 1909) (WMQ53820), respectively. Putative traces of alien DNA were investigated in a similar way to what is described in Justine *et al.* (2022a) using a database appended with mitogenomic sequences. Annotation of the protein coding genes (PCG) of the mitogenome was done with the help of ORFFINDER (Wheeler *et al.* 2003), rRNA were found by alignments with reference sequences and tRNA were found with ARWEN v1.2.3 (Laslett & Canbäck 2008). A map of the organellar genome was drawn on the OGDRAW online portal (Lohse *et al.* 2013). Alignment of the two types of *18S* was done using Clustal omega (Sievers *et al.* 2011) and visualised with Jalview 2.11.3.0 (Waterhouse *et al.* 2009).

Phylogeny

Mitochondrial phylogeny was conducted using IQ-TREE 2.2.0 (Minh *et al.* 2020) with 1,000 ultrafast bootstrap replicates after appending existing datasets (Gastineau *et al.* 2024) with the sequences from *M. scharffi*. Protein sequences of the 12 PCG were aligned separately by MAFFT 7 (Katoh & Standley 2013), trimmed using trimAl (Capella-Gutiérrez *et al.* 2009), concatenated with Phyutility 2.7.1 (Smith & Dunn 2008), and the best model of evolution was evaluated on the concatenated alignment using ModelTest-NG (Darriba *et al.* 2020). The phylogeny inferred from the β -CATENIN-1 proteins was prepared the same way, except for the concatenation. The sequence of the putative protein of *M. scharffi* was extracted after annotation of the introns and exons and aligned with 15 sequences listed in Vila-Farré *et al.* (2023). The sequence from *Camerata robusta* Vila-Farré, Sluys, D'Aniello, Cebrià, Ferrer & Romero, 2009 (infraorder Maricola) was used as outgroup.

Results

The mitochondrial genome and its characteristics

The contig corresponding to the mitogenome was found as a 15,297 bp contig with a 246.19X coverage with redundant endings and as such, considered complete and circular. After trimming, the mitogenome is 15,291 bp long (GenBank: PP711816). It codes for 12 protein coding genes (PCGs), 2 rRNAs and 21 tRNAs (Figure 2). It is colinear with those of other Geoplanidae concerning the PCGs and the rRNAs. Regarding the tRNAs, three features are noteworthy. First of all, the position of the *tRNA-Cys* (between *tRNA-His* and *rrnS*) is consistent with what has been observed among Geoplaninae (Solà *et al.* 2015; Justine *et al.* 2020b) and Bipaliinae (Gastineau *et al.* 2019; Justine *et al.* 2022a), but differs from Rhynchodeminae among which *tRNA-Cys* is between *cox3* and *tRNA-Ile* (Gastineau *et al.* 2020; Gastineau & Justine, 2020; Gastineau *et al.* 2024). Secondly, it was possible to find a *tRNA-Thr*, although the candidate is missing its D-loop, while this tRNA is frequently reported as hard to find if not missing among Geoplanidae (Soo *et al.* 2023; Gastineau *et al.* 2024). Finally, it was not possible to find a *tRNA-Ala* using ARWEN. In other species, this tRNA is located between *ND3* and *ND2*. By aligning *tRNA-Ala* sequences from published Bipaliinae, it was, however, possible to find matches in the *ND2-ND3* intergenic part, containing the conserved TGC anticodon. The aligning portion of the mitogenome of *M. scharffi* was extracted and folded on the UNAFold Web Server, with a percentage of suboptimality of 10%. Out of the 4 putative conformations returned, none was congruent with a tRNA, with none of them presenting the anticodon loop.

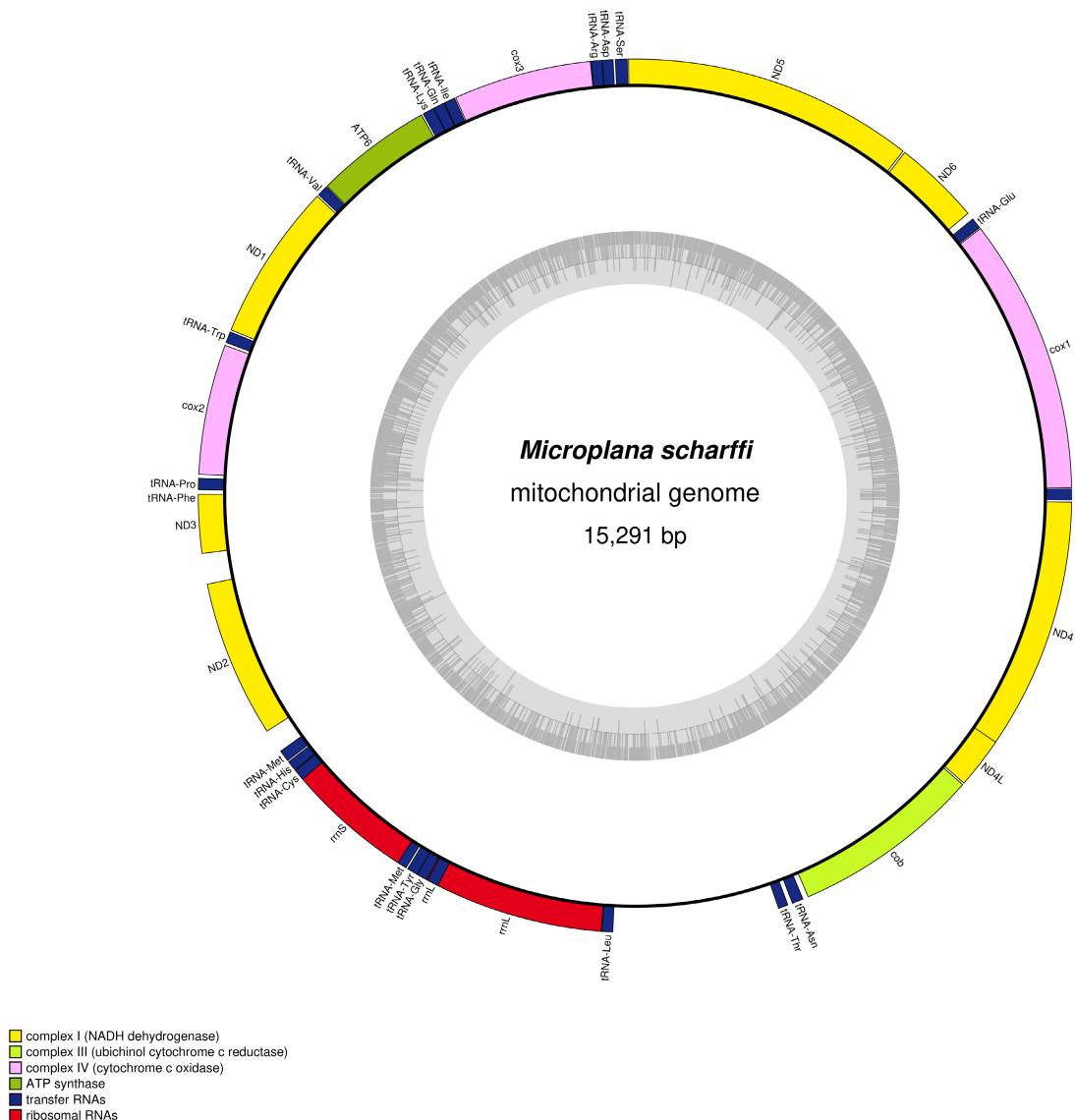


FIGURE 2. *Microplana scharffi*, map of mitochondrial genome. The mitogenome codes for 12 conserved protein-coding genes, 21 tRNA and 2 rRNA. No *tRNA-Ala* could be found. *ND4L* and *ND4* overlap by 32 bp.

It was not possible to find a canonical start codon for the *cox1* gene using genetic code 9. The first ATG-encoded Methionine that could be used as a start would lead to a 494 amino-acid long protein that would lack the first conserved tyrosine residue of the D-pathway and also the first leucine residue assigned to the polypeptide binding site of the subunit I/VIIc interface. By alignment with reference sequences of Bipaliinae, the most congruent start codon seems to be a TTG, a feature discussed below. Megablast query of the *cox1* gene returned 100% identity with *M. scharffi* isolates MS1 (KR906623), 542Y269 (KR906630) and 1188U466 (KR906638), the two first from the United Kingdom (Cumbria and North Yorkshire) and the third from Bulgaria.

The size of the Cox2 protein is 246 amino-acids (AA), which compares well with the Bipaliinae and Geoplaninae, and it does not display the extra-size observed among all Rhynchodeminae so far (Gastineau *et al.* 2024). However, like Geoplaninae and Rhynchodeminae, there is a conserved 32 bp overlap between *ND4L* and *ND4* that has never been noticed until now among the seven species of Bipaliinae whose mitogenome has been sequenced.

Data-mining the nuclear genes of interest

From the contigs file, it was possible to obtain the complete 1,395 bp *EF1 α* gene (GenBank: PP729467). Megablast queries and alignments showed that it is 98.96% conserved with KU872694 from *M. scharffi* isolate MS3, from United Kingdom (Mateos *et al.* 2017), but that out of the six differences detected, four were in fact undefined 'N' nucleotides found in sequence KU872694.

The full gene coding for the β -CATENIN-1 protein was also obtained. As a nuclear gene (in opposition to the mRNA used for data-mining), this gene is 4,421 bp long and contains five introns (GenBank: PP729465). The encoded mRNA is 2,835 bp long, which is similar to the sequences obtained by Vila-Farré *et al.* (2023) on various planarians.

It was not possible to obtain the complete adenomatous polyposis coli protein (APC) gene. Successive assemblies with various lower k-mer, extensive data-mining and attempts to extend the contigs *in silico* all failed. The gene seemed to be split into several contigs that we failed to align. Generally speaking, this gene seems to have large intronic portions. Results of data-mining suggested that some of these non-coding parts might be repeated in the genome, which would explain why it was not possible to resolve them with short reads. It was, however, possible to retrieve a large intronless part coding for a conserved 896 AA long ORF (GenBank: PP729466) that easily aligns with the references from Vila-Farré *et al.* (2023).

The peculiar case of the paralogous nuclear rRNA

It was not possible to successfully assemble the two different paralogous clusters of rRNA with short-read sequencing, as has already occurred with different models (Soo *et al.* 2023), and this would have required the use of long-read sequencing (Gastineau *et al.* 2024). However, two contigs containing the complete *18S* gene were retrieved after assembly. The type I *18S* (GenBank: PP708608) was found in a 3,832 bp long contig with a coverage of 243.857837X. Megablast queries returned as best result the type I *18S* from *Microplana nana* Mateos, Giribet & Carranza, 1998 (GenBank: AF033042) with 98.03% of identity, as it should be noted that no type I *18S* was available on GenBank for *M. scharffi* (Carranza *et al.* 1998). Type II (GenBank: PP708613) was part of a 6,317 bp contig with a 1737.973191X coverage. Megablast queries of the type II returned 100% identity with *M. scharffi* isolate 914I443 (GenBank: KR906679) from Sluys *et al.* (2016), followed by AF050435, a sequence ascribed to the type II *18S* of *M. scharffi* by Carranza *et al.* (1998), with 99.72% identity. It should be noted that all five differences between our sequence and AF050435 were not SNPs but nucleotides that were not accurately identified and instead labelled as 'N' or 'R' on GenBank. Lengths of the *18S* were 1,784 bp and 1,791 bp for type I and II, respectively and their sequences were only 90.57% identical when aligned with Clustal omega. The alignment between both two types is available as Supplementary Figure 1, as explained below in the data availability statement.

Phylogenies

The best model of evolution returned for the mitochondrial multiprotein alignment was MTZOA+I+G4+F. The mitochondrial phylogeny strictly positions *M. scharffi* as sister to the clade containing all Bipaliinae, with maximum support (Figure 3). This larger clade of Microplaninae + Bipaliinae appears as sister-group to Rhynchodeminae, while Geoplaninae appear as a sister-group to them. The best model of evolution returned for the alignment of the putative β -CATENIN-1 proteins was JTT+G4+F. The phylogeny strictly distinguishes between Planarioidea and Geoplanoidea (Figure 4).

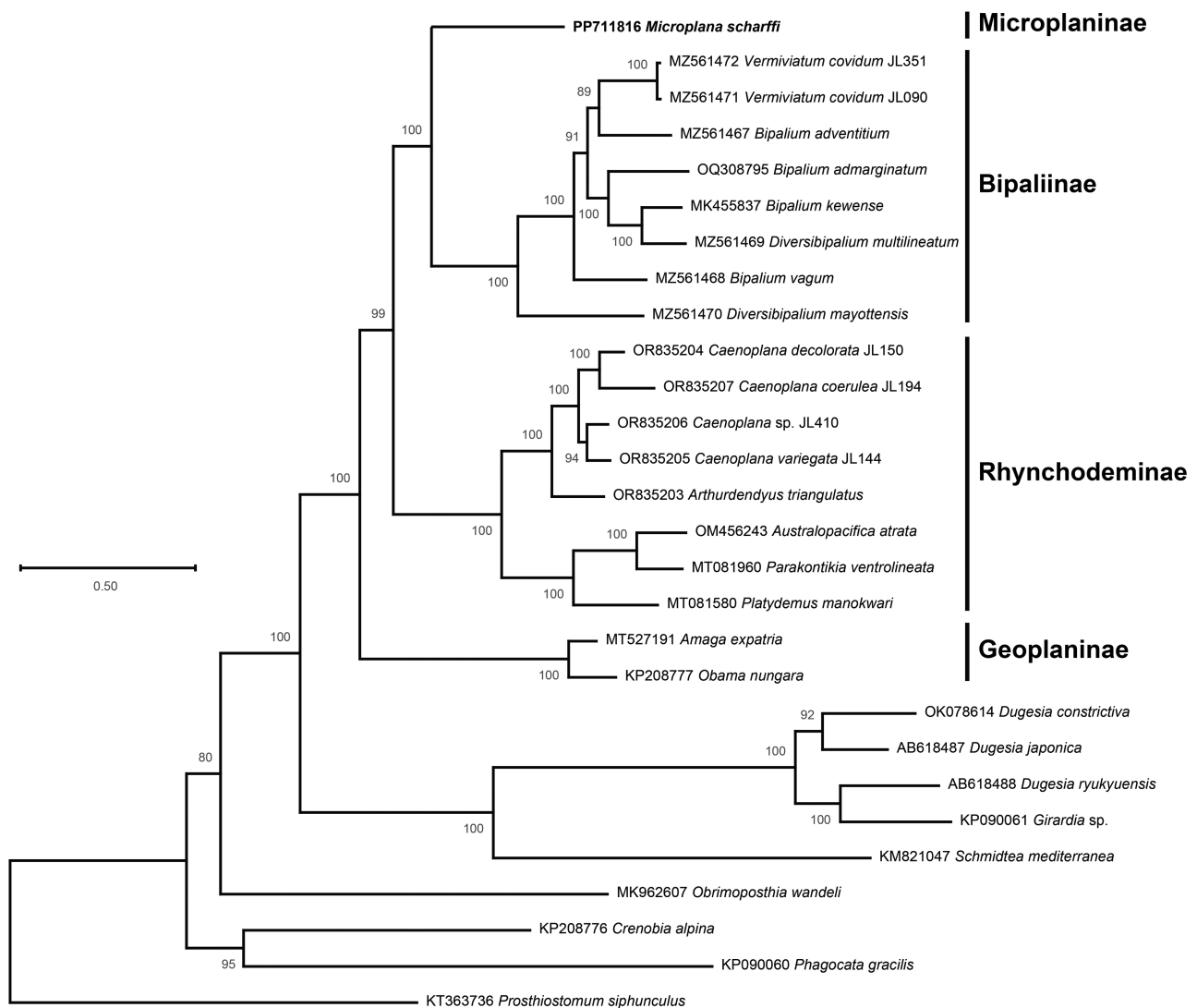


FIGURE 3. Maximum likelihood phylogenetic tree obtained from concatenated amino-acid sequences of the mitochondrial proteins of *Microplana scharffi* and other flatworms, obtained using the MTZOA+I+G4+F model of evolution. Bootstrap values are indicated at the nodes and subfamilies of Geoplanidae are noted on the right.

Food

After assembly, we also retrieved a 15,238 bp contig with a coverage of 12.84X which returned as best megablast query the mitogenome of the native humus earthworm *Lumbricus rubellus* Hoffmeister, 1843 (GenBank: OX243829) from a specimen collected in Cardiff in the framework of the Darwin Tree of Life Project, with a coverage of 100%, e-value 0.0 and identity 99.15%. This contig can be accessed as indicated in the data availability statement.

Presence of *Microplana scharffi* in France

The three specimens collected in France and barcoded had 100% *coxI* identity with our specimen from Northern Ireland and other specimens from Bulgaria, United Kingdom and Italy (KR906632) available in GenBank, thus ascertaining with molecular data the presence of the species in France, with a wide geographical range from north-west to south-west and south-east France.

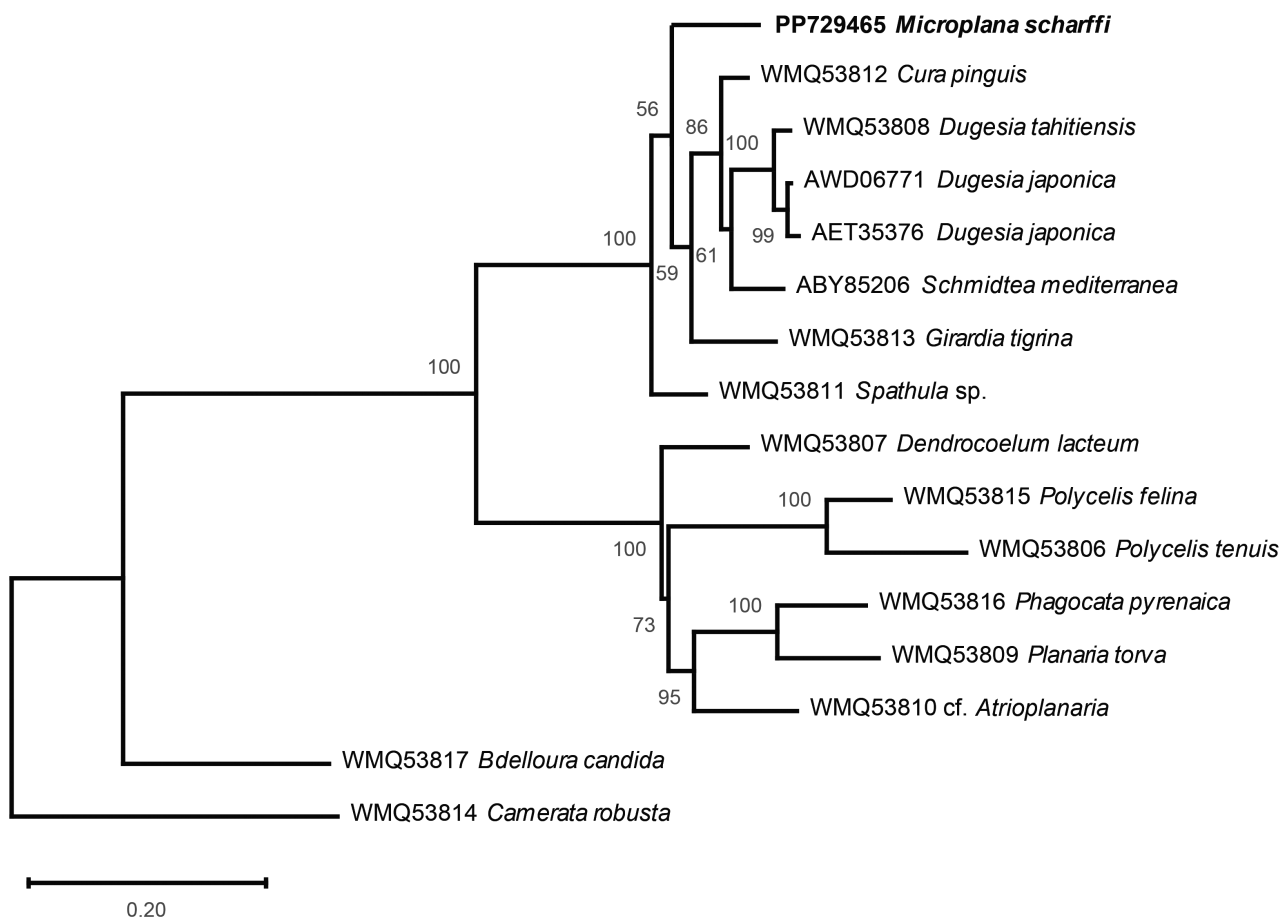


FIGURE 4. Maximum likelihood phylogenetic tree obtained from an alignment of the β -CATENIN-1 proteins, using the JTT+G4+F model of evolution. Bootstrap values are indicated at the nodes.

Discussion

The current study makes the case for additional sequencing of the less studied groups of Geoplanidae. Within a single run of a ‘genome skimming’ approach of sequencing, it was possible to retrieve a massive amount of information.

The mitochondrial genome displays an interesting distribution of characteristics observed among other groups. The position of the *tRNA-Cys* and the *ND4L/ND4* overlap seems common to Microplaninae and Geoplaninae, although the phylogenetically closer Bipaliinae never displayed this overlap in any of the species sequenced until now. This is the first time that *tRNA-Ala* seems poorly conserved and possibly not functional among Geoplanidae. This is also the first occurrence of an alternative TTG start codon in the *cox1* gene among Geoplanidae, although the situation has occurred with species of the sister family Dugesiiidae, namely *D. japonica* (AB618487) and *D. ryukyensis* Kawakatsu, 1976 (AB618488) (Sakai & Sakaizumi 2012). For these two species, the *cox1* gene also seems to start with a TTG codon. It is worth noting that among Eukaryota, TTG has been known for decades in genetic code 5 (Invertebrate mitochondrial code) to be a possible alternative start codon, as exemplified by model nematode species *Caenorhabditis elegans* (Maupas, 1900) and *Ascaris suum* Goeze, 1782 (Okimoto *et al.* 1990). There are more recent reports suggesting that TTG could also act as a start codon in echinoderms, whose mitogenome is also translated by genetic code 9 (Quek *et al.* 2021), and also among other flatworms (Ross *et al.* 2016). All in all, this advocates for more sequencing on an extended sample of taxa of Microplaninae. The Microplaninae presently comprises eight genera (*Amblyplana* Graff, 1896, *Diporodemus* Hyman, 1938, *Geobenazzia* Minelli, 1974, *Incapora* Du Bois-Reymond Marcus, 1953, *Microplana* Vejdowsky, 1890, *Othelosoma* Grey, 1869, *Pseudoartiocotylus* Ikeda, 1911 and *Statomicroplana* Kawakatsu, Froehlich, Jones, Ogren & Sasaki, 2003, which is a collective genus). Many species in these genera were described towards the end of the 19th century but possibly never subsequently observed; it is noteworthy that only the genus *Microplana* is documented in GenBank.

We were also able to obtain insights on the type of prey consumed by *M. scharffi*, confirming the previous observations by McDonald & Jones (2007).

This is the first time that type I of the 18S has been obtained for *M. scharffi*, and in a similar manner to that which was recently observed in *A. triangulatus*: what was previously labelled as type II corresponds to the contig assembled with the highest coverage (almost 8 times the coverage of type I) (Gastineau *et al.* 2024).

The current study is also our first attempt to data-mine our sequencing results for genes related to regeneration, and with regard to this, might be considered successful, especially concerning the gene coding for β -CATENIN-1. The relevance of such a gene in determining the phylogeny of Geoplanidae is debatable considering the few references that are presently available. In future sequencing, we aim to perform a more systematic investigation of these genes, keeping in mind that they might require a deeper sequencing depth when compared to the sole sequencing of a mitogenome. We hope that these data will help the members of the community who work on the regenerating properties of flatworms.

Data availability

All the sequences obtained in the course of this study and the supplementary figure 1 can be downloaded from Zenodo following this link: <https://doi.org/10.5281/zenodo.11237992>.

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