



Molecular and morphological evidence for two new species of terrestrial planarians of the genus *Microplana* (Platyhelminthes; Turbellaria; Tricladida; Terricola) from Europe

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Abstract

Three species of terrestrial planarian were found in a garden in Yorkshire, *Microplana scharffi* (Graff 1896), *M. terrestris* (Müller 1774) and a third *Microplana* species similar in size and shape to *M. terrestris* but brown (khaki) rather than grey/black. Mitochondrial cytochrome oxidase I sequences show that the three are distinct species, but that the sequences of the khaki specimens are identical with a GenBank sequence under the name "*Microplana terrestris*". Photomicrographs of the copulatory apparatus of several specimens of each species, including type material, are presented which confirm that the three species are distinct and show the variation within each species. The khaki species is named as *Microplana kwiskea* n. sp. Specimens from Spain relating to the GenBank "*Microplana terrestris*" sequence have been examined and are not of that species, nor are they *M. kwiskea*. They are considered to be a further species, *M. groga* n. sp.

Key words: Cytochrome oxidase I; *cox1*; COI; *Microplana scharffi*; *Microplana groga*; *Microplana kwiskea*; *Microplana terrestris*; Rhynchodemidae; Microplaninae

Introduction

During the course of observations in a domestic garden in Yorkshire, UK, three species of terrestrial planarian were found (McDonald and Jones 2007). Two of these, *Microplana scharffi* (Graff 1896) and *M. terrestris* (Müller 1774), are well known and widely distributed across Europe (Minelli 1977; Jones 1998; 2005). *Microplana scharffi* (Fig. 1a) is up to 7 cm long. Its colour varies, dependent on recent prey, from cream/yellow to pink or even greyish, with a small dark patch anteriorly (McDonald and Jones 2007). *Microplana terrestris* (Fig. 1b & c, lower) is up to about 2 cm long and is normally uniformly dark grey, almost black, dorsally and laterally but with a pale ventral creeping sole. The third species found is of similar size and shape to *M. terrestris* but brown (khaki) (Fig. 1b & c, upper) and was referred to as "khaki *Microplana*" by McDonald and Jones (2007). Also found were specimens again similar to *M. terrestris* but with non-uniform "granular" dark and pale grey pigmentation (Fig. 1d). Both the khaki and granular specimens were clearly distinct from *M. scharffi*, but we were unsure whether they were colour variations of *M. terrestris* or different species. Other brown species have been previously described from Europe, e.g. *Rhynchodemus henrici* Bendl 1908, *R. attemsi* Bendl 1909 (these were synonymised by Minelli 1977) and *R. carli* Fuhrmann 1914. The GenBank sequence under the name "*M. terrestris*" originates from one of the "reddish to brown" specimens found near Barcelona, Spain, and identified as *M. terrestris* by Mateos *et al.* (1998). One of us (HDJ) had earlier collected similar khaki specimens from other localities (near Southport, Lancashire, the site of the studies in Jones *et al.* 1998; 2001, and in Manchester) but they had not been identified.

In order to identify the khaki and granular specimens, specimens of these and of *M. scharffi* and *M. terrestris* were collected from the same site for comparative purposes. Taxonomy and identification of this group is classically based on general morphology but particularly that of the copulatory apparatus. Thus specimens were partially sectioned to compare morphological details with published descriptions and with type material. The remaining portions of the same specimens were used for DNA extraction to compare with each other and with existing sequences in GenBank. The use of the same specimens for morphological and molecular studies ensures that we can be certain of our identification and the attribution of the genetic sequences. Since the main issue was the identity of *M. terrestris* and the only available GenBank sequence apparently of that species (AF178318) is the mitochondrial cytochrome oxidase 1 (*cox1* or COI) sequence, that sequence was determined for each of our specimens. One problem with type material, published descriptions and GenBank data is that most are based on a single specimen. Obviously there is a single holotype (or neotype) and there may or may not be further paratype material. The majority of GenBank sequences (at least for the Platyhelminthes) are from a single specimen of each species. Thus there is little or no information on individual variation within a species for either the morphology (which may in any case vary with maturity, fixation and storage) or the

molecular results, even if the species has been accurately identified. The use of more than one specimen of each species in this investigation gives some indication of intraspecific variation.



FIGURE 1. *Microplana* spp. Colour photographs of live specimens (stills from digital video). Scale line = 1 cm (the circular depressions on the background stone are 1 cm apart and about 2 mm in diameter): a. *Microplana scharffi* (about 35 mm long, anterior to the right). b. *Microplana kwiskea* n. sp. a khaki flatworm (upper, about 23 mm long, anterior to the right) and *M. terrestris* (lower, the dark grey specimen, about 20mm long, anterior to the bottom). c. The same specimens as (b) when contracted and lying on their dorsal side. The narrow end of both is anterior. The ventral creeping sole is visible in both. d. A “granular” specimen of *M. terrestris* (about 15 mm long, anterior to the right). All specimens from Kirby Wiske, Yorkshire.

Notwithstanding that *M. terrestris* and *M. scharffi* are widely distributed across Europe, examination of our material and of other deposited material, including type material, has shown that published descriptions of both are inadequate in some ways. Though there are published descriptions and illustrations of the copulatory apparatus (variously complete) of specimens identified as *M. terrestris* (to be discussed later), the neotype of

M. terrestris designated by Minelli (1977) was not described or figured by Minelli. The holotype of *M. scharffi* is partially figured in Graff (1899) but certain crucial details are not illustrated or unclear. Illustrations in subsequent papers (again to be discussed later) can be confusing. Thus the type material of both *M. terrestris* and *M. scharffi* has been re-examined. We have also attempted to trace and examine other material relating to published papers of *M. scharffi* and *M. terrestris* and synonyms of both. Minelli (1977) revised the European species of land planarians and synonymised several species with each of the above. Digital micrographs have been made of as much material as possible for future reference. Thus identification of the specimens collected above has been verified and it is possible to provide micrographs of *M. scharffi* and *M. terrestris* based on type material and several other specimens, giving an indication of intraspecific variation. As will be shown, there has been some apparent misidentification of specimens in the literature and it is critically important that specimens are identified correctly, particularly those used for molecular analysis. Taxonomic opinions expressed here are largely those of HDJ.

Material and methods

Specimen collection and treatment

Eight *M. scharffi* (S1-S8), 15 *M. terrestris* (T1-T15), and nine khaki specimens (K1-K9), were collected over several days prior to 25 October 2004 from beneath wood and plastic traps set in a domestic garden at Kirby Wiske, Yorkshire, YO7 4ER (OS grid reference SE375849), the site of the studies reported in McDonald and Jones (2007). They were initially stored alive in small jars with damp leaf mould. Each was examined, photographed and videotaped when alive, individually killed in warm 100% ethanol and preserved in the same. Three mature specimens, evidenced by a visible gonopore, of each species were selected, those that were least coiled (to facilitate sectioning). In two of the specimens, the posterior portion containing the copulatory apparatus was removed for longitudinal sections (LS). A small mid-portion of the third specimen of each species was removed for transverse sections (TS) and the anterior used for LS. All sections were 10 µm thick. The remaining portion (approximately half the body) was preserved in 100% ethanol and subsequently used for DNA extraction. Six "granular" specimens (G1-6) were collected around 23 August 2005 and similarly treated. The anterior portions of two of these were used for DNA extraction. The reference number of the selected specimens was engraved on the microscope slides and is referred to during subsequent analysis and descriptions. All slides, remaining portions and intact specimens are deposited in the Natural History Museum, London (NHML). The full list of specimens with their reference numbers and NHML accession numbers follows.

Microplana scharffi. Accession numbers: NHML 2006.6.28.22-29.

S1: 8 slides posterior LS including copulatory apparatus; anterior DNA extraction. S2: 9 slides posterior LS including copulatory apparatus; anterior DNA extraction. S4: 6 slides posterior HLS including copulatory apparatus; anterior preserved in 100% ethanol. S5: 3 slides TS mid-portion of body; 7 slides LS anterior; posterior DNA extraction. S3, S6, S7, S8 all preserved in 100% ethanol.

Microplana terrestris. Accession numbers: NHML 2006.6.28.1-15.

T1: 7 slides posterior LS including copulatory apparatus; remainder (damaged) preserved in 100% ethanol. T2: 8 slides posterior LS including copulatory apparatus; remainder DNA extraction. T4: 9 slides posterior LS including copulatory apparatus; remainder DNA extraction. T7: 3 slides TS mid-portion of body; 2 slides LS anterior; posterior DNA extraction. T3, T5, T6, T8, T9, T10, T11, T12, T13, T14, T15 all preserved in 100% ethanol.

Granular specimens. Accession numbers: NHML 2006.6.28.16-21.

G1: posterior preserved in 100% ethanol; anterior DNA extraction. G4: posterior preserved in 100% ethanol; anterior DNA extraction. G2, G3, G5, G6 all preserved in 100% ethanol.

Khaki specimens—*Microplana kwiskea* n. sp. Accession numbers: NHML 2006.6.28.30-38. Holotype: K9, syntypes: all others.

K1a: 8 slides posterior LS including copulatory apparatus; anterior preserved in 100% ethanol. K2: 9 slides posterior LS including copulatory apparatus; remainder DNA extraction. K8: 2 slides TS mid-portion of body; 5 slides LS anterior; posterior used for DNA analysis. K9: 6 slides posterior LS including copulatory apparatus; remainder DNA extraction. K4, K5, K6, K7 all preserved in 100% ethanol.

Morphology

Selected portions of specimens as listed above were embedded in paraffin wax after automated impregnation, serially sectioned at 10 µm, simultaneously stained in Ehrlich's haematoxylin and eosin in an automated staining machine and mounted in Canada balsam. Representative sections and material borrowed from other institutions were digitally photographed at NHML. HDJ visited the Natural History Museum, Vienna (NHMW) and The Zoological Museum, University of Copenhagen (The Natural History Museum of Denmark, NHMD) to examine type and other material. This was examined and digitally photographed using facilities at the host institution. The NHMW has an extensive collection of land planarian specimens and microscope slides originating from the University of Graz and L. von Graff and his students (a full list of specimens is available from HDJ and from the NHMW).

Some images have been corrected (levels, contrast and colour only) using Photoshop CS. Other material examined will be listed later under each species. Digital images are available from HDJ.

DNA extraction and gene amplification

Genomic DNA (gDNA) was extracted from the 11 specimens listed above. Ethanol was removed by evaporation and specimens were rehydrated by soaking in 1M Tris-EDTA (pH 8) buffer for 5 min. gDNA was extracted using a Qiagen DNeasy tissue kit following the manufacturer's protocols to give a final concentration of ~10-20 ng/µl of gDNA. Partial fragments (451bp, including primers) of mitochondrial cytochrome oxidase I (*cox1*) were amplified using PCR. PCRs were carried out in a volume of 25µl containing Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads each containing ~1.5 units Taq DNA polymerase, 10 mM Tris-HCl at pH 9, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP and stabilizers, including BSA, 1-2µl of gDNA, 10 mM of each PCR primer (Forward: pr-a2/CO1a2 5'AGCTGCAGTTTTGGTTTTTTGGACA TCCTGAGGT3'), (Reverse: pr-b2/CO1b2 5'ATGAGCAACAACATAATAAGTATCATG3') and double distilled water. See Pongratz *et al.* (2003) for further details concerning primers. Thermal cycling was performed in a Perkin Elmer 9600 Thermal Cycler using the following conditions: 3 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 48°C, 2 min at 72°C and a final elongation step of 7 min. PCR amplicons were purified directly using Qiagen Qiaquick columns, cycle-sequenced from both strands using the two original PCR primers, ABI BigDye™ chemistry, alcohol-precipitation, and run on an ABI Prism 377™ automated sequencer.

Gene alignment, comparison and phylogenetic analysis

Completed *cox1* were aligned along with published sequences (Table 1) with reference to their inferred amino acid translations. There were no insertions or deletions. Primer sequences and the first position at the 5' end (where there was one indel from previously published sequences) were removed prior to phylogenetic analysis, leaving an alignment of 381 bp for 15 taxa. Trailing gaps at the 3' end for *Neppia montana* (32bp) and *Geoplana ladislavi* (20bp) were treated as missing characters. Pairwise distance analysis, between all

cox1 sequences for all taxa, was performed with PAUP* (Swofford, 2002) in order to quantify within and between sequence differences among the ingroup taxa.

TABLE 1. List of species, isolates and associated GenBank accession numbers of *cox1* (COI) sequences used in this study; § - indicates new sequences.

Species (isolate)	GenBank accession	Locality and voucher details
INGROUP		
<i>Microplana scharffi</i> (S1)	§EU334577	NHML 2006.6.28.22
<i>Microplana scharffi</i> (S2)	§EU334578	NHML 2006.6.28.23
<i>Microplana scharffi</i> (S5)	§EU334579	NHML 2006.6.28.26
<i>Microplana kwiskea</i> (K2)	§EU334574	NHML 2006.6.28.31
<i>Microplana kwiskea</i> (K8)	§EU334575	NHML 2006.6.28.37
<i>Microplana kwiskea</i> (K9)	§EU334576	NHML 2006.6.28.38
<i>Microplana terrestris</i> (T2)	§EU334580	NHML 2006.6.28.2
<i>Microplana terrestris</i> (T4)	§EU334581	NHML 2006.6.28.4
<i>Microplana terrestris</i> (T7)	§EU334582	NHML 2006.6.28.7
<i>Microplana terrestris</i> (G1)	§EU334583	NHML 2006.6.28.16
<i>Microplana terrestris</i> (G4)	§EU334584	NHML 2006.6.28.20
" <i>Microplana terrestris</i> "	AF178318	Font Groga, Spain. NHML 1998.2.9.3-5
<i>Microplana nana</i>	AF178317	Les Alberes, Spain. NHML 1998.2.9.1
OUTGROUPS		
<i>Platydemus manokwari</i>	AF178320	
<i>Arthurdendyus triangulatus</i>	AJ405984	Manchester, UK.
<i>Artioposthia testacea</i>	AF178305	
<i>Geoplana ladislavii</i>	AF178315	
<i>Spathula</i> sp.	AF178324	
<i>Dendrocoelum lacteum</i>	AF178312	
<i>Bipalium</i> sp.	AF178307	
<i>Bipalium adventitium</i>	AF178306	
<i>Neppia montana</i>	AF178319	

All phylogenetic analyses were conducted using PAUP* (Swofford 2002). For maximum parsimony (MP) a branch and bound search was used, exploring all tree space. The program Modeltest (version 3.6) (Posada and Crandall 1998) was used to select an appropriate model of genetic evolution for maximum likelihood (ML) analysis, employing the AIC (Akaike Information Content) criterion. The GTR+I+G model (rates set to gamma, with 6 substitution types) was used and a heuristic search was employed with 10 replicates and random addition of sequences. Nodal support was estimated with bootstrap analysis (MP, n=1,000 reps; ML, n=100 reps). Trees were rooted against the outgroup taxa (see Table I).

Abbreviations

IRSNB	Institut Royal des Sciences Naturelles de Belgique, Brussels, Belgium.
MHNG	Muséum d'histoire naturelle, Geneva, Switzerland.
MNHNP	Museum National d'Histoire Naturelle, Paris, France.
NHMD	The Natural History Museum of Denmark (Zoological Museum, University of Copenhagen).
NHML	The Natural History Museum, London (UK), formerly The British Museum of Natural History (BMNH).
NHMW	Naturhistorisches Museum, Wien (Vienna, Austria).

Abbreviations on figures

a = antrum; b = bursa; bc = bursal canal; cs = creeping sole; csd = combined sperm duct; csd-ed = junction of combined sperm duct and ejaculatory duct; ed = ejaculatory duct; edcm = circular muscle surrounding the ejaculatory duct; esd = expanded sperm duct; gic = genito-intestinal canal; gic-i = genito-intestinal connection; gp = gonopore; i = intestine; jsd = junction of sperm ducts; lm = longitudinal muscle; m = mouth; o = ovary; ovd = ovo-vitelline duct; p = penis; sd = sperm duct; sd-ed = sperm duct junction with ejaculatory duct; t = testis; v = vagina; v-gic-ovd = junction of vagina, ovo-vitelline duct and genito-intestinal canal; vnc = ventral nerve cord; v-ovd = junction of vagina and ovo-vitelline duct.

Results

Molecular data

The new *cox1* sequences were resolved (Fig. 2) into three distinct clades: (i) sequences for all K isolates were identical with one another and with GenBank AF178318 under the name “*Microplana terrestris*”; (ii) sequences for all S isolates were identical to one another; (iii) sequences of T and G isolates differed from one another by only three base positions. In all analyses each of these three groups were resolved as distinct from one another and from *Microplana nana* Mateos *et al.* 1998 and the outgroup taxa. For the entire alignment, of the 381bp utilised in the analyses, 187 positions were variable and of these 142 were informative under the principles of parsimony. Gene sequences differed between all major clades at both silent and non-silent positions. The inferred amino acid translation for this portion of *cox1* differed from 8–11 amino acids between the major ingroup clades, including the fourth ingroup lineage, *Microplana nana*.

Maximum parsimony analysis found two equally parsimonious trees (CI=0.547, RI=0.670, length=543), differing from one another only by the relative position of T2 and T4 (either as sister groups, or with both collapsed and unresolved in the G+T clade). Maximum likelihood analysis found a single tree entirely congruent with MP. Figure 2 shows the results using the ML solution (with ML and MP bootstrap support). Nodal support for each of three clades listed was consistently high, although the interrelationships of the clades was poorly resolved. Results strongly indicate that: 1, the K isolates are monospecific and the same species as GenBank AF178318 “*Microplana terrestris*”; 2, the S isolates are monospecific; 3, the T and G isolates are monospecific; 4, *Microplana nana* is a distinct species.

In view of these molecular results, it was necessary to be certain that specimens identified as *M. scharffi* and *M. terrestris* were correctly identified, particularly the latter. What follows are comments on published descriptions and illustrations of each species, based, where possible, on examination of the original material of each author, of the specimens listed above and of further specimens.

Order Terricola Hallez, 1890

Family Rhynchodemidae Graff 1896

Subfamily Microplaninae Pantin, 1953

Genus *Microplana* Vejdovsky, 1890

Microplana scharffi (Graff 1896)

Though the identity of this species amongst European species seems clear, examination of several specimens and the material of some authors, has shown that published descriptions and illustrations are in some ways

unclear, deficient or inaccurate, even the fairly comprehensive description of Graff (1899). Descriptions and figures of the copulatory apparatus may be found in Graff (1896, 1899, as *Rhynchodemus scharffi*), Percival (1925), Eastham (1933) and Adam and Leloup (1944) (all as *R. britannicus*), Beauchamp (1934, as *R. hovassei*), Battalgazi (1945, as *R. decennii*), Minelli (1977) and Ball and Reynoldson (1981) (both as *M. scharffi*). Minelli (1977) synonymised the above species as *Microplana scharffi* (Graff 1896). Minelli (1977) also considered *R. monacensis* Heinzl 1929 to be a synonym of *M. scharffi*. It will be shown that this is not the case.

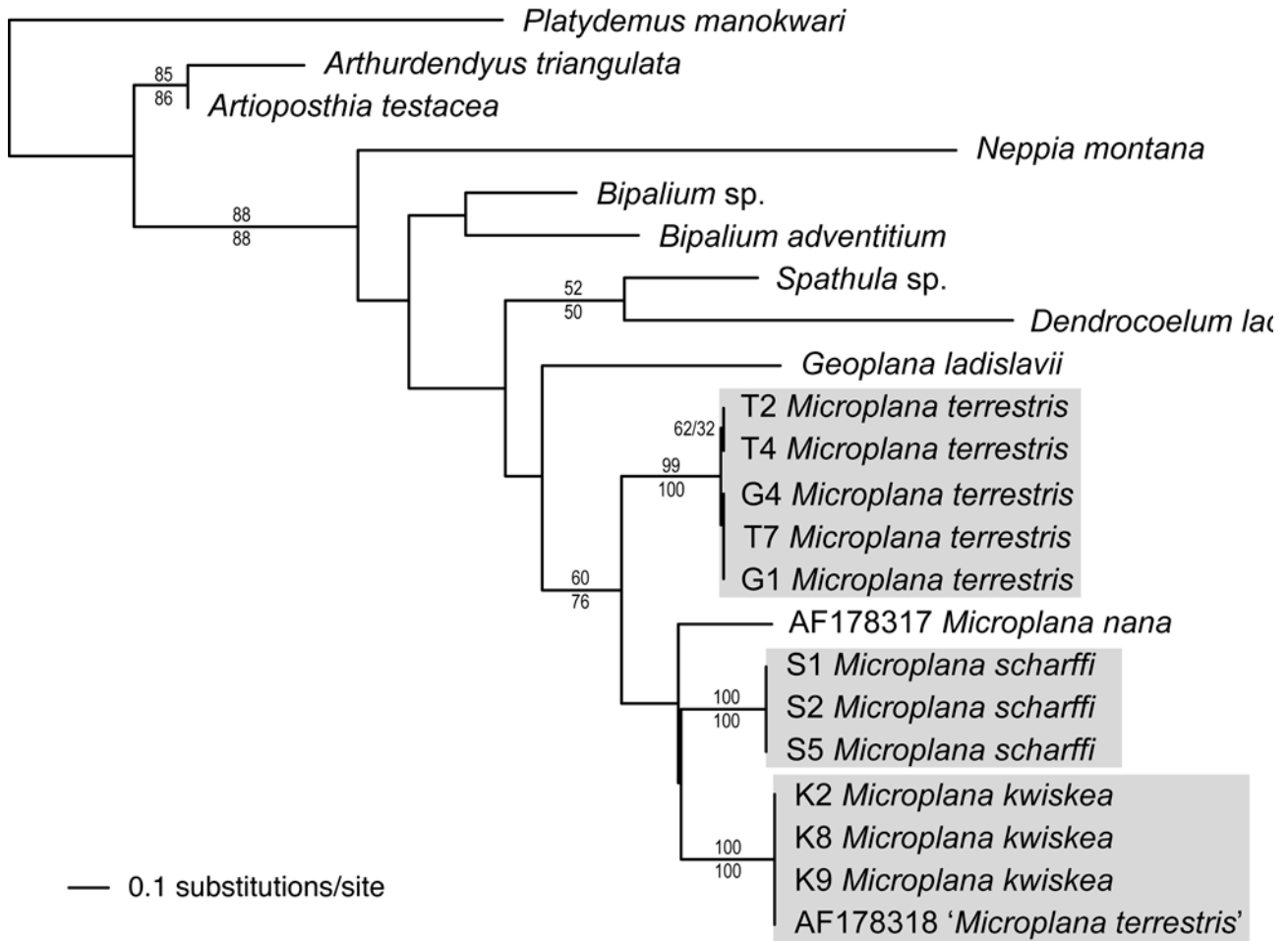


FIGURE 2. Phylogenetic analysis of *cox1* genes showing results from maximum likelihood (ML) analysis. The topology of the tree was identical with maximum parsimony (MP). Nodal support indicated is from bootstrap resampling (ML/MP; n=100/1000 respectively; =50% only shown).

Rhynchodemus scharffi Graff 1896. Holotype, NHMW, L154/M254, Inventory number 2832: I, 9 slides, anterior TS; II, 2 slides, mid-body LS; III, 9 slides, posterior LS. This is of a single specimen found by R.F. Scharff in Dublin, Ireland and is undoubtedly the original specimen. It is in excellent condition. It was designated as holotype by Minelli (1977).

In general shape and proportion, the copulatory apparatus of this specimen is as figured by Graff (1896, Fig. 9; 1899, Text Fig. 53). The same diagram is used in both, but with slightly different labeling. However, after examination of the slides, the diagrams are in some respects misleading as follows.

1. The bulk of the penis (Fig. 3) consists of dense layers of radial muscle, giving a stratified appearance in section. This is not conveyed by the Graff diagrams. The outer ends of these fibres turn towards the tip and become longitudinal (as figured by Graff, 1899, Taf. XLVII, Fig. 2).

2. The sperm ducts each widen (as in most terrestrial planarians) to form a sperm storage organ anterior to the penis, turn dorsally and enter the base of the penis separately as narrow ducts. They continue separately as even narrower ducts, internal diameter about 5µm, external diameter about 15µm. They are difficult to follow through the penis because of their narrowness and the dense musculature of the penis, but they can be traced. They join nearly half way along the penis to form a short common sperm duct of the same width (Fig. 3). These ducts within the penis musculature are narrower and their junction is closer to the bulb of the ejaculatory duct than suggested by the Graff (1896, 1899) diagrams. The common sperm duct opens into the ejaculatory duct which has a broad proximal bulb and narrows towards the pointed penis tip. The internal wall of this bulb and duct is smooth, with no projections. There is a layer of circular muscle surrounding the ejaculatory duct, not very clear in the type specimen, but clearer in other specimens.
3. Graff (1896) labeled the penis with a chitinous tip though Graff (1899) corrected this in the diagram legend and the text. The tip is not chitinous.
4. The female copulatory apparatus is as illustrated by Graff, with the exception that it is possible to discern a narrow connection from the anterior of the bursa (labeled "uterus" by Graff) to the intestine on the right side (Fig. 3, a & d), not noted or figured by Graff. Thus there is a genito-intestinal connection. As Minelli (1977) commented, there is a pencil note (author unknown) on slide III.5 of the holotype series indicating a genito-intestinal connection. The canal leading to this bursa is strongly ciliated.

Rhynchodemus britannicus Percival 1925. Percival's material was mostly from Yorkshire, UK, the same county as specimens S1-8. It was described as a new species but Percival did not clearly distinguish between that and *R. scharffi*. Percival worked at the University of Leeds, UK, and then at the University of Canterbury, New Zealand. Neither can trace any of Percival's material (pers. comms., Baker 2007; Johns 1983; Norris 1982). Percival (1925) figures the copulatory apparatus, in outline, of three specimens. The penis of specimens A and C is rooted dorsally and is quite long and in one case flexed, similar to S1, S2 and S4. However, the penis of specimen B is illustrated as short and conical and seems rather less typical. Unfortunately both Minelli (1977) and Ball and Reynoldson (1981) used this diagram as the basis for their diagrams, which are thus misleading. The sperm ducts of all three specimens are shown as converging at or near the base of the penis, which, from other specimens, may not be accurate. Percival considered the ciliated genito-intestinal duct (= bursal canal) to open directly into the gut. As we have shown, there is a genito-intestinal connection in the type specimen of *M. scharffi* via an expanded bursa.

Eastham (1933) figures the copulatory apparatus of a specimen identified as *R. britannicus*. His material is lost (pers. comm., Eastham 1977). He listed several differences between *R. scharffi* and *R. britannicus* but most can be discounted as resulting from different fixation or individual variation. The most significant is the genito-intestinal connection in *R. britannicus* but its apparent absence in *R. scharffi*. But as we have seen there is a genito-intestinal connection in *R. scharffi*. Thus we can be confident that the specimens of both Percival (1925) and Eastham (1933) were of *M. scharffi* (Graff 1896).

Rhynchodemus britannicus, material of Adam and Leloup (1944) from Belgium. The material is extant, IRSNB, IG 13876 (5 slides, longitudinal sections of posterior half including copulatory apparatus, examined), IG 14582 (one specimen in alcohol, not examined). The diagram in Adam & Leloup (1944) is mostly accurate, the main discrepancy being that they indicate a direct connection from the genito-intestinal canal to the intestine (their Fig. 1, c.g.i. and d.i. respectively). Examination of the slides confirms that there is a constriction between the bursa (labeled d.i. by Adam & Leloup) and the intestine (Fig. 4), as in other specimens of *M. scharffi*. The penis also has dense, layered musculature and narrow sperm ducts joining within the penis just before entering the ejaculatory duct. We are confident that this specimen is of *M. scharffi* (Graff 1896).

Rhynchodemus hovassei Beauchamp 1934. The type specimen was from Turkey, but Beauchamp (1957) reports further specimens (as *Microplana hovassei*) from Madeira. The slides of the specimens from both

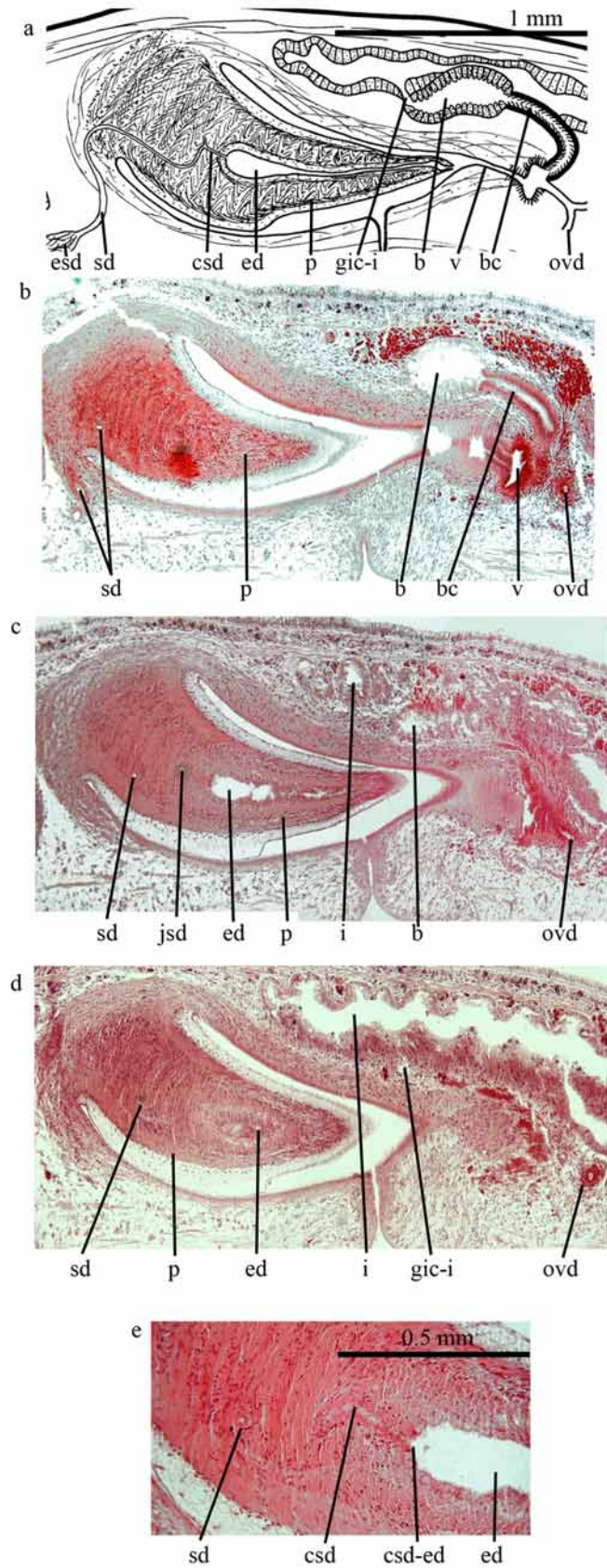


FIGURE 3. *Microplana scharffi* (Graff 1896). Holotype (as *Rhynchodemus scharffi*), NHMW, L154/M254, Inventory number 2832. a. Diagram of the copulatory apparatus, based on the following micrographs. b–d. Micrographs of the copulatory apparatus at different planes through the serial sections. e. Micrograph at larger magnification of the penis showing the combined sperm duct entering the ejaculatory duct. Anterior to the left.

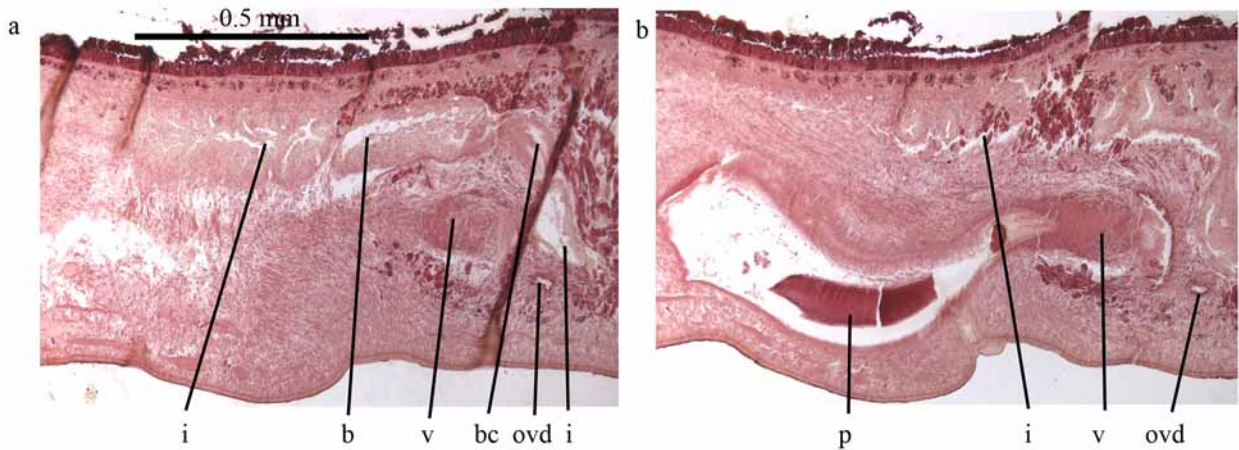


FIGURE 4. *Microplana scharffi* (Graff 1896). Specimen of Adam & Leloup (1944) from Belgium (as *Rhynchodemus britannicus* Percival 1925), IRSNB, IG 13876. a – c. Sections through the copulatory apparatus at different planes. Anterior to the left.

locations are extant and have been examined. Turkey: MNHNP, 1 slide, longitudinal sections of copulatory apparatus labeled “*Rhynchodemus hovassei* de B, type, Balta Liman (illegible) 29.IV.32”, label on reverse: AO 29. As Beauchamp (1934, 1957) comments, the sections are poor and badly folded. The penis is as figured by Beauchamp (1934), quite well developed (Fig. 5a) and has the dense layered muscular core typical of *M. scharffi*, though is rather horizontal compared with other specimens. There is a well-defined duct running dorsally from the posterior of the vagina, similar to the bursal duct of other specimens, but the extent of the bursa and any connection to the gut is obscured by folding of the sections. However, enough is visible to be reasonably certain that this was a specimen of *M. scharffi* (Graff 1896) but probably not fully mature.

Microplana hovassei Madeira specimens (Beauchamp 1957): MNHNP, 7 slides, 2 TS, 5 LS, variously labeled “Madé a”, labels on reverse AO30-36. Paper label on AO 33 reads “*Microplana hovassei* (Beau. Coi.) Madère a, IV.57”. (“Coi” is a reference to M. Coiffait, the collector.) The sections are apparently of a single mature individual. As well as TS sections of this specimen, AO 31 also has a whole mount of an immature specimen 11 mm long. The paper label reads: “*Microplana hovassei* (Beauchamp) Madère (Coiffait), IV.57, Monte Gueymadas C. de d Rabaçal”. This seemingly refers to the whole mount which must be the “non sexue” specimen mentioned by Beauchamp (1957). The sectioned specimen is mature and, as Beauchamp comments, the structure of the penis leaves no doubt that this is of the same species as the specimen from Turkey (Fig. 5b). The bursal canal, bursa and genito-intestinal connection are as the holotype of *M. scharffi* and there is no doubt that these specimens are of that species.

Rhynchodemus decennii Batalgazzi 1945. Minelli (1977) considered this to be a synonym of *M. scharffi*. We have been unable to trace the material of Batalgazzi. From the diagram of the copulatory apparatus in Batalgazzi (1945) there are some apparent differences from *M. scharffi*. The penis is shown as horizontal; the two sperm ducts are shown separately entering the bulb of the ejaculatory duct (labeled as Vesicula seminalis); it is not clear if there is any bursa in the genito-intestinal connection. Nevertheless it otherwise seems similar to the specimen from Turkey described by Beauchamp (1934) and we can be reasonably certain that this was a specimen of *M. scharffi*.

Thus we concur with Minelli (1977) in considering *Rhynchodemus britannicus* Percival 1925, *Rhynchodemus hovassei* Beauchamp 1934 and *Rhynchodemus decennii* Batalgazzi 1945 as junior synonyms of *Microplana scharffi* (Graff 1896).

Microplana scharffi (Graff 1896), Specimens S1, S2, S4 and S5 (see above). Specimens S1, S2 and S4 each have a well-developed penis with the dense layered radial musculature typical of this species (Figs. 6, 7 & 8). The penis of both S1 and S2 has a dorsal origin and they are both flexed at the level of the bulb of the

ejaculatory duct. The two sperm ducts are very narrow and join just before entering the bulb of the ejaculatory duct. The bulb and canal of the ejaculatory duct are surrounded by circular muscle within the body of the penis. The bursal canal opens into the bursa which has a narrow anterior connection to an intestinal diverticulum. Examination with polarised light reveals that the intestinal epithelium has many refractive granules (of unknown origin or significance, but possibly a product of prey digestion). These granules are also found in the epithelium of the bursa but only in the region near to the intestinal connection, not in the end near the bursal canal (Figs 6c, 7c & 8d). This suggests that digestive products enter the bursa from the intestinal connection.

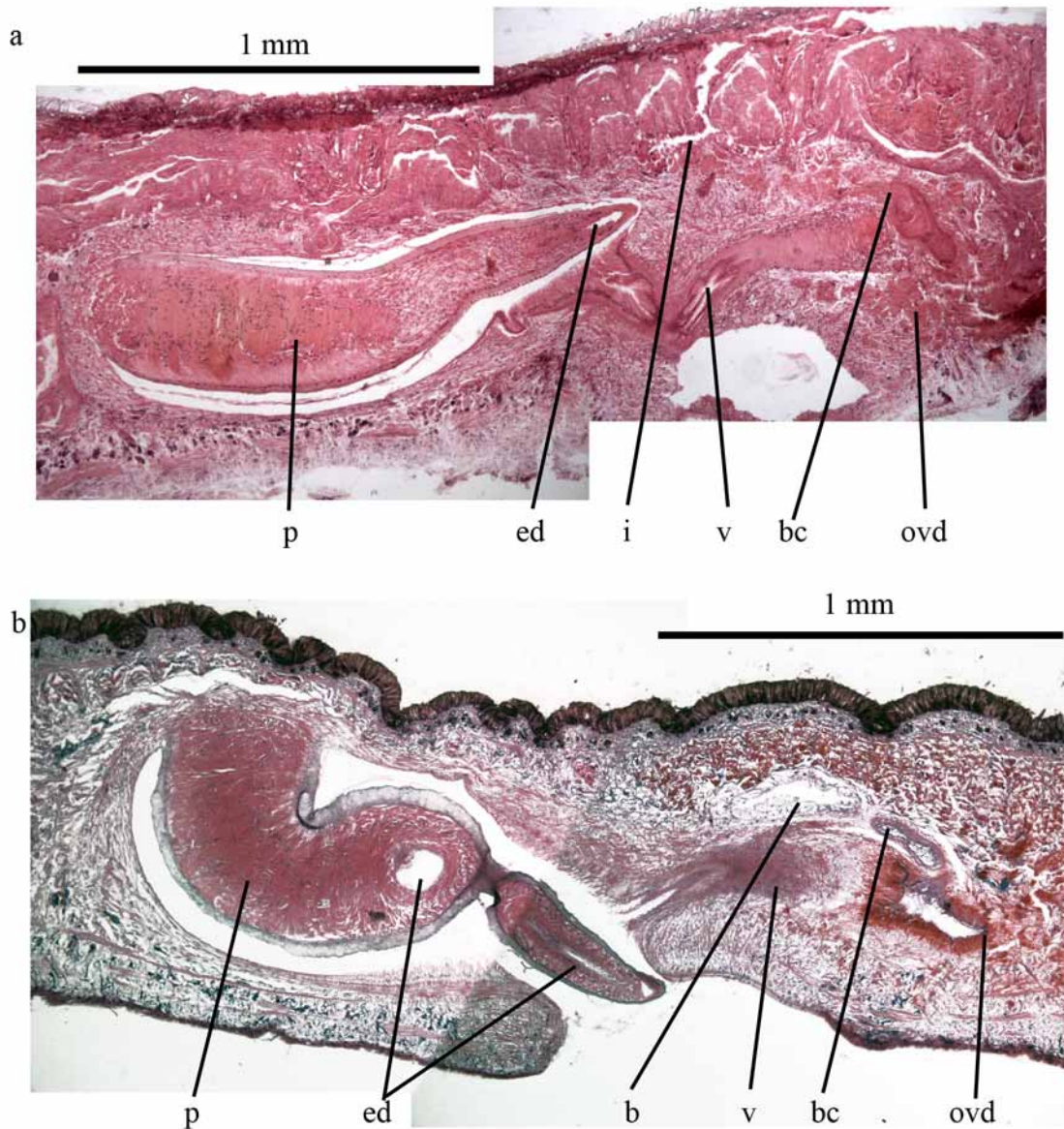


FIGURE 5. *Microplana scharffi* (Graff 1896). a. The holotype specimen of *Rhynchodemus hovassei* Beauchamp 1934 from Turkey, MNHNP, AO 29. b. Specimen from Madeira identified as *Microplana hovassei* by Beauchamp (1957), MNHNP, AO 30-36. Sections through the copulatory apparatus, both of merged adjacent images. Anterior to the left.

Discussion of *Microplana scharffi*. Amongst the European land planarian species, *M. scharffi* is characterised when alive by its length and elongate, slender shape when extended, its extensibility and its colour, though colour is variable due to different prey (McDonald & Jones 2007). Internally it is characterised by the unusual length of the penis which may have a dorsal origin and is free for most of its length, by its dense, layered penis musculature, the narrow sperm ducts within the penis which join before discharging into the ejaculatory duct, and by the nature of the genito-intestinal connection with a narrow duct leading to a broad bursa

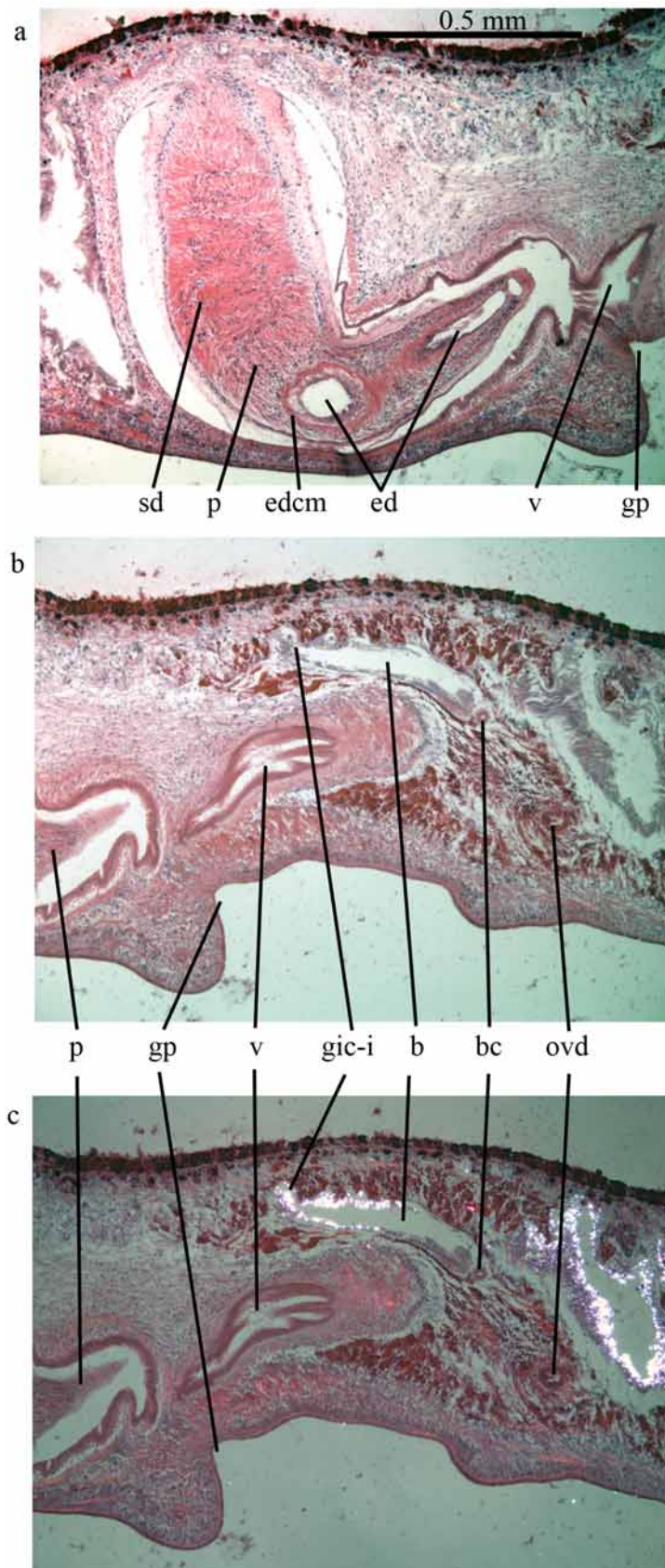


FIGURE 6. *Microplana scharffi* (Graff 1896). Specimen S1, NHML 2006.6.28.22. a. Section through the penis. Note the circular muscle around the ejaculatory duct. b. Section through the female part of the copulatory apparatus. c. Same section as (b) viewed with polarised light, showing the refractive granules (white) in the intestinal epithelium and in the bursal epithelium nearest to the entry to the intestine. Anterior to the left.

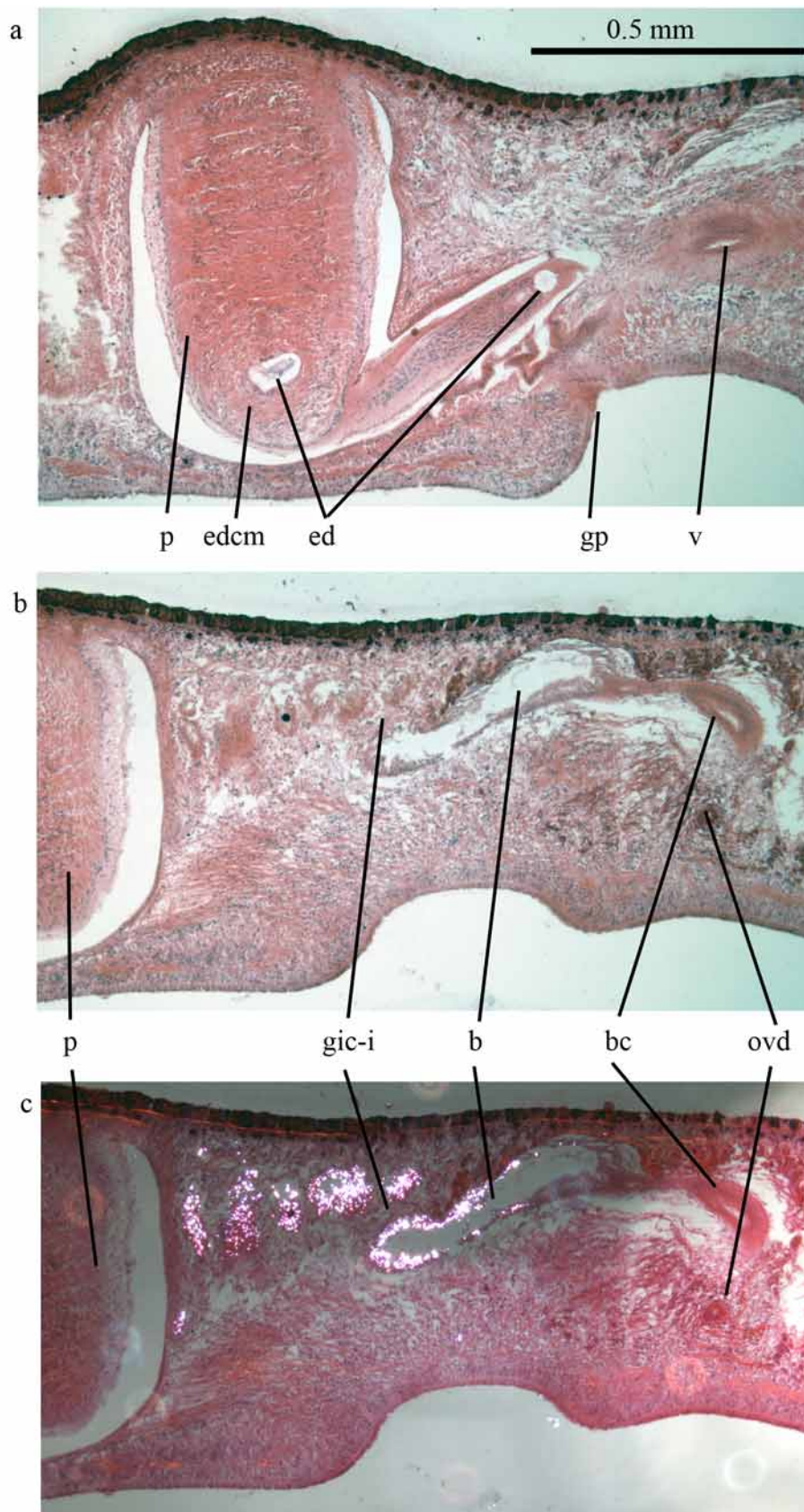


FIGURE 7. *Microplana scharffi* (Graff 1896). Specimen S2, NHML, 2006.6.28.23. a. Section through the penis. Note the circular muscle around the ejaculatory duct. b. Section through the female part of the copulatory apparatus. c. Same section as (b) viewed with polarised light, showing the refractive granules (white) in the intestinal epithelium and in the bursal epithelium nearest to the entry to the intestine. Anterior to the left.

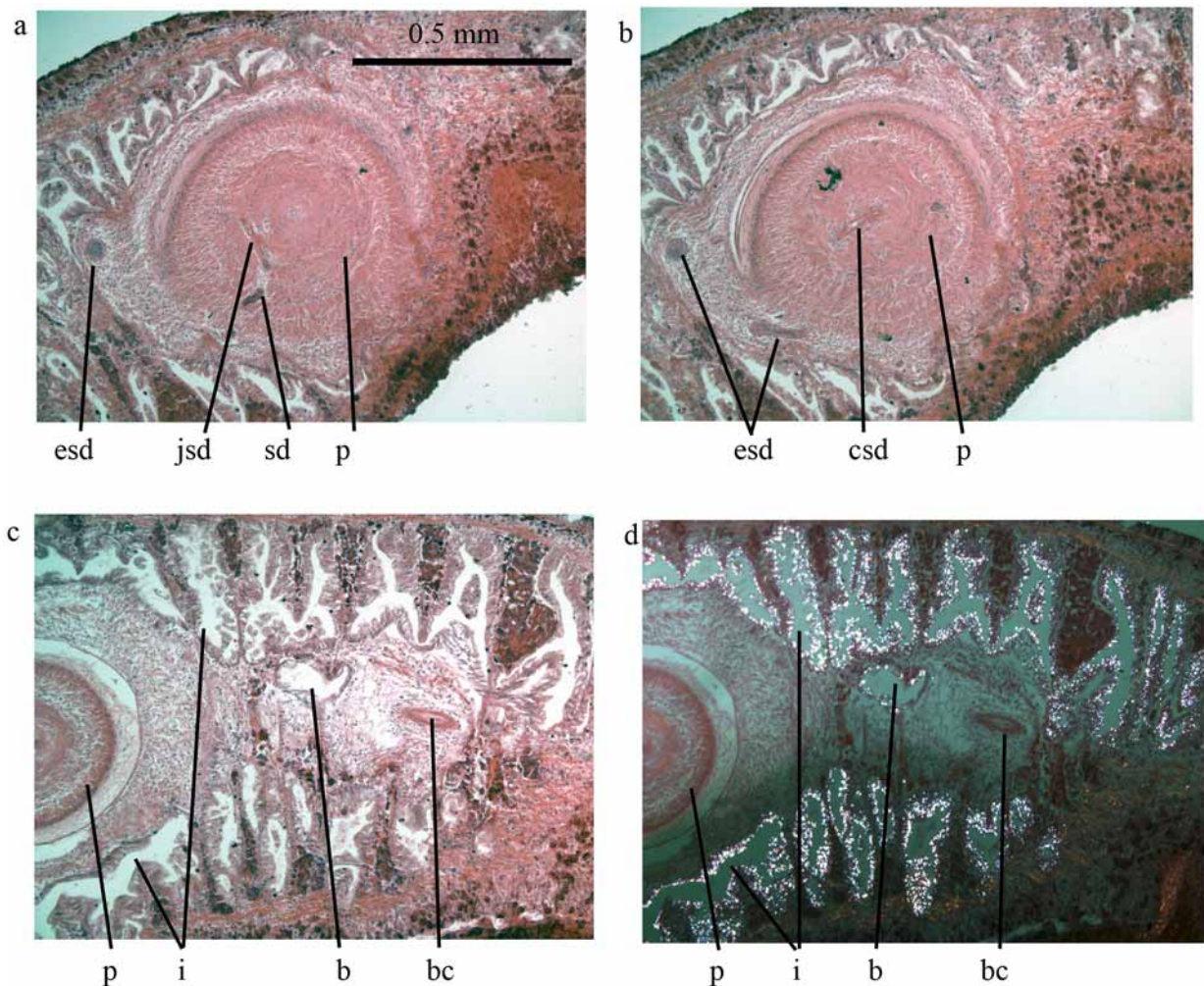


FIGURE 8. *Microplana scharffi* (Graff 1896). Specimen S4. NHML, 2006.6.28.25. a & b. HLS sections through the penis showing the junction of the sperm ducts (a) and the single combined sperm duct (b). c & d. Sections showing the bursal canal and bursa (genito-intestinal duct) d. is in polarised light showing the refractive granules (white) of the intestinal epithelium and in the bursa.

which may receive digestive products. Thus it is a relatively easily recognisable and distinctive species both when alive and when sectioned.

Rhynchodemus monacensis Heinzl 1929. Minelli (1977) also considered this to be a synonym of *M. scharffi*. The specimen, from Monaco, exists (not destroyed as assumed by Minelli 1977), NHMW, L153/M249, Inventory number 2849. I 1-2, 2 slides TS anterior end; II 1-2, 2 slides TS mid-body; III 1-3, LS posterior end. Sections cut at 5 μ m. Heinzl (1929) states that the specimen was identified as *R. scharffi* by Bendl. All slides are labeled "Bendl 1908" and labeled by him "*Rhynchodemus scharffi* Graff" with "*scharffi*" crossed out and replaced by "*monacensis*", presumably by Heinzl. The copulatory apparatus (Fig. 9) is as figured by Heinzl (1929, Taf. 10, Fig. 4). The specimen was only 5 mm long, fully mature, and the penis, bursa and genito-intestinal connection are very different from any specimen of *M. scharffi* (compare Fig. 9 with Figs 3–8). In view of these differences, it is surprising that either Bendl or Minelli (1977) should consider this to be conspecific with *M. scharffi*. Thus we consider the proposal of Minelli (1977) to be invalid and that *Microplana monacensis* (Heinzl 1929) should be reinstated as a valid species. Beauchamp (1961) figures the copulatory apparatus of *M. monacensis* (his Fig. 59a) but the legend incorrectly states that it is of *M. terrestris*.

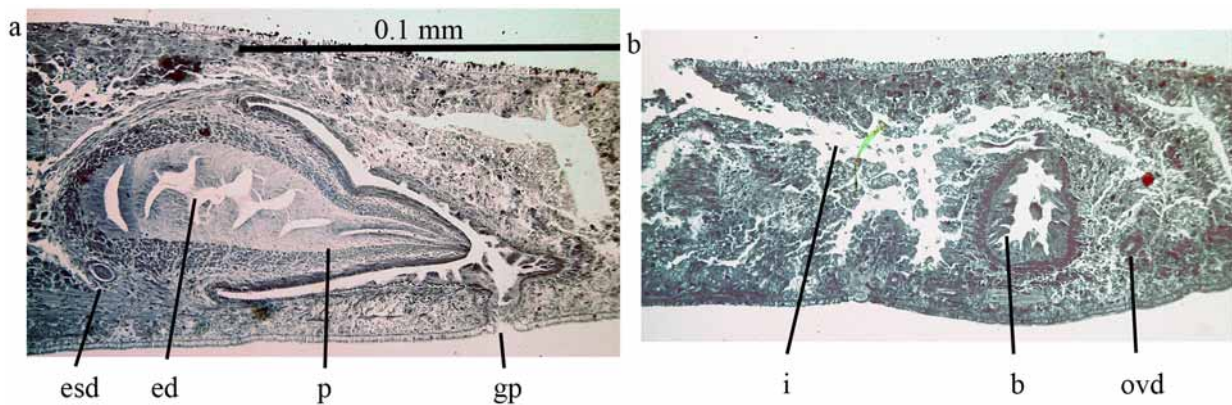


FIGURE 9. *Microplana monacensis* (Heinzel 1929). Holotype. NHMW, L153/M249, Inventory number 2849. a. Section through the penis. b. Section through the bursa. Anterior to the left.

Microplana terrestris (Müller 1774)

Müller's (1774) original description (Fig. 10) is minimal and none of his specimens remain. Müller did not specify any location or even country for the three specimens he examined, though he was Danish and worked in Denmark. Minelli (1977) designated a specimen from Denmark as neotype for the species but gave no figure or description of this specimen. Probably the best descriptions and diagrams of the copulatory apparatus of this species are by von Kennel (1882) and Bendl (1908; 1909), though there are differences apparent in the two Bendl diagrams.

Microplana terrestris (Müller), neotype (see Minelli, 1977), NHMD, TUR-17.1-8 (8 slides LS anterior including pharynx); TUR-19.1-12 (12 slides LS posterior including copulatory apparatus). Labels read: "*Microplana terrestris* (O.F. Müll.); det. Minelli 1976; Kildeskoven; I Ellestøb; 6.X.1918; Hj. Ditlevsen". Google Earth shows two places with the name Kildeskoven, one near Fredericia (Jutland), the other near Kalundborg (Zealand). Figure 11 shows a series of sections through the copulatory apparatus of this specimen. Each sperm duct expands to form a sperm storage organ immediately before entering the muscular bulb of the penis. The sperm ducts discharge separately into the base of the penis. The penis consists of two distinct parts, a basal bulb which does not project into the antrum, and a tapering, muscular, penis papilla which projects into the antrum. The basal bulb has an external diameter about 0.4 mm and there are several broad internal projections into its cavity. This cavity is equivalent to the "seminal vesicle" of many authors, though it is uncertain to what extent it is glandular. The projecting penis papilla has a basal diameter of about 0.2 mm and is about 0.5 mm long. A narrow ejaculatory duct, diameter about 25 µm, runs from the bulb to the distal end of the penis through the muscular part. The vagina opens directly off the antrum, is narrow and about 0.3 mm long. The paired ovo-vitelline ducts open separately but close together into its posterior extremity ventrolaterally. Also opening at its posterior extremity, but from the dorsal side is the genito-intestinal connection. This opens into a broad, sinus-like, cavity which, in this specimen, has a single narrow connection antero-dorsally to an intestinal diverticulum.

Rhynchodemus terrestris NHMW, 2831 M257. This collection consists of 151 slides of 20 specimens, some immature, from various European localities. The material originates from L von Graff and his collaborators at the University of Graz and some slides can be recognized as the source of illustrations in Graff (1899) and Bendl (1908, 1909). Some labels are apparently in Bendl's handwriting. Figure 12a shows a section of a specimen from County Galway, Ireland. There is a spherical basal bulb with internal projections, and a short, conical penis papilla projecting into the antrum. The vagina is short and, as above, the two oviducts and broad

genito-intestinal canal open into its posterior end. Transverse sections of another specimen (no locality on label) show the broad genito-intestinal canal and its opening into the intestinal diverticulum (Fig. 12b & c).

196. FASCIOLA TERRESTRIS,

FASCIOLA linearis, supra convexa, cinerea, sub-
tus alba.

Dan. JORD - FLAD - ORMEN.

long. 6 - 8 lin.

lat. $\frac{2}{3}$ lin.

Primo intuitu juniorem *Limacem* crederes.

Animalculum elongatum, simplicissimum, viscidum, opa-
cum, nigricans, supra teres, subtus planiusculum; antica pars solo
incessu a postica distingvitur. Quoties se in crassum volumen con-
trahit, annuli subtilissimi, sive striæ annulares pallidæ ope lentis
passim conspiciuntur. *Intestina* nullo modo visibilia. *Oculis*
quidem instruitur, punctis nempe duobus minimis in apice antico
(quodvis in areola pallida) at lentis quoque ope difficillime videntur.

More *Hirudinum* sese contrahit & extendit, more *Limacum*
progreditur vestigiaque viscositate sua notat.

Tria individua examini subjeci, in unius dextro latere ma-
culam parvam subluteam duas lineas ab antica parte distantem re-
peri, an *porus* excrementorum aut genitalium, aut utriusque?

In

Fasciola terrestris HIRUDINES & LIMACES quadricorn-s, *capitata* FA-
SCIOLAS & LIMACES *bicornes* affinitate jungunt.

FASCIOLA.

69

In asseribus muscisque humidis, qua terram spectant, Septem-
bri & Novembri reperi. Extra humiditatem posita minorum
intervallo perit, nec in humidum missa, aut aqua adpersa, revi-
viscit. Hinc sensu strictissimo ne hic quidem vermis terram, nisi
humidam, inhabitat.

FIGURE 10. *Microplana terrestris* (Müller 1774). Müller's description of 1774 (as *Fasciola terrestris*). This is the first published description of any terrestrial planarian.

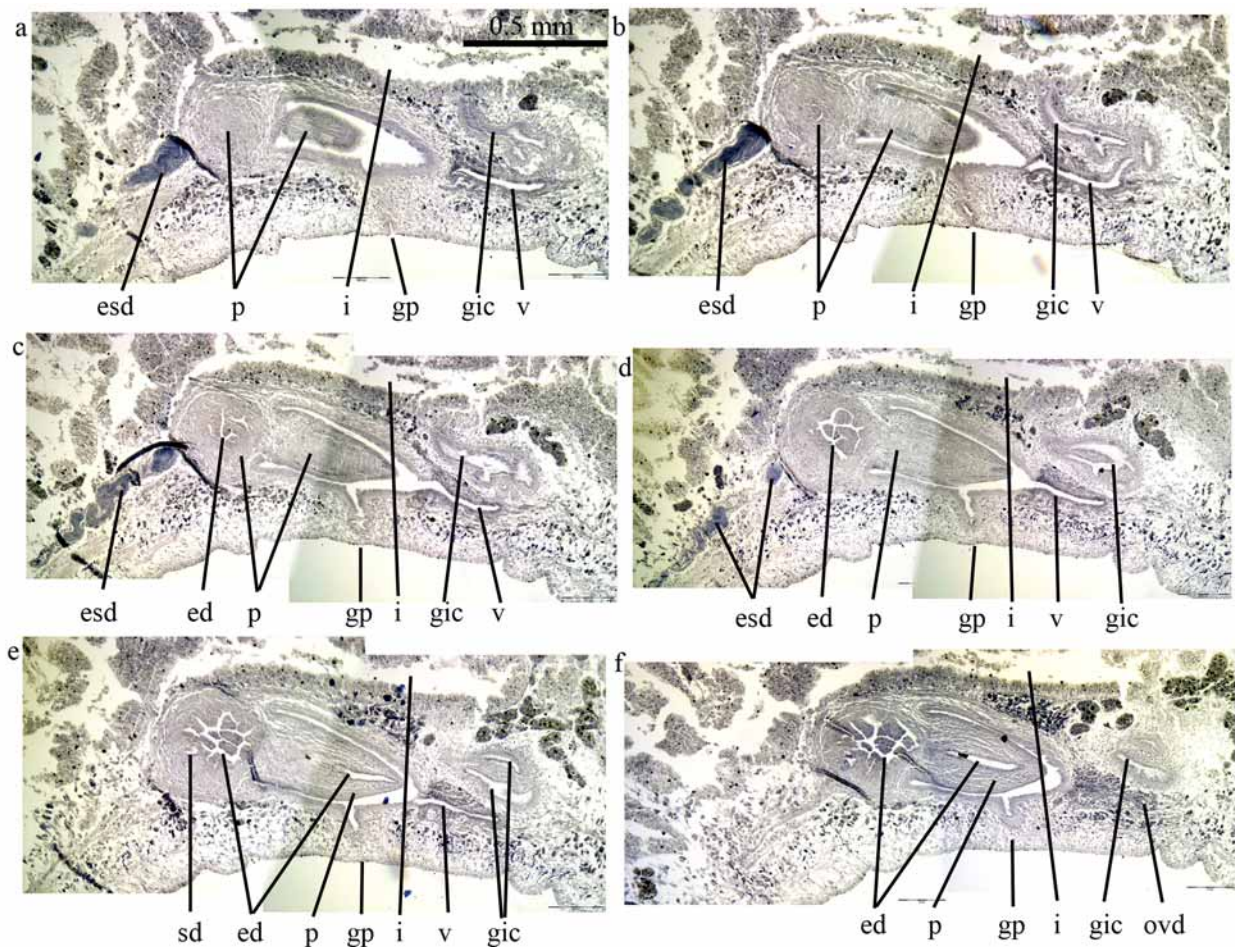


FIGURE 11. *Microplana terrestris* (Müller 1774). Neotype (designated by Minelli, 1977). NHMD, TUR-19.1-12. a–f. Series of sections of the copulatory apparatus at different planes through the serial sections. Each is a merged image of two adjacent images. Anterior to the left.

Rhynchodemus carli Fuhrmann 1914. NHMG (transferred from the University of Neuchâtel). 5 slides. Minelli (1977) considered this to be a synonym of *Microplana terrestris*, but as Minelli comments, “the type specimen is of very little help in assessing the true nature of the species”. The specimen was 10.5 mm long (preserved) and uniform brown/yellow in colour. Sections of the copulatory apparatus are poor, incomplete and not clear enough for a definitive identification. The ovaries are only partially mature and no ovo-vitelline duct is visible in transverse sections. However, numerous testes are visible. Expanded sperm ducts with stored sperm are visible posteriorly, but the specimen was damaged and nothing of the penis or other parts of the copulatory apparatus is present. The colour of the specimen suggests that this may not have been a specimen of *M. terrestris*, but we cannot be certain of its identity.

Microplana terrestris MNHNP, AO38-49. Labelled “Madé C_{1,3}”. This specimen is one of those reported by Beachamp (1957) from Madeira. The penis is as described above (Fig. 13a) thus its identification is confirmed.

The copulatory apparatus of two specimens of *M. terrestris* from Shirdley Hill, Lancashire (HDJ collection) are shown in Fig. 13, b & c.

Microplana terrestris T1, T2, T4, T7 (see above). The copulatory apparatus of T1, T2 and T4 all have a basal penial bulb with internal projections, and a short muscular penis papilla (Figs. 14 & 15). The vagina opens directly from the antrum and the genito-intestinal canal is broad and sinus-like. There are two openings from the genito-intestinal canal into the intestine in T1 and T2 but only one in T4. Refractive granules in the

digestive epithelium do not enter the genito-intestinal canal (Figs 14c, 15b, 15d). We are confident that our identification of these specimens as *M. terrestris* is accurate.

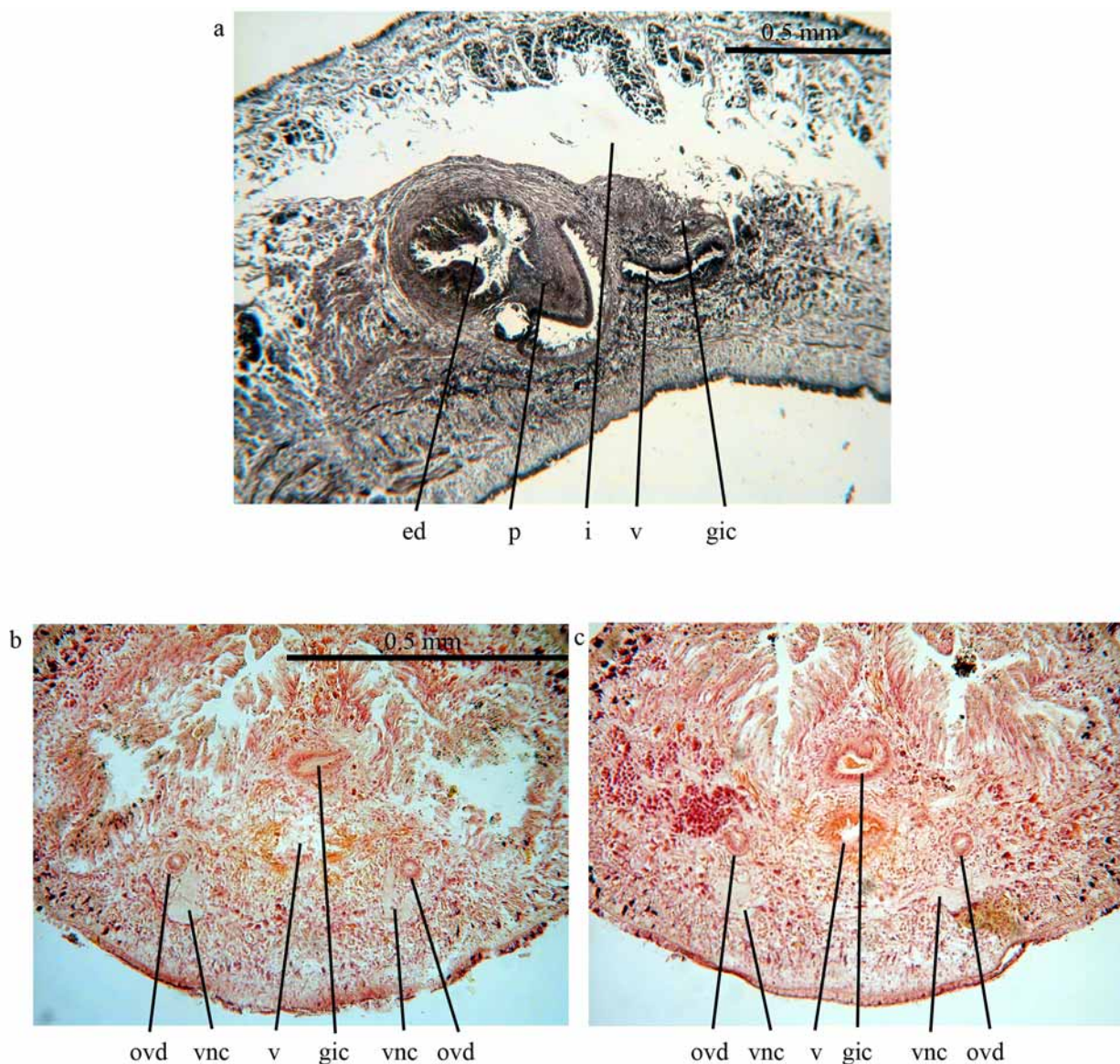


FIGURE 12. *Microplana terrestris* (Müller 1774). Specimens from NHMW, originating from the University of Graz. a. Longitudinal section through the copulatory apparatus of a specimen from County Galway, Ireland. b & c. Transverse sections through a specimen of unknown origin.

Discussion of *Microplana terrestris*. The size, grey or black colour and broad white ventral creeping sole are quite good discriminating factors for live specimens of this species. However the colour may be non-uniform as in the granular specimens. The copulatory apparatus is distinctive, the penis having a spherical basal ejaculatory duct (seminal vesicle) with internal projections, and a distal, muscular penis papilla projecting into the antrum. The genito-intestinal duct opens via a broad, sinus-like chamber into a branch of the intestine via one or two openings. Both Graff (1899) and Bendl (1908; 1909) show that there may be more than one such connection. Minelli's (1977) figure, which is after Bendl (1908), is generally accurate with the exception that the entry of the sperm ducts (labelled "deferens") into the penis is misleading. The figure in Ball & Reynoldson (1981) is generally accurate with the exception that the cavity in the basal bulb of the penis (labelled "seminal vesicle") is shown with a smooth lining without any of the internal projections visible in all the above specimens.

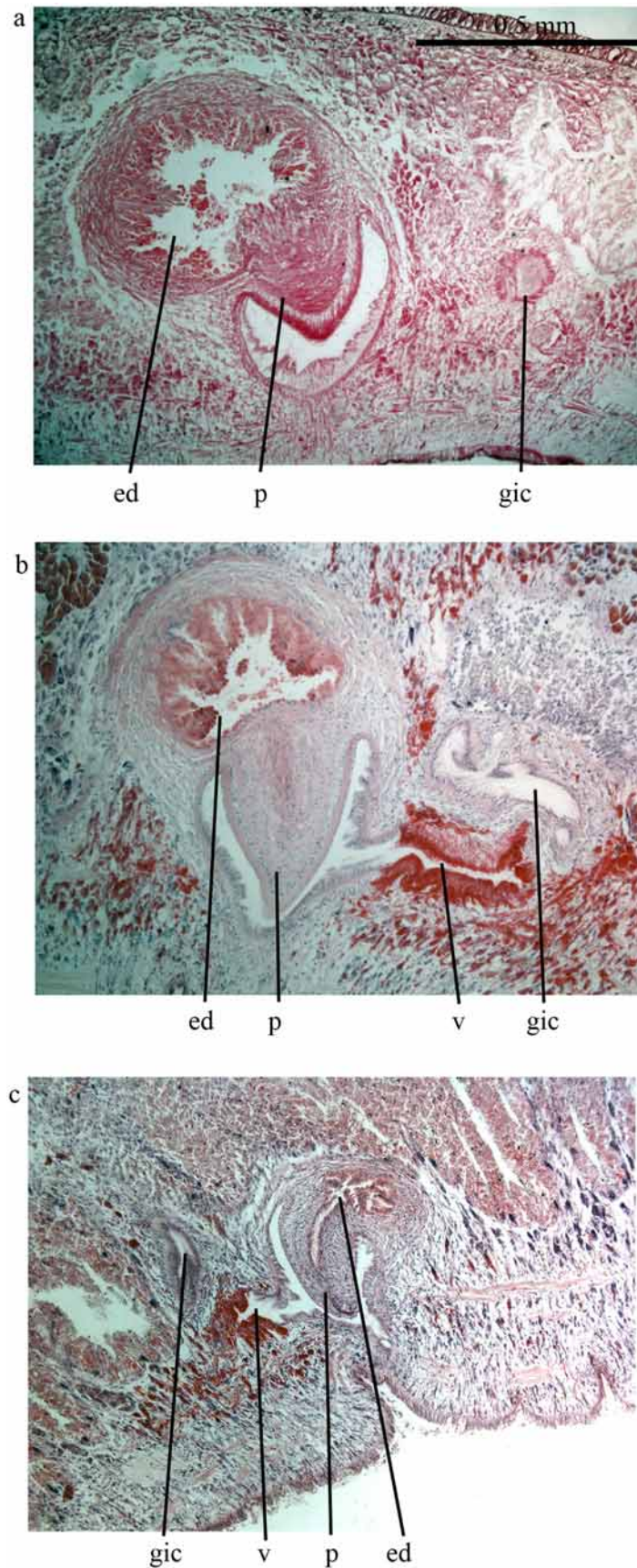


FIGURE 13. *Microplana terrestris* (Müller 1774). Copulatory apparatus of specimens from: a. Madeira collected and identified by Beauchamp (1957) MNHNP, AO 38-40; b & c. Two specimens from Shirdley Hill, Lancashire collected 1992 by HDJ. Anterior to the left in a & b, to the right in c. All to same scale.

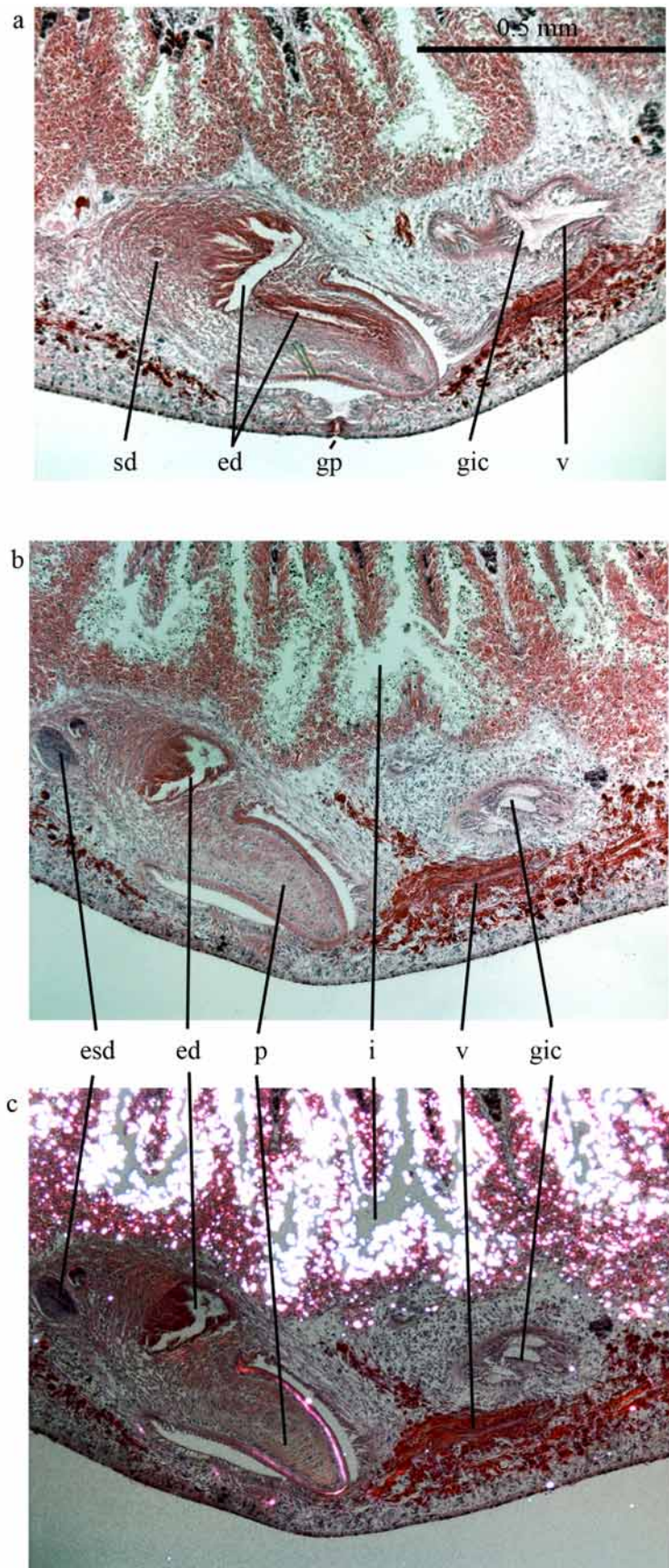


FIGURE 14. *Microplana terrestris* (Müller 1774). Specimen T1, NHML, 2006.6.28.1. Sections through the copulatory apparatus. b & c are of the same section, c is viewed with polarised light showing the refractive granules (white) limited to the intestinal epithelium. Anterior to the left.

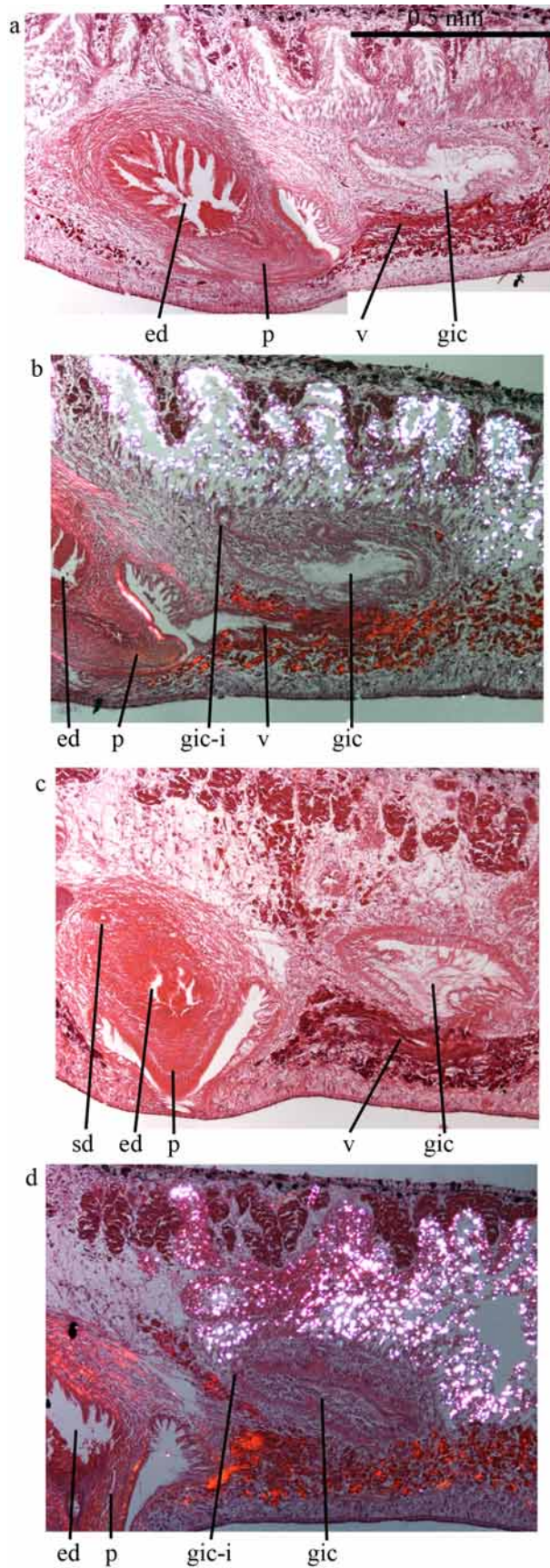


FIGURE 15. *Microplana terrestris* (Müller 1774). a & b. Specimen T2, NHML, 2006.6.28.2. c & d. Specimen T4, NHML, 2006.6.28.4. Sections through the copulatory apparatus, b & d viewed with polarised light showing the refractive granules (white) limited to the intestinal epithelium.

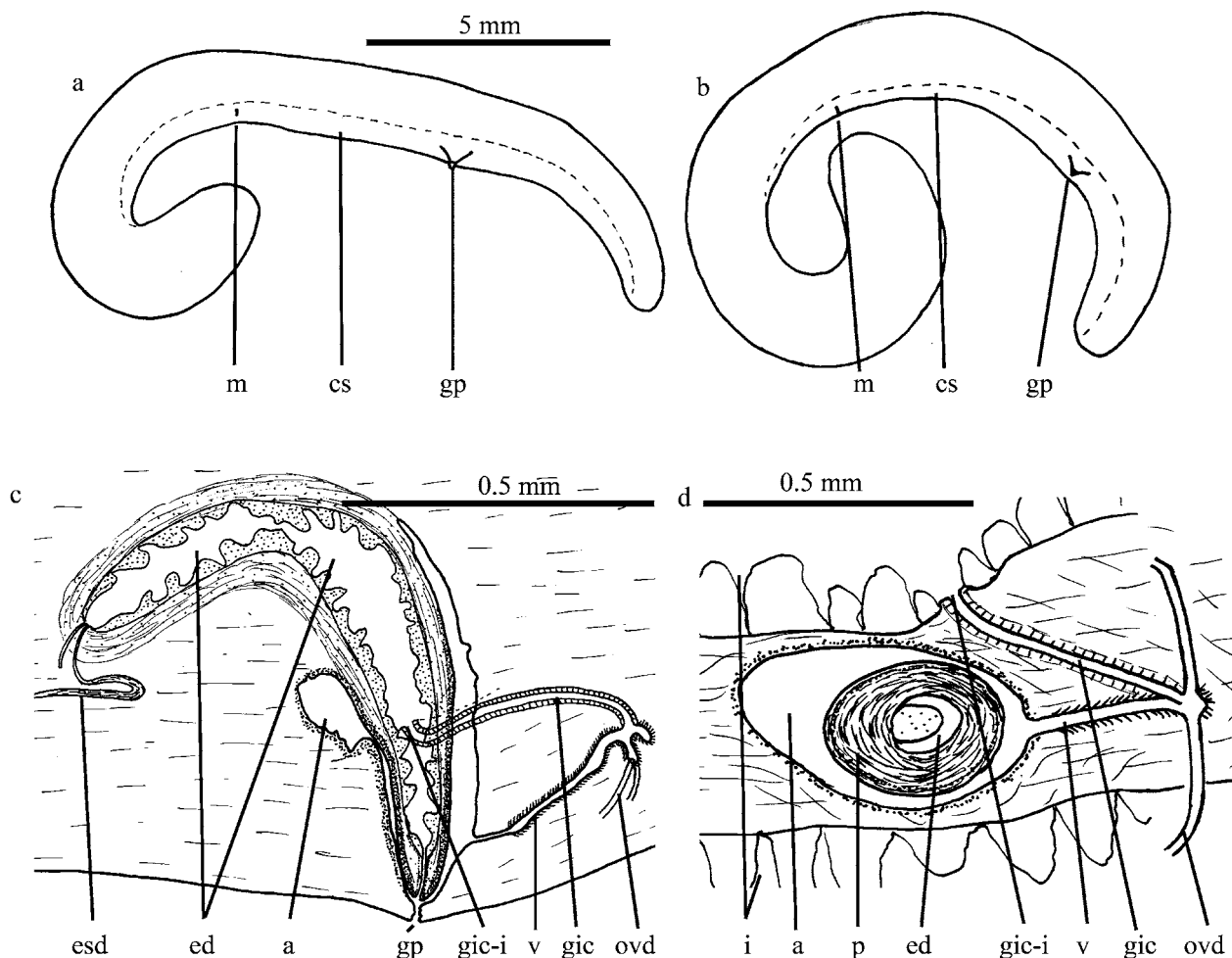


FIGURE 16. *Microplana kwiskea* n. sp. a & b. Outline drawings of preserved specimens K7 and K6 respectively, both to the same scale. Anterior end to the left in both. c. Diagram of the copulatory apparatus in longitudinal section, based on Specimen K9 (Fig. 18). d. Diagram in horizontal section through the copulatory apparatus, based on Specimen K1a (Fig. 19).

***Microplana kwiskea* n. sp.**

Material examined: Holotype K9. Syntypes K1a to K8 (see above). All collected a few days prior to, and fixed on, 25 October 2004.

Type locality: Bridge House, Kirby Wiske, Thirsk, Yorkshire, United Kingdom (Ordnance Survey Grid Reference: SE375849).

Etymology: The specific epithet “*kwiskea*” is an abbreviation of Kirby Wiske, the type locality.

Description: Live animals (Fig. 1b, upper) are up to 2.5 cm long and about 2 mm in diameter at the widest point when extended. When contracted they are about 6 mm long and 3 mm wide (Fig. 1c, upper). They are cylindrical and taper towards the anterior and posterior ends both of which are blunt. They are a uniform mid-brown (khaki) colour, even ventrally. There is a creeping sole mid-ventrally in the form a narrow ridge. Observations on creeping animals through transparent tubes showed little muscular distortion of the sole suggesting that locomotion is primarily by means of the cilia of the sole. Two small eyes, visible as black spots, are present at the anterior end. Otherwise there are no markings or orifices visible on live specimens. Preserved specimens are up to 18 mm long, the anterior end particularly being contracted and curled ventrally

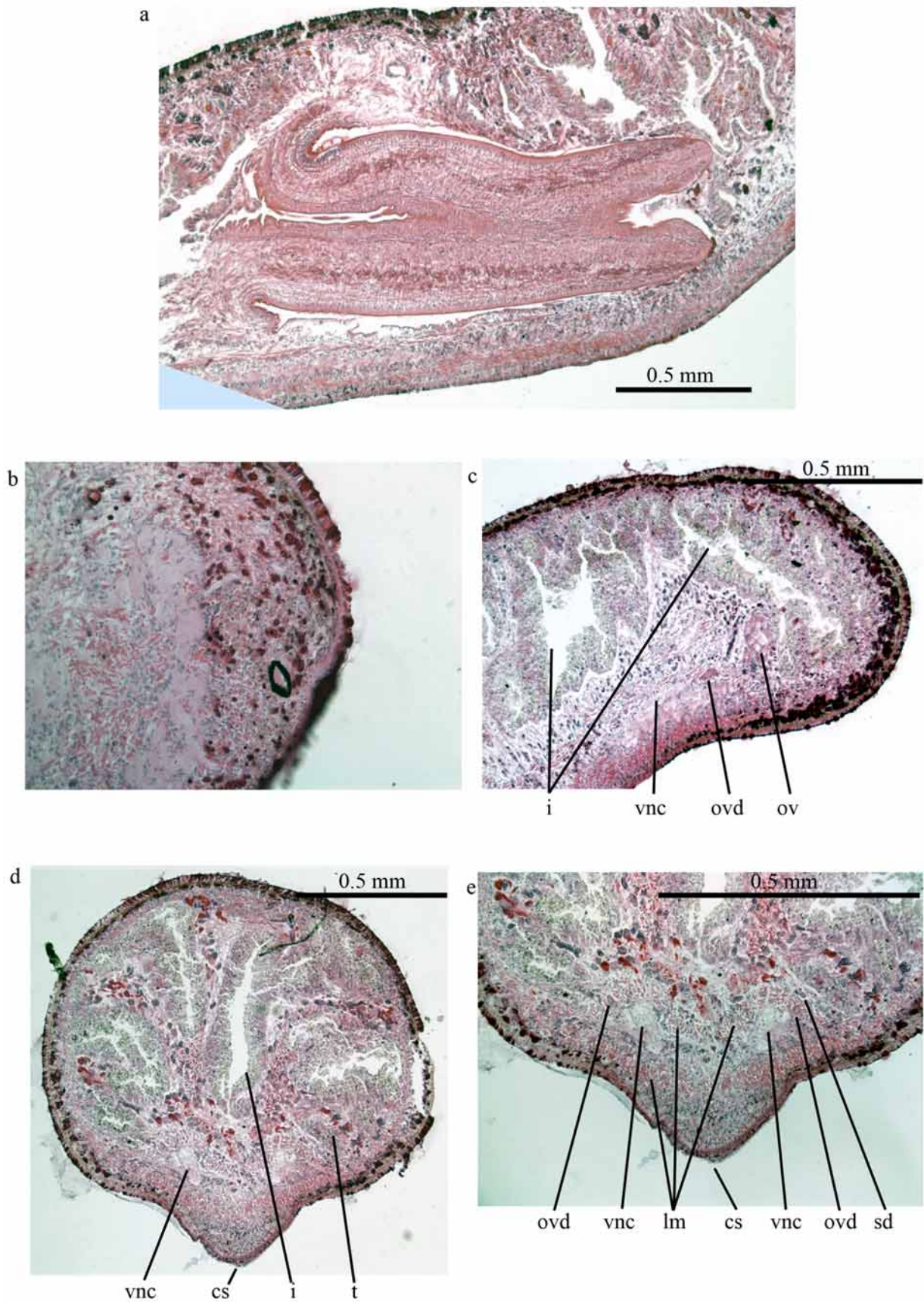


FIGURE 17. *Microplana kwiskea* n. sp. a. Specimen K2, NHML, 2006.6.28.31. Section through the pharynx. Anterior to the left. b-e. Specimen K8, NHML, 2006.6.28.37. b. Section through an eye. c. Section through an ovary near the anterior end. d. Transverse section, anterior to the pharynx. e. Enlarged view of the ventral portion of (d)

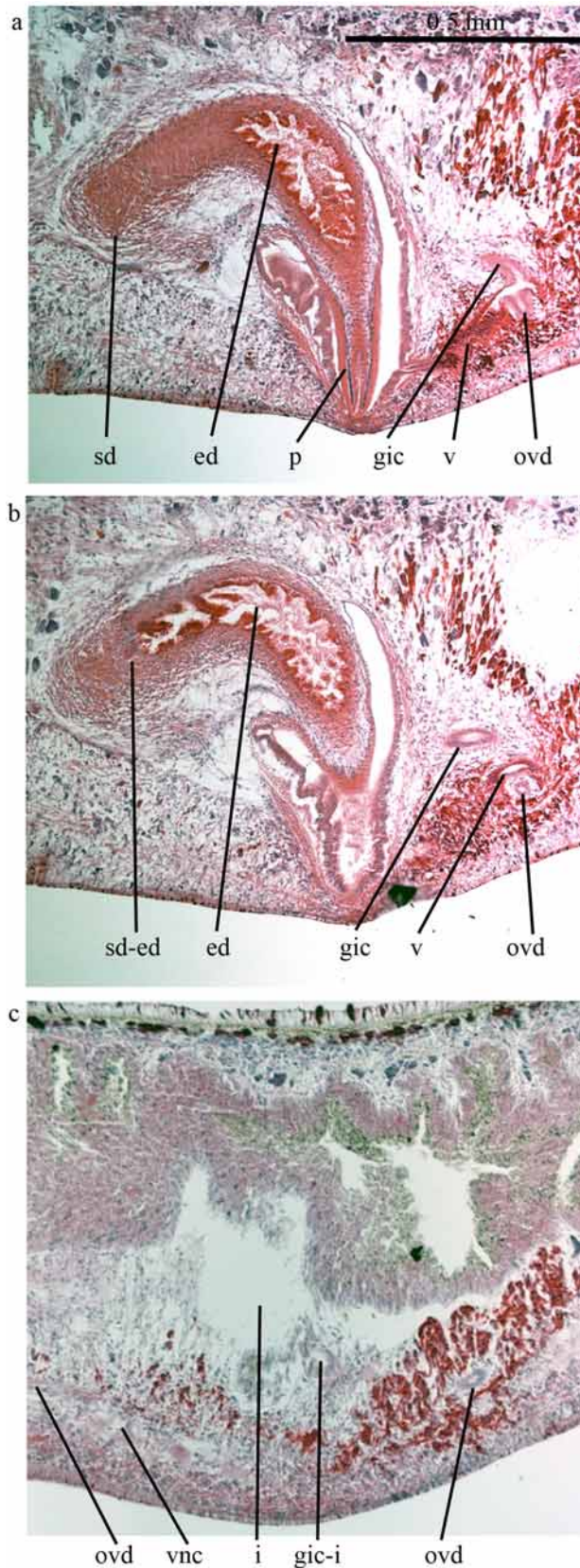


FIGURE 18. *Microplana kwiskea* n. sp. Holotype, specimen K9, NHML, 2006.6.28.38. Longitudinal sections through the copulatory apparatus. Anterior is to the left and the sections are progressively from approximately the midline towards the right side. a. Through the tip of the penis and also the junction of the ovo-vitelline ducts, vagina and genito-intestinal canal. b. Through the base of the penis showing the entry of the sperm ducts to the ejaculatory duct. c. Through the opening of the genito-intestinal duct to the intestine.

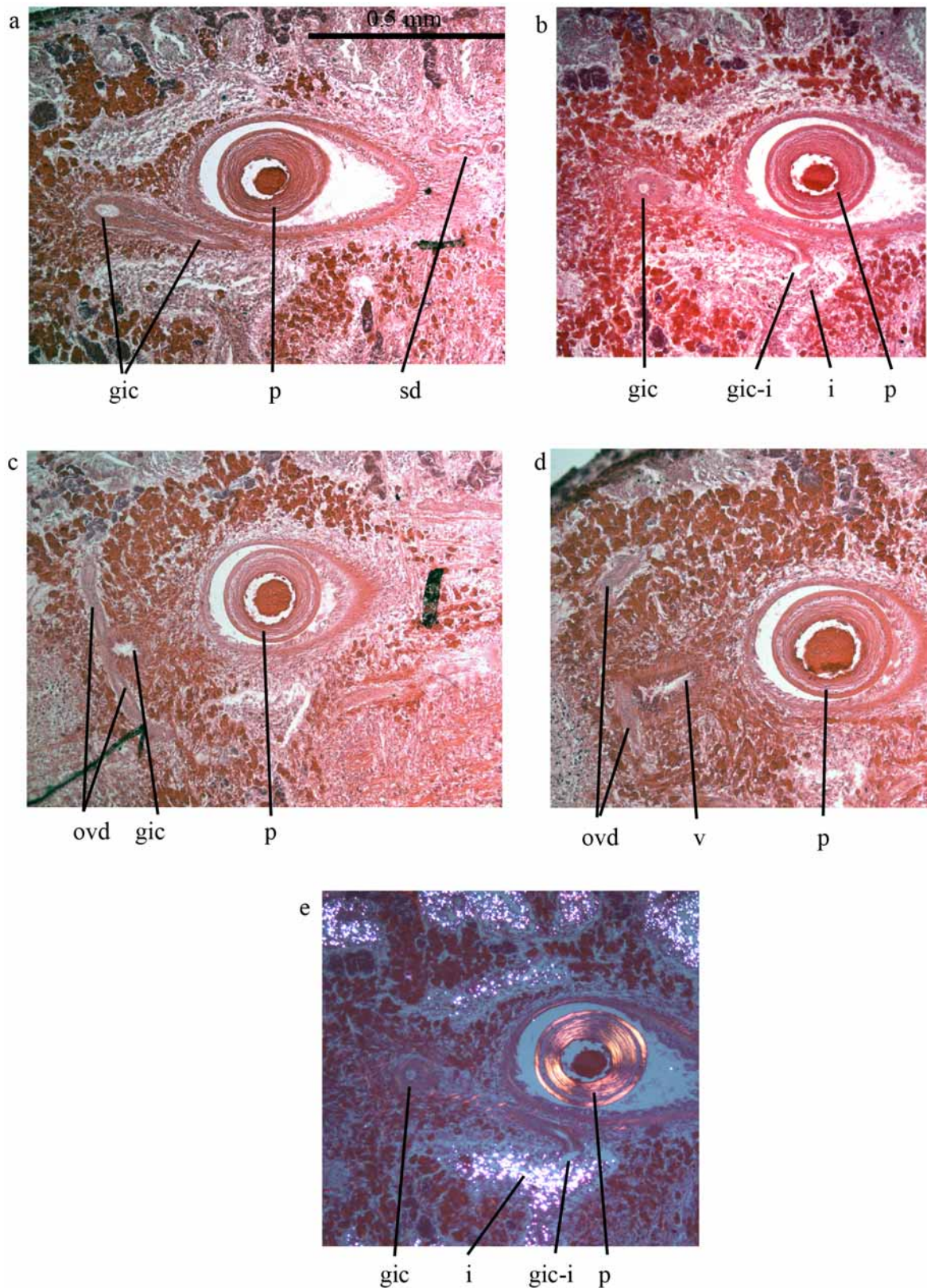


FIGURE 19. *Microplana kwiskea* n. sp. Specimen K1a, NHML, 2006.6.28.30. Horizontal longitudinal sections through the copulatory apparatus. Anterior to the right. a–d are progressively more ventral. a. The penis and genito-intestinal canal. b. The penis and, to its right, the opening of the genito-intestinal canal into a digestive diverticulum. c. The conjunction of the genito-intestinal canal and the ovo-vitelline ducts. d. The vagina and penis. e. Micrograph in polarised light of the section in (b) showing the circular muscle of the penis and the highly refractive granules (white) present in the digestive epithelium. There are no granules in the genito-intestinal canal (cf. *M. terrestris* and *M. scharffi*).

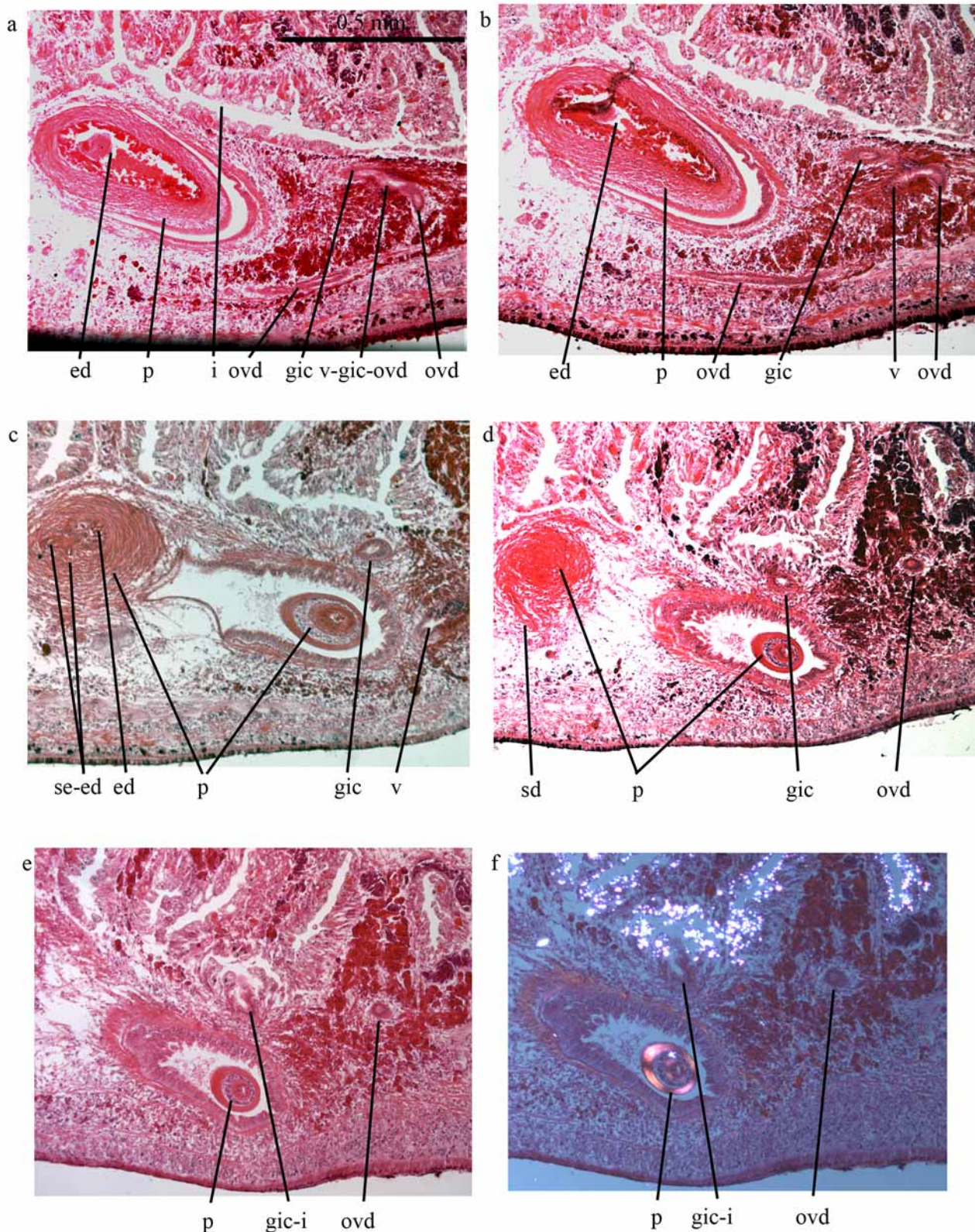


FIGURE 20. *Microplana kwiskea* n. sp. Specimen K2, NHML, 2006.6.28.31. Longitudinal sections at an oblique angle. Anterior to the left. a–e show the course of the genito-intestinal canal from its origin at the posterior of the vagina (a) to its opening into the digestive diverticulum (e). c. shows the opening of the sperm ducts into the ejaculatory duct. f. Micrograph in polarised light of the section in (e) showing the circular muscle of the penis and the highly refractive granules (white) present in the digestive epithelium. There are no granules in the genito-intestinal canal (cf. *M. terrestris* and *M. scharffi*).

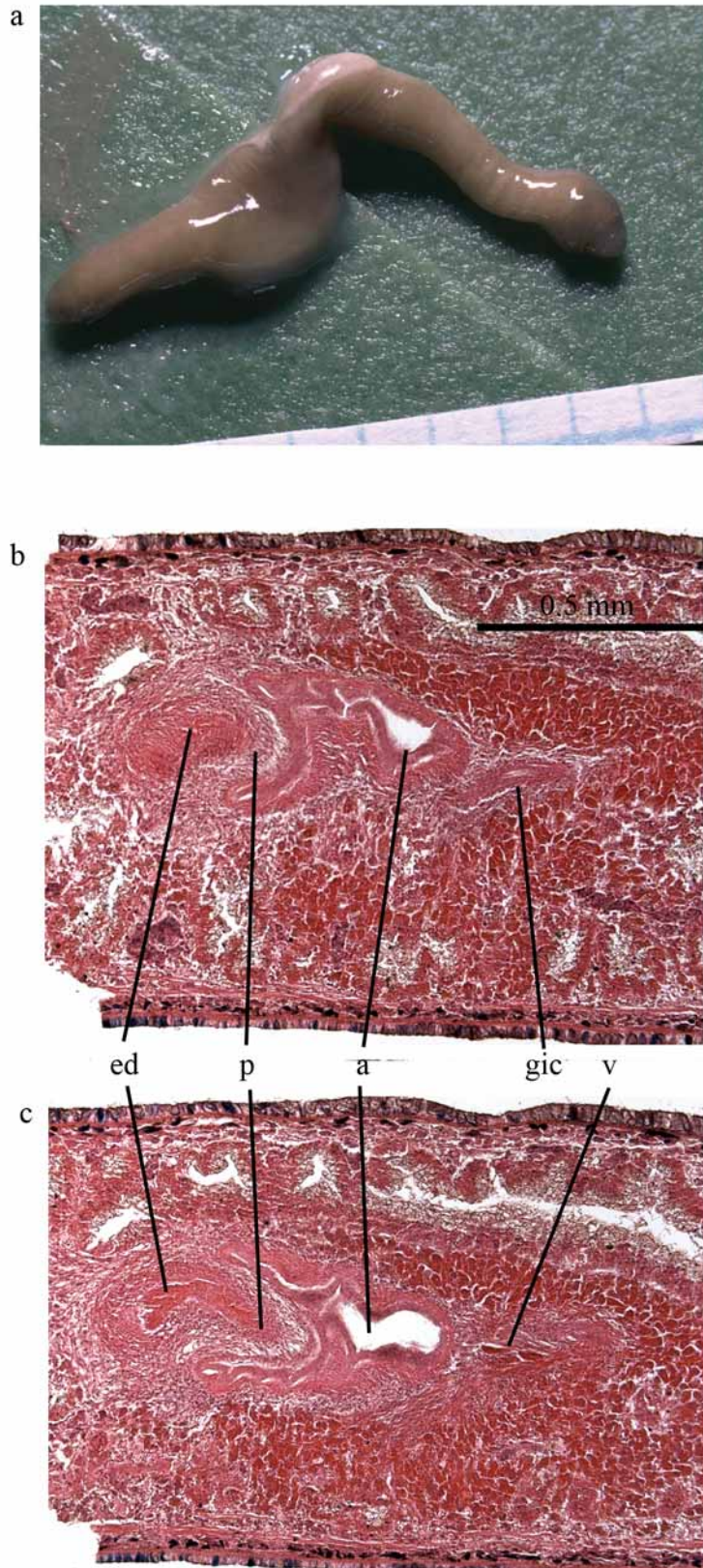


FIGURE 21. *Microplana kwiskea*. Manchester (1985) specimens (HDJ collection). a. Photograph of a live (partly auto-digested) specimen. Anterior to the right. 2 mm graph paper. b & c. Sections through the copulatory apparatus. Anterior to the left.

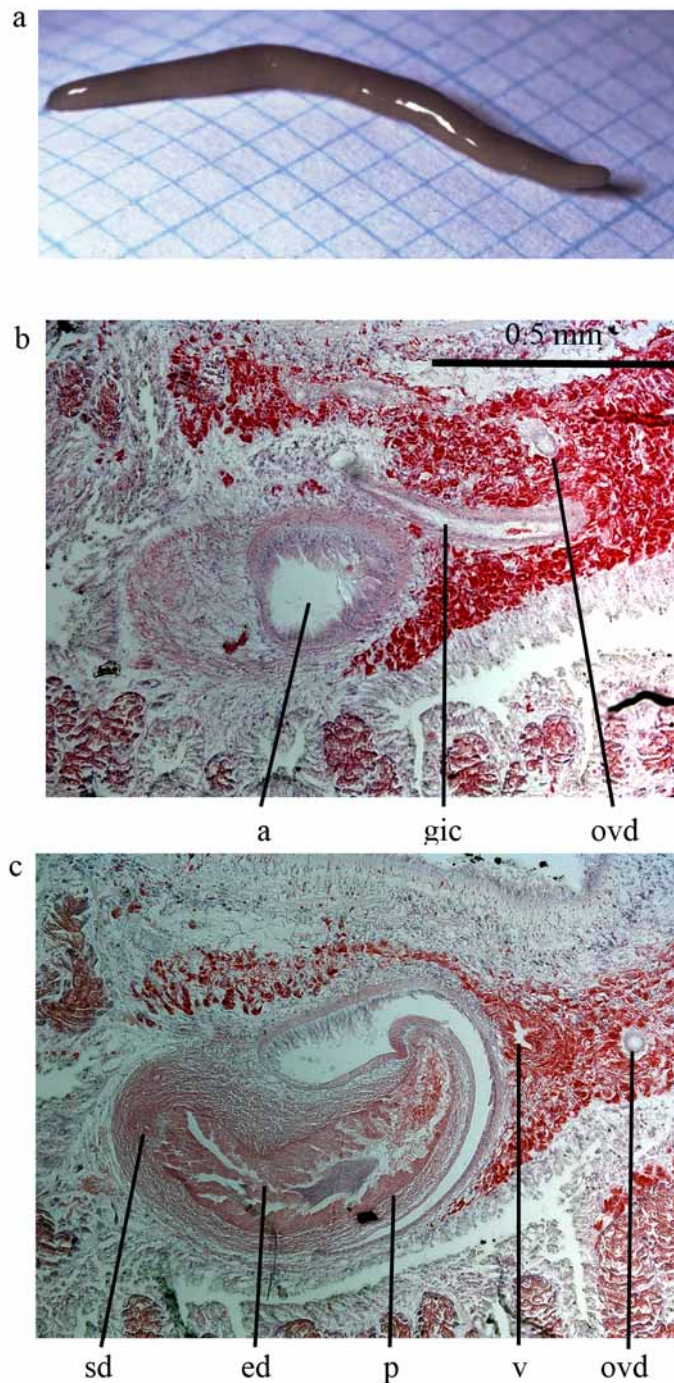


FIGURE 22. *Microplana kwiskea*. Shirdley Hill (1992) specimens (HDJ collection). a. Live specimen, anterior to the right, on 2 mm graph paper, thus the worm was about 2.2 cm long and 2 mm wide. b & c. Sections through the copulatory apparatus of a further specimen. Anterior to the left.

(Fig. 16a & b). The mouth (pharyngeal aperture) is about 8 mm from the anterior end (44% of body length) and the gonopore is about 12 mm from the anterior end (67%), but these must be regarded as approximate due to contraction. The gonopore in the remaining preserved specimens (Fig. 16a & b) and in the sectioned specimens is raised and prominent with a slightly protruded penis, presumably from muscular contraction at fixation.

The general anatomy is typically rhynchodemid. The two eyes of K8 have a diameter about 35 μm and length about 50 μm (Fig. 17b). The gut is triclad and there is a cylindrical pharynx. The pharynx of K2 is about 1.5 mm long and 0.5 mm in diameter (Fig. 17a). The wall of the pharynx is about 200 μm thick. The

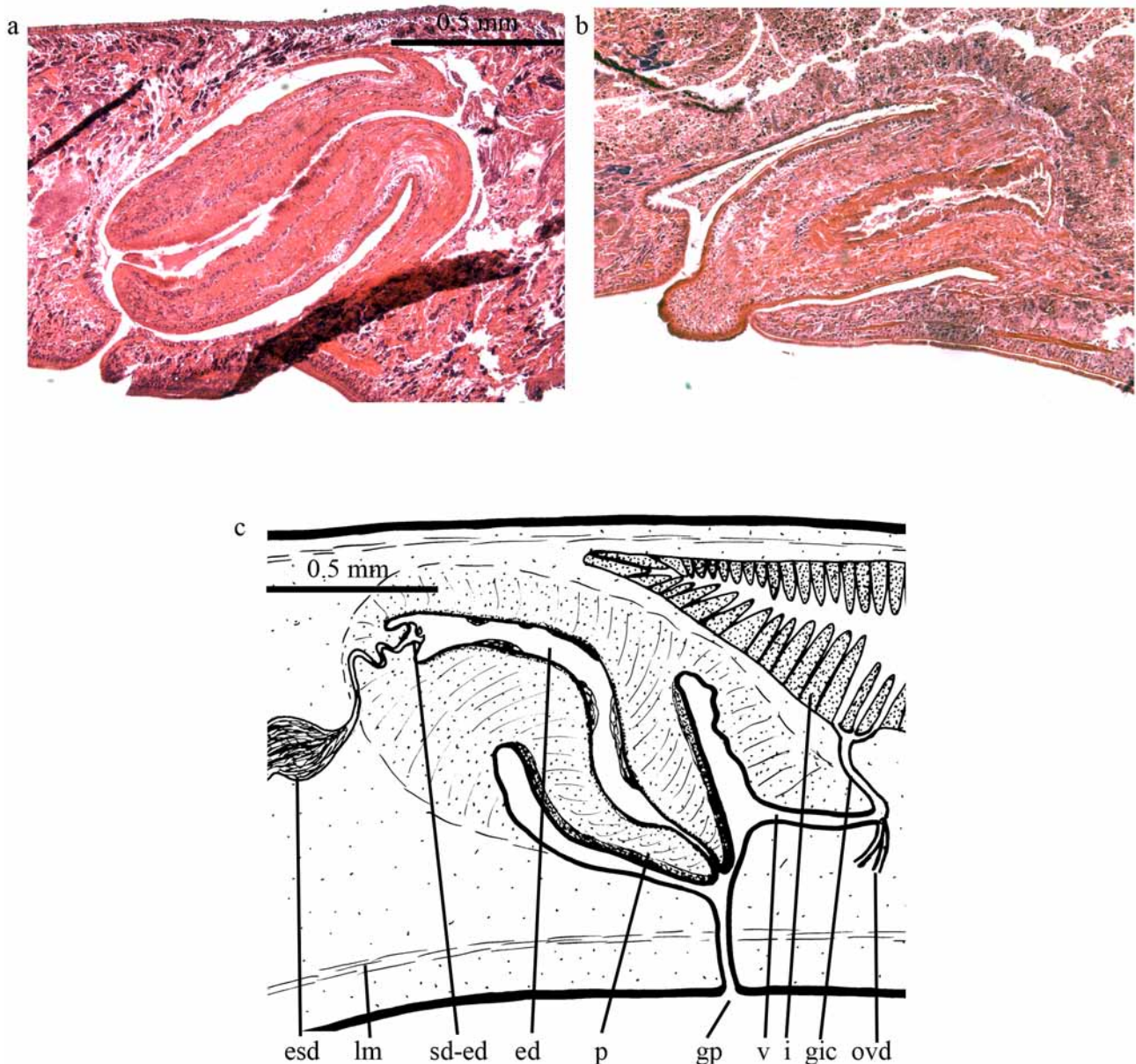


FIGURE 23. *Microplana groga* n. sp. a & b, the pharynx of specimen FG2. a is of the whole pharynx and (b) is a high magnification view of part of (a). c & d, similar low and high magnification views of the pharynx of specimen FG3. Anterior to the right. e. Composite diagram of the copulatory apparatus, based mostly on specimen FG1 (cf. Figure 24). Anterior to the left.

outer epithelium of the pharynx is about 2.5 μm thick and densely ciliated, the cilia being about 2.5 μm long. Inside this is a loose layer of radial and longitudinal muscle about 160 μm thick with an inner dense layer of circular and longitudinal muscle about 40 μm thick. The inner wall is not ciliated. The creeping sole (Fig. 17d & e) is distinct, narrow (0.5 mm wide, about one third of the total width) and raised as a mid-ventral ridge (0.25 mm high). The epidermis of the creeping sole is about 20 μm thick, has few, narrow, rhabdites and is densely ciliated with cilia about 5 μm long. Dorsal and lateral epithelium is about 30 μm thick with copious broad rhabdites but apparently devoid of cilia. Sub-epidermal muscle is indiscernable. Rhabdites are formed sub-epidermally, just inside the epidermis, about 50 μm from the outer surface. Inside the rhabdite-forming zone is a layer of parenchymal longitudinal muscle, about 50 μm thick dorsally and laterally, thickening ventrally over the creeping sole to about 100 μm . There are further longitudinal muscle bundles dorsal to the ventral nerve cords (Fig. 17d & e). The paired ventral nerve cords are joined by occasional transverse

commissures (Fig. 17d & e). The epithelium of the intestinal diverticula is between 50 µm and 100 µm thick and contains numerous very small refractive granules. The paired ovaries of K8 are 500 µm from the anterior end (Fig. 17c). The paired ovo-vitelline ducts run posteriorly dorso-lateral to the ventral nerve cords. Testes lie ventrally between the intestinal diverticula. They are oval, about 100 µm by 70 µm. It is not possible to discern the sperm ducts in transverse sections of the anterior of the body, but posterior to the pharynx they run dorso-lateral to each ventral nerve cord in the vicinity of the ovo-vitelline ducts. The two sperm ducts broaden to about 30 µm in diameter just before they reach the penis to form sperm storage organs which are reflexed forwards (Fig. 18). Presumably these straighten on penis eversion. Each then narrows to about 10 µm external diameter, 5 µm internal diameter, enters the muscular base of the penis and the two ducts discharge separately but close together into the ejaculatory duct. The penis is long, about 1 mm, and the distal half is free in the antrum (Figs 16c & 18). For most of its length it is roughly parallel-sided, about 200 µm in diameter. The ejaculatory duct is broad, about 100 µm in diameter and has numerous internal short projections or folds. At its distal end (the last 250 µm) the penis narrows to a blunt point and the ejaculatory duct in the distal 100 µm is narrow, about 10 µm in diameter, and has a smooth internal wall. The free portion of the penis has a dense outer layer of circular muscle fibres about 15 µm thick. Transverse sections of the penis show it to consist mostly of dense circular muscle fibres with a thin layer of longitudinal muscle near the outer epithelium (Figs 16d & 19). The vagina is narrow and short, 320 µm long. The posterior end of the vagina receives the openings of the two ovo-vitelline ducts ventrally and the genito-intestinal duct dorsally. The genito-intestinal duct is clearly defined, strongly ciliated internally and has an internal diameter of 10 µm and external diameter of 30 µm for its entire length. It runs dorsally from the vagina, forwards and to the right to open into the right posterior intestinal diverticulum at about the level of the penis. The refractive granules of the digestive epithelium do not enter the genito-intestinal duct (Figs 19e & 20f).

Specimen from Manchester. Collected 20 October 1985, Ecology Study Area, campus of the University of Manchester (Grid Reference: SJ846968). This specimen (Fig. 21) partially autodigested before it was fixed. It is not quite fully mature in that the penis is not very large, but the ejaculatory duct extends for most of its length and has internal folds or projections, similar to the above. The genito-intestinal duct is well-defined, narrow and runs directly to a digestive diverticulum on one side. Its identity was uncertain until compared with the above specimens from Kirby Wiske and we are confident that it is a specimen of *M. kwiskea*.

Specimens from Shirdley Hill, Lancashire. Collected October 1992 at the site of population studies of *Australoplana sanguinea* (sensu Jones 1981) reported by Jones *et al.* (1998 and 2001) (Grid reference: SD360129). Two specimens have been sectioned (in addition to undoubted *M. terrestris* from the same site). Both have the same penis structure and direct genito-intestinal duct typical of *M. kwiskea* (Fig. 22) and again we are confident these are of that species.

Discussion on *Microplana kwiskea*. The presence of a single pair of eyes and of a genito-intestinal connection means that these specimens are undoubtedly of the genus *Microplana*. There was little doubt about *M. kwiskea* being different from *M. scharffi*, the shape, size and colour being sufficient to distinguish the species. The different anatomy of the copulatory apparatus simply confirms this distinction. However, the size and shape of *M. kwiskea* is broadly similar to that of *M. terrestris*, though the colour is different, hence our original uncertainty. The molecular results are unequivocal, the specimens are of a different species to *M. terrestris*. The anatomy of the male ducts, penis and of the genito-intestinal duct are quite different in the two species. The expanded ejaculatory duct with internal projections (seminal vesicle of some authors) runs most of the length of the penis in *M. kwiskea*, but in *M. terrestris* it occupies only the basal half of the penis. In *M. kwiskea*, the genito-intestinal duct is of constant diameter for its entire length and it runs noticeably forwards to open into the intestine to one side (the right) of the penis. In *M. terrestris* the genito-intestinal duct broadens to a sinus-like cavity before narrowing to its opening or openings to the intestine which, though forwards of its origin, are somewhat dorsal and not as far forwards as in *M. kwiskea*. The pharyngeal opening (mouth) and gonopore are further apart in *M. kwiskea* than in *M. terrestris*.

Apart from their different colour, probably the best discriminating feature between the two species when alive is the ventral creeping sole. This is best seen when the animals are viewed from the ventral side when placed in a transparent container. In *M. terrestris* the sole is wide, almost half the ventral width, hardly raised and almost white. In *M. kwiskea* the sole is narrow, ridged and virtually the same colour as the rest of the body.

Microplana groga n. sp.

Material examined: Specimens labelled “*M. terrestris* Font Groga”, NHML 1998.2.9.3-5, deposited by Mateos *et al.* (1998). Three specimens, FG1 (3 slides), FG2 (3 slides), FG3 (4 slides).

Type locality: “Font Groga”, Massis de Collserola (Sant Cugat del Vallès, Barcelona, Spain).

Etymology: the specific epithet is after the type locality.

Holotype: Specimen FG1, three slides FG1.1, FG1.2 and FG 1.3, labelled Font Groga, *M. Terrestris*, NHML 1998.2.9.3. Co-types FG2 (4 slides, NHML 1998.2.9.4) and FG 3 (4 slides, NHML 1998.2.9.5), similarly labelled.

Mateos *et al.* (1998) identified five specimens as *M. terrestris*. Three were sectioned and are the specimens deposited above. A further two were sacrificed for nucleic acid extraction and one of these is the source of the GenBank sequence AF178318 (Carranza, pers. comm.) which is identical to that of our specimens of *M. kwiskea* (K2, K8, K9) (Fig. 2). The worms were described as “reddish to brown with black granules all over the surface: anterior end darker; creeping sole white” (Mateos *et al.* 1998). Representative sections of these three specimens are shown in Figs 23–26. The distance between the mouth (pharyngeal aperture) and the gonopore can be measured in all three specimens. In FG1 it is 1.4 mm, in FG2 it is 1.7 mm, in FG3 it is 1 mm. The pharynx is cylindrical, 1.1 mm long in FG2 and 0.9 mm in FG3 (it is reflexed in FG1), and about 0.4 mm in diameter in all three specimens (Fig. 23a–d). The pharyngeal aperture is two-thirds along the pharyngeal cavity. The outer epithelium of the pharynx is densely ciliated, cilia about 2.5 μm long. The pharyngeal musculature consists of an outer thin, sub-epidermal layer of circular muscle about 10 μm thick, a loose layer of longitudinal and radial fibres about 130 μm thick and an inner, dense layer of mainly circular fibres, but with some longitudinal fibres, about 40 μm thick. The inner epithelium is not ciliated. Though there are no transverse sections of the whole body, it can be seen in longitudinal sections that there is no longitudinal muscle immediately sub-epidermal but that longitudinal muscle fibres are present 30 to 60 μm inside the epidermis (Fig. 23e). There are few longitudinal fibres dorsal to the ventral nerve cords. In all three specimens, the sperm ducts are exceptionally broadly expanded between the pharynx and the anterior end of the penis (Fig. 23e). The ducts narrow, internal diameter about 5 μm , enter the penis musculature where they are convoluted, and discharge separately into the base of the ejaculatory duct (which is labeled “seminal vesicle” by Mateos *et al.* 1998). The penis is muscular and free in the antrum for about three-quarters of its length. The penis musculature is quite dense with, from outer to inner: dense circular muscle about 15 μm thick; dense longitudinal muscle about 10 μm thick; more loose radial muscle about 100 μm thick; circular muscle about 10–20 μm thick around the ejaculatory duct. The ejaculatory duct is broad, about 70 μm in diameter, for most of the length of the penis but narrows at the distal end of the penis to about 10 μm . There are no internal projections or folds on the wall, though there are some possible secretory products. There is a short vagina which at its posterior end receives the two ovo-vitelline ducts ventrally and the genito-intestinal canal dorsally (Mateos *et al.* label this as “bursa copulatrix”). In FG1 and FG2 the single genito-intestinal canal opens into one of the digestive diverticula via a confused, plate-like opening, possibly with more than one opening (Figs 24, 25). In FG3 the genito-intestinal canal forks to become Y-shaped with each upper branch opening into a digestive diverticulum on the opposite side (Fig. 26). These openings are more distinct than in FG1 and FG2.

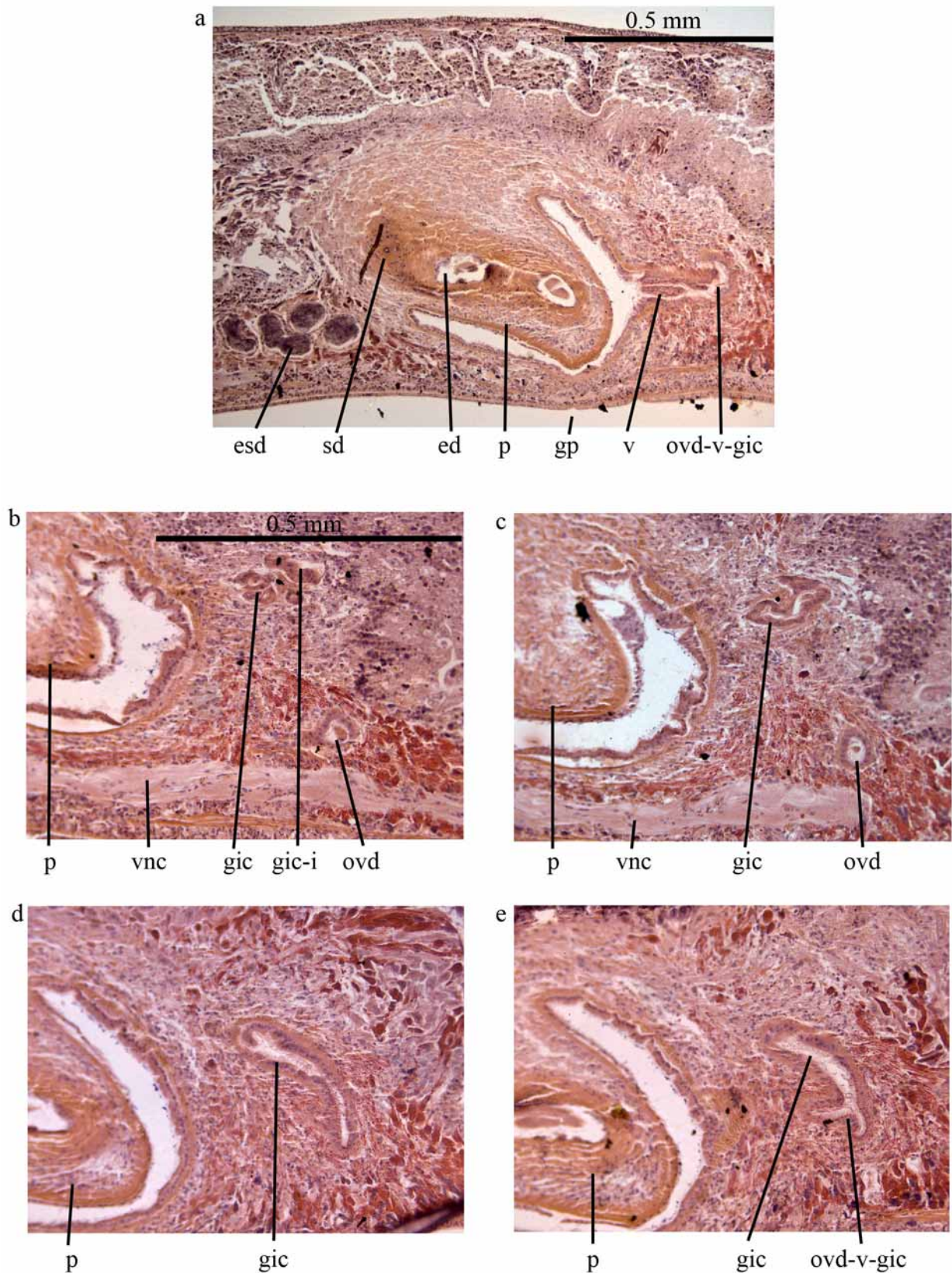


FIGURE 24. *Microplana groga* n. sp. Specimen FG1 of Mateos *et al.* (1998). NHML, 1998.2.9.3. Anterior to the left. a. Longitudinal section showing the expanded sperm duct, penis and vagina. b–e. Sections at intervals through the vagina and genito-intestinal canal. All to the same scale.

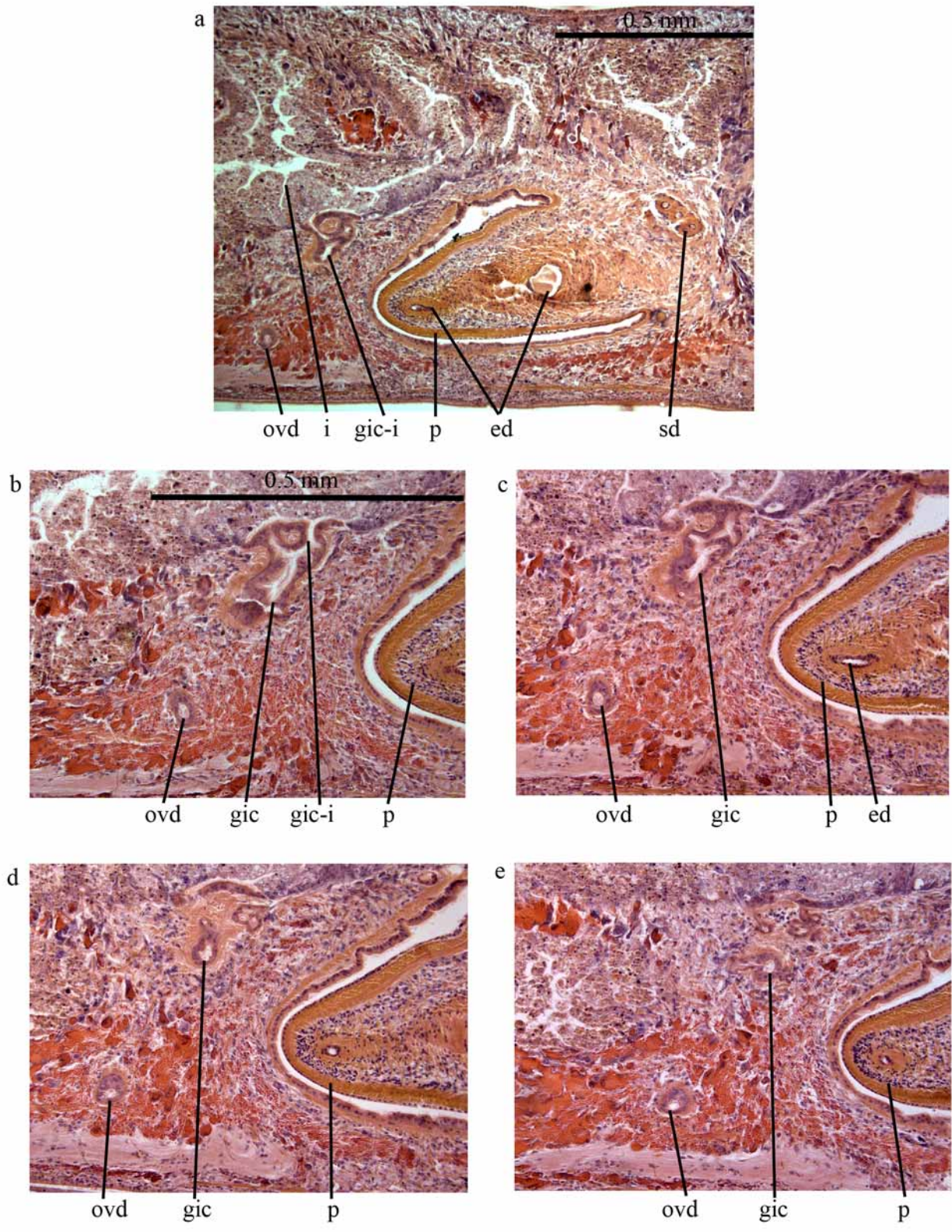


FIGURE 25. *Microplana groga* n. sp. Specimen FG2 of Mateos *et al.* (1998). NHML, 1998.2.9.4. Anterior to the right. a. Longitudinal section showing the penis and genito-intestinal canal. b–e. Sections at intervals through the genito-intestinal canal. The serial sections are incomplete. All to the same scale.

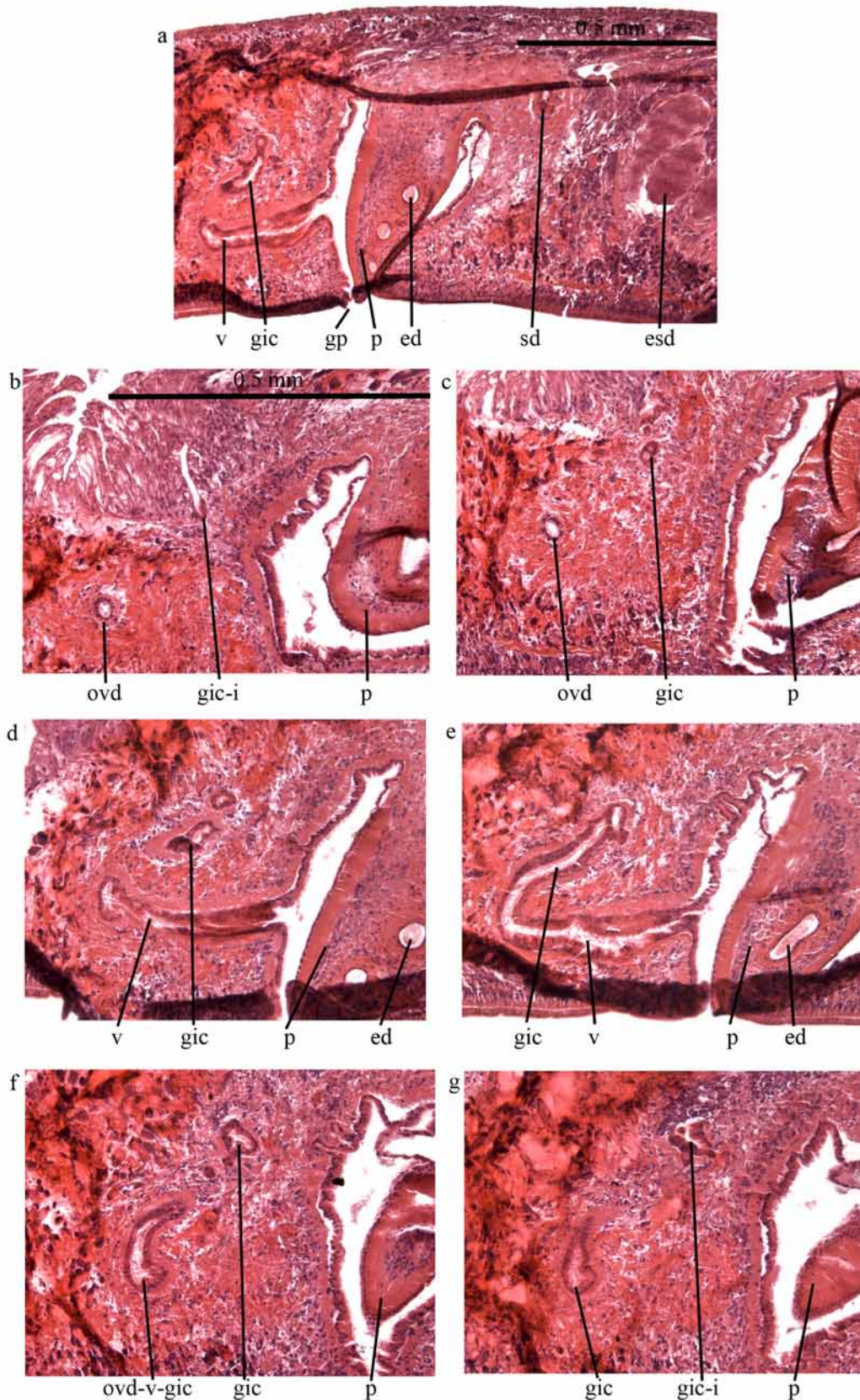


FIGURE 26. *Microplana goga* n. sp. Specimen FG3 of Mateos *et al.* (1998). NHML, 1998.2.9.5. Anterior to the right. a. Longitudinal section showing the expanded sperm duct, penis and vagina. b–g. Sections at intervals through the vagina and genito-intestinal canal. Note that the genito-intestinal canal forks and there are two openings to the intestine, one on either side. (b) shows the opening of the genito-intestinal canal on one side, and (g) on the other side. All to the same scale.

Discussion of *M. groga*. The presence of a single pair of eyes and of a genito-intestinal connection means that these specimens also are of the genus *Microplana*. However, they are distinct from both *M. terrestris* and *M. kwiskea*. The penis of *M. terrestris* has a broad, spherical proximal ejaculatory duct with internal projections (“seminal vesicle” of many authors) and a narrow, distal, muscular penis papilla. The ejaculatory duct of *M. groga* lacks such a differentiated portion. The genito-intestinal canal of *M. terrestris* broadens to a sinus-like cavity before narrowing to its opening or openings to the intestine. That of FG1 and FG2 is narrow for its entire length. That of FG3 is narrow but forked. The penis of FG1, FG2 and FG3 are similar, so the significance of the forked genito-intestinal canal in FG3 is uncertain. Its openings into the intestine on either side are rather different from FG1 and FG2. The lack of a distinct layer of longitudinal muscle dorsal to the ventral nerve cord also distinguishes these specimens from *M. terrestris* and also from *M. kwiskea*.

The *cox1* results (Fig. 2) suggest these specimens are identical with *M. kwiskea*. However, Mateos *et al.* (1998) noted that their specimens had a white sole, but the sole of *M. kwiskea* is of the general body colour. The mouth and gonopore are much closer together in the Font Groga specimens (1–1.7 mm apart) than in *M. kwiskea* (4 mm apart). The internal surface of the ejaculatory duct appears smooth in the Font Groga specimens, unlike *M. kwiskea* in which there are internal folds or projections. The musculature of the penis of the Font Groga specimens (outer to inner circular, longitudinal, radial and circular fibres) is different from that of *M. kwiskea* (dense circular fibres with a longitudinal layer). In the Font Groga specimens the genito-intestinal canal is narrow for its entire length and in two of the three specimens the duct divides near its entry to the digestive diverticulum so that there are two or three openings into the diverticulum (Figs 24 & 25, and Fig. 12 of Mateos *et al.* 1998). FG3 has a forked, Y-shaped genito-intestinal canal (Fig. 26). These openings are posterior to the penis and antrum and somewhat dorsal. The genito-intestinal canal of *M. kwiskea* is narrow for its entire length and opens into the intestine on one side at the level of the penis via a distinct papillate opening. These differences lead us to conclude on morphological evidence that these specimens are of a different species to *M. kwiskea*. That is despite the apparently unambiguous identity of the *cox1* sequence from the specimen AF178318. Thus they are a previously undescribed species, *Microplana groga*.

How can the identical *cox1* sequences reported above (Fig. 2, GenBank AF178318, K2, K8 and K9) be explained? There are two possibilities. Firstly, since different individuals were sectioned and sequenced by Mateos *et al.* (1998) there is the possibility that the sequenced individual (AF178318) was in fact not of the same species as FG1, FG2 and FG3. Secondly, although less likely, it is possible that the small fragment of *cox1* characterized is identical between *M. kwiskea* and *M. groga*. Although widely popular as a ‘barcoding’ gene, partial *cox1* is not always capable of discriminating between species with absolute accuracy (e.g. Meyer and Paulay 2005). Also, the fragment of *cox1* characterized by Mateos *et al.* (1998) and herein, is approximately half the size of the fragment (~650 bp) suggested by Hajibabaei *et al.* (2007) for DNA barcoding. Whilst it remains possible that the fragment of *cox1* used in the current study fails to differentiate between what are clearly distinct species (*M. kwiskea* and *M. groga*), there is a need to sequence new specimens of *M. groga* as detailed in this study; namely to section part of the worms for species verification and sequence the remaining tissues. If the sequences remain identical, or insufficiently different to separate them in a phylogeny, the possibility that the fragment of *cox1* is incapable of differentiating these taxa remains and additional sequence data will be required for further verification.

General discussion

This work illustrates both the power of, and the danger inherent in, molecular taxonomy. Undoubtedly, molecular techniques in general and the mitochondrial *cox1* gene in particular may be highly discriminatory for identification at the species level (and maybe at other taxa), though there are exceptions. But such techniques must be coupled with reliable traditional taxonomy and particularly with accurate identification to species

level. We have shown that the specimens relating to GenBank sequence AF178318 were not correctly identified. However, to their credit, Mateos *et al.* (1998) did deposit further specimens in a recognized collection which has allowed their identity to be checked. It should be essential in molecular studies that further voucher specimens or part specimens of poorly known species, should be deposited in a referenced collection. This is particularly important in a group where there is limited taxonomic expertise, such as in the present case. The need for voucher specimens has been emphasized for other animal groups (e.g. Peterson *et al.* 2007). Meanwhile GenBank sequence AF178318 must be re-named.

We would strongly recommend that in similar cases to ours, several specimens are used and where possible parts of the same individual specimens are used for molecular studies and for morphological comparison. In the case of terrestrial flatworms, we suggest that the posterior half including the pharynx and copulatory apparatus is sectioned longitudinally, a small mid-portion sectioned transversely and the remaining anterior portion used for molecular analysis. We used portions of the same specimens for both morphological and genetic comparison and we have made detailed comparisons with type and other material. Thus we can be sure that our *cox1* sequences refer to accurately identified species.

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