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Source: Zoological Science, 17(4) : 505-516

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/0289-0003(2000)17[505:ATSOEJ]2.0.CO;2

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ZOOLOGICAL SCIENCE **17**: 505–516 (2000) © 2000 Zoological Society of Japan

A Taxonomic Study of *Enchytraeus japonensis* (Enchytraeidae, Oligochaeta): Morphological and Biochemical Comparisons with *E. bigeminus*

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ABSTRACT—*Enchytraeus japonensis*, a terrestrial enchytraeid, reproduces asexually by fragmentation and subsequent regeneration. Because of its extreme ability to regenerate, the species has recently been proposed as a new experimental material for regeneration study, and a method has been developed for inducing sexual reproduction in the laboratory. The inducibility of sexual reproduction allowed us to perform, for the first time, a taxonomical analysis of *E. japonensis* based on the morphology of the genital organs, and to compare it with the morphology of the highly similar *E. bigeminus*, another fragmenting enchytraeid. Comparisons between the two species were also made for general protein patterns and isozyme patterns of seven enzymes using PAGE-IEF. The morphological investigation revealed that the two species are discriminable by only one character; glandular bodies associated with the male copulatory organs that are present in *E. bigeminus* but absent in *E. japonensis*. In contrast to this morphological similarity, protein patterns were highly divergent between the two species; 29 (55%) out of 53 distinguishable bands of general proteins and 25 (86%) out of 29 isozymes of the enzymes examined (EST, MDH, PGM, GPI, LDH, ME, and HE) were species-specific. These results suggest that *Enchytraeus japonensis* and *E. bigeminus* are closely related but different species.

INTRODUCTION

Enchytraeids (potworms) are small, whitish oligochaetes that occur in almost all soil types (Didden *et al*., 1997). They can reach high values of abundance and biomass, and critically influence soil conditions. Biological studies on enchytraeids, however, are hampered by difficulties with their taxonomic identification (Schmelz, 1999; Schmelz *et al*., 1999); species-discriminating morphological differences are often minute, many important characters are intraspecifically variable, and observable only in live specimens. Moreover, most species can be identified with certainty only at sexual maturity, based on the morphology of the reproductive organs.

Worldwide, several hundred species of enchytraeids have thus far been described. Among these, eight have been reported to reproduce asexually by fragmentation and subsequent regeneration (Bell, 1959; Christensen, 1959; Nielsen and Christensen, 1963; Bouguenec and Giani, 1987; Nakamura, 1993; Dózsa-Farkas, 1995). *Enchytraeus japonensis* is the only fragmenting enchytraeid reported from Japan (Nakamura, 1993). The description of the species confined to somatic characters because sexual specimens were not found and the species was thought to reproduce only by fragmentation. Nonetheless, *E. japonensis* was described as a new species separate from *E. bigeminus* (Nielsen and Christensen, 1963), one of the eight fragmenting enchytraeids, by a single character; the absence of the fourth pair of pharyngeal (septal) glands. However, Nielsen and Christensen (1963) have reported that the fourth glands are occasionally absent even in *E. bigeminus*, and Bouguenec and Giani (1987) have reported that sexually mature specimens of *E. bigeminus* usually have three pairs of pharyngeal glands while asexual specimens typically have four. We consider, therefore, that the number of pharyngeal glands may be too variable to be used as a criterion for separating the two species.

Recently, taking notice of its high potential as a new material for regeneration study, Myohara *et al*. (1999) investigated regeneration and reproduction in *E. japonensis* and found that sexual reproduction could be induced by changing the culture conditions. This enabled us to scrutinize the taxonomy of *E. japonensis* in terms of a new morphological description that included the sexual organs. A laboratory population of *E. bigeminus* was also available for comparison.

In order to distinguish interspecific differences from intraspecific variability, greatly detailed morphological studies

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were carried out. Rota *et al*. (1998), Rota and Healy (1999), and Schmelz (1999) have shown that elaborate species descriptions are beneficial to the solution of many intricate problems in Enchytraeidae taxonomy. We extended the catalogue of taxonomically relevant characters, studied specimens reared under different conditions, and compared the anatomy of live and preserved specimens. The detailed account of the anatomy of *E. japonensis* will serve as an information base for future studies on the species.

The availability of a live culture of *E. bigeminus* allowed a further interspecific comparison of the protein patterns of the two species. In a series of studies, Brockmeyer (1991), Westheide and Brockmeyer (1992), and Schmelz (1996, 2000) have shown that protein analysis is a valuable additional tool in Enchytraeidae species-level taxonomy, and, in a previous report, a particular species was described and defined solely on the basis of protein data (Westheide and Graefe, 1992). We used isoelectric focusing in flat horizontal polyacrylamide gels (PAGIF) (Righetti *et al*., 1990) with subsequent specific staining of isozymes or unspecific silver staining of all water-soluble proteins (“general protein”) (Heukeshoven and Dernick, 1983).

The results of our investigation show that *E. japonensis* is morphologically almost identical with *E. bigeminus*, though their protein patterns are highly divergent. We suggest that *Enchytraeus japonensis* and *E. bigeminus* are closely related but different species.

MATERIALS AND METHODS

Materials

Specimens of *E. japonensis* were taken from mass cultures reared in Petri dishes in 0.6 % (w/v) agar medium or commercial garden soil. The culture conditions are described in Myohara *et al*. (1999). Cultures of *E. japonensis* are descended from one specimen of the population from which the species has been described (Nakamura, 1993). Eighty-nine asexual specimens (65 alive and 24 fixed and stained) and 31 sexually mature specimens (4 alive and 27 fixed and stained) of *E. japonensis* were examined. A culture of *E. bigeminus*, the origin of which is unknown, was reared under identical conditions. From this culture, 40 asexual (all alive) and 14 sexual specimens (4 alive and 10 fixed and stained) were examined. In addition, 15 orcein-stained and whole mounted specimens (10 mature and 5 submature) of *E. bigeminus* from the original culture, kindly provided by Dr. Bent Christensen of University of Copenhagen, were examined.

Morphological investigation

Living specimens and whole mounted preparations were examined by interference contrast light microscopy. Living specimens were observed in a drop of tap water between a slide and a coverslip that were gently pressed to reduce motility by removing some of the interstitial water with filter paper. For preparation of permanent whole mounts, specimens were fixed in hot Bouin’s fluid (70°C), stained with paracarmin, passed through an ethanol-xylol dehydration series, then mounted in Malinol between two coverslips to make the specimen examinable from both sides. In some cases, specimens were stained with orcein and mounted in 50% glycerol as described in Myohara *et al*. (1999). For purposes of histochemistry for alkaline phosphatase, specimens were fixed in 100% methanol, stained in NBT/X-phosphate color substrate, counterstained with orcein, and mounted (Myohara *et al*., 1999). Observations were documented photographically, or as drawings accomplished by the use of a drawing tube.

Protein investigation

Protein electrophoresis and subsequent staining followed the protocol established for enchytraeids in the laboratory of Osnabrück (Brockmeyer, 1991; Westheide and Brockmeyer, 1992, including references therein). Live specimens were removed from the cultures and kept in water to allow defecation before processing. Isoelectric focusing was performed with homogenates of the entire live specimen in 0.5% (w/v) DTT buffer solution (general protein staining) or distilled water (enzyme staining) in polyacrylamide gels (0.5×125×265 mm for enzymes, 0.5×165×265 mm for general protein) on a horizontal system (LKB Multiphor II). Separation distance was 9 cm for enzymes and 15 cm for general protein. Proteins were focused for 90 min. (enzymes) and approximately 230 min. (general protein), over a range of pH4–9. The silver staining of proteins was performed according to the method of Heukeshoven and Dernick (1983). Isozyme staining followed Brockmeyer’s method (1991) with modifications of current techniques (Murphy *et al*., 1996). Seven enzyme systems were examined, including EST, MDH, GPI, PGM, HE, LDH, and ME (nomenclature after Murphy *et al*., 1996). In the case of EST, α-naphthyl acetate was used as substrate.

RESULTS

Morphological description of *Enchytraeus japonensis* Nakamura, 1993

Somatic characters

Habitus: The worms are transparent to whitish with the gut contents shining through. Sexually mature individuals are recognized at low power magnification by the large white eggs and whitish-reflecting masses of developing sperm at 1–2 mm from the anterior end.

Behavior: Compared with other species of the genus, the worms of this species are very active and difficult to investigate alive microscopically due to rapid and persistent movements even under pressure between a slide and a coverslip. Movement in cultures is also comparatively rapid.

Body dimensions: Body length varies extremely, from 10–15 mm in full-grown specimens down to 0.4 mm in fragments of asexually reproducing cultures. The number of segments per fragment varies from 2 to 14 or more. Body diameter varies between 0.13 and 0.25 mm, depending on the culture conditions and body lengths. In the clitellar region of sexually mature specimens, the body diameter is about 0.35 mm. Fixation reduces body lengths and enlarges body diameters.

Segment numbers: A maximum of 60-80 segments is typical in full-grown asexuals, and a minimum of 2 segments in freshly fragmented specimens. Sexual animals usually have 40–45 segments, though often fewer, down to 16.

The *prostomium* (Figs. 1A, C, 2B, pr) is bluntly triangular, with a number of knob-like cuticular sense organs on the outer surface, and the dorsal pore is at 0/I (Fig. 3A, arrowhead), as is common in the genus.

Chaetae: There are typically two chaetae per bundle (Figs. 1B, C, lc, vc, 3B) from segment II on, diverging distad, though single bundles may contain none, one, three, four, or,

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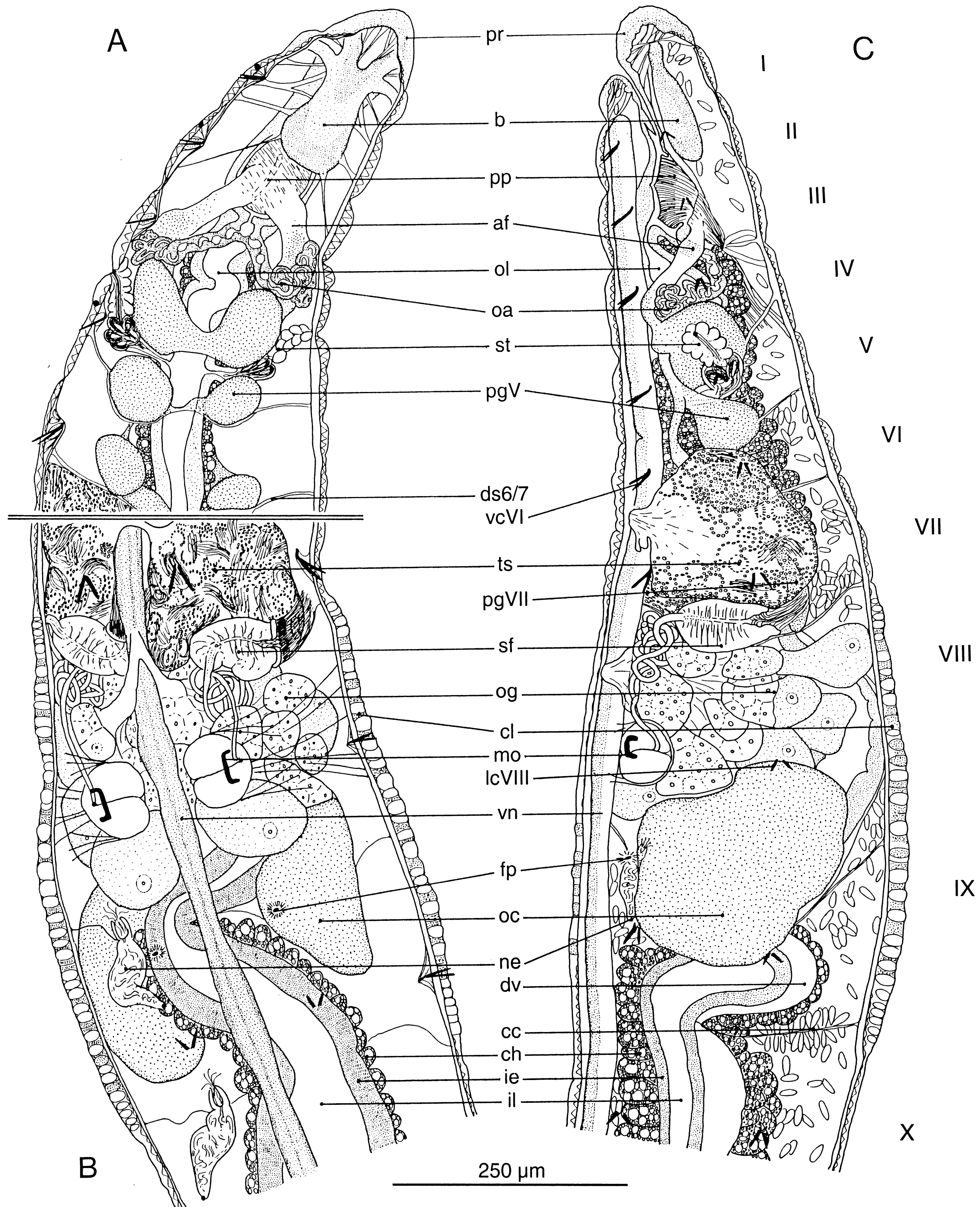

Fig. 1. Anterior body region of sexually mature specimens of *E. japonensis*. (**A**) Dorsal view. (**B**) Ventral view. (**C**) Lateral view. All from stained whole mounts, **A** and **B** from the same specimen. Anterior dorsal blood vessel and retractor muscles of pharyngeal pad are omitted in **A** and **C**; coelomocytes omitted in **A** and **B**. Roman numerals indicate segments, arabic numerals indicate segment boundaries. **af**, afferent fascicle of pharyngeal gland cell processes; **b**, brain; **cc**, coelomocytes; **ch**, chloragocytes; **cl**, clitellum; **ds**, dissepiment; **dv**, dorsal blood vessel; **fp**, female pore; **ie**, intestinal epithelium; **il**, intestinal lumen; **lcVIII**, lateral chaetae of segment VIII; **mo**, secondary male opening, bursal slit; **ne**, nephridium; **oa**, oesophageal appendage (peptonephridium); **oc**, mature oocyte; **og**, oogonia; **ol**, oesophageal lumen; **pgV**, pharyngeal (septal) gland of segment V; **pgVII**, pharyngeal (septal) gland of segment VII; **pp**, pharyngeal pad; **pr**, prostomium; **sf**, sperm funnel; **st**, spermatheca; **ts**, testis with sperm sac; **vcVI**, ventral chaetae of segment VI; **vn**, ventral nerve chord.

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rarely, six chaetae. Their shape is straight with a pointed distal tip and a conspicuous proximal bend. They are of equal size laterally and ventrally throughout the entire animal, 35–40 μm long and ca. 3 μm thick, except in the most anterior segments where they are 28–32 μm long. The arrangement of chaetae in bundles with chaetal numbers other than two is most often irregular; e.g. one chaeta is added laterally to a pair, or two pairs are juxtaposed to one another or lying one on top of the other, though regular fan-shaped bundles with three and four chaetae are also present. In sexually mature specimens, chaetae are absent ventrally in the segment of the male copulatory organ, as is typical in the genus.

Chaetal follicle cells: The chaetae are proximally enveloped by a number of cells of variable shape and size (Figs. 2B, 3B, arrowheads). They can be pale, inconspicuous, and completely embedded in the chaetal follicle, or voluminous and larger than the chaetae, projecting outside the chaetal follicle into the coelomic cavity, measuring up to one fourth of the body diameter. These cells are floppy with a yellowish tinge, and they follow the movements of the coelomic fluid. The differing cell size is apparently dependent on the environment; it can be induced by changing the medium. Specimens reared in agar with abundant food supply contain large cells. Cell size is reduced within a few days after the animals have been transferred to garden soil.

Epidermal gland cells (Fig. 2C, eg) are present as long, narrow, and finely granulated fields arranged transversely in two to four rows in anterior segments. They are absent dorsomedially and ventromedially; their numbers are reduced in posterior segments. They show most conspicuously in live specimens but are also visible in stained whole mounted specimens. Some animals lack epidermal gland cells.

The *brain* (Figs. 1A, C, b) occupies the dorsal cavity of segments I and II with a maximum length of 130 μm and a length:width ratio of about 3:2. Its posterior margin is rounded or slightly concave, and the lateral sides converge towards the anterior end, where four nerves branch off. The outer nerves are the circumoesophageal connectives, the inner two project anteriad and branch out to innervate the prostomium. The medial anterior margin of the brain is concave. Two neurosecretory areas located postero-dorso-laterally are visible in most but not all specimens examined alive.

The *oesophageal appendages* (peptonephridia) (Figs. 1A, C, 2B, oa, 4A) commence as a single dorsomedial tubular invagination of the oesophageal epithelium posteriorly and adjacent to the pharyngeal pad in segment III (Fig. 4A, arrowhead). It bifurcates after a short distance into two very long tubes that coil and taper increasingly towards the blind distal end. The distal coiling and folding is pronounced, the organs appear more as spongy masses than tubes (Fig. 4A, arrow). The appendages are moving freely in the coelom and they fill up a considerable part of the space between the pharyngeal pad and the first pair of pharyngeal glands in segment IV. The tubes are highly contractile and of differing shape due to their position and orientation in the body cavity, and due to the actual state of contraction. When contracted completely—this is usually the case in fixed specimens - they appear as a pair of long solid club-shaped bodies projecting from a common dorsal origin in a postero-latero-ventral direction, with a larger diameter distally than proximally (see also Nakamura, 1993, Fig. 1C). In the state of slight contraction, the proximal tube lumina appear as a series of interconnected subspherical chambers of varying size (Fig. 1A). Chamber sizes decrease distad, the unpaired proximal one being the largest. The distal half of the oesophageal organs then takes on the appearance of a spongy multilobed mass with small but isolated lumina. The complete canal is visible only when the live animal is relaxed or stretching forward (Fig. 4A). The inner lumen surface becomes slightly wavy and decreases distad in diameter, as do the tube walls. Apart from the proximal bifurcation, no branchings occur in the organ, although its intricate foldings (Fig. 1C) give the impression of branches or twigs.

The *pharyngeal* or *septal glands* (Figs. 1A, C, 2A, pg, 2E) are arranged in three or four pairs in segments IV–VI or IV–VII, respectively, in both asexual (Fig. 2E) and sexually mature specimens. The fourth (hindmost) pair in VII is often rudimentary (Fig. 1C, pgVII). The glandular bodies are not subdivided into dorsal and ventral lobes, though a ventral posterior projection is often present. The lobes may be connected dorsally by a small commissure, but they are often separate. The lobes of each side of the body are interconnected by a common ventrolateral afferent strand or fascicle (Fig. 1A, C, af) that consists of proliferations of the pharyngeal gland cells, which transport the secretory products to the surface of the eversible pharyngeal pad (Figs. 1A, C, pp). The fascicles are particularly conspicuous in segments IV and III, where they rise to enter the pharyngeal pad from a dorsal direction. Here they are often wider than the adjacent oesophageal appendages, and can easily be confused with them.

Chloragocytes (Figs. 1A–C, 2A, ch, 4B) are present as a dense and conspicuous layer from the beginning of segment IV, with considerable portions appearing between the pharyngeal glands. They are absent in segment VIII of sexually mature specimens. Their size and opacity depend on the nutritional state of the specimen. In specimens that have or had an abundant food supply, they fill up most of the coelom in segments posterior to IX, measuring 15–30 μm in height, and they obscure the body anatomy by their numerous refractile vesicles of different sizes. Under conditions of reduced food supply, cell sizes go down to 9 μm. The opacity disappears in dehydrated preparations.

Coelomocytes (Figs. 1C, 2A, cc, 3C, 4B) are always numerous, and in well-fed animals they fill up the entire coelomic cavity or what is left of it by the chloragocytes. The cells are flat with a central nucleus, 15–25 μm long, and their shape ranges from ovally elongated, up to twice as long as wide and slightly tapering at one end, to broadly oval and almost spherical. Shape and size are influenced by the availability of nutrition. Many cells are attached to the peritoneum in dense aggregations by their narrowed ends, especially at the postclitellar septa. Others are moving freely in the coelo-

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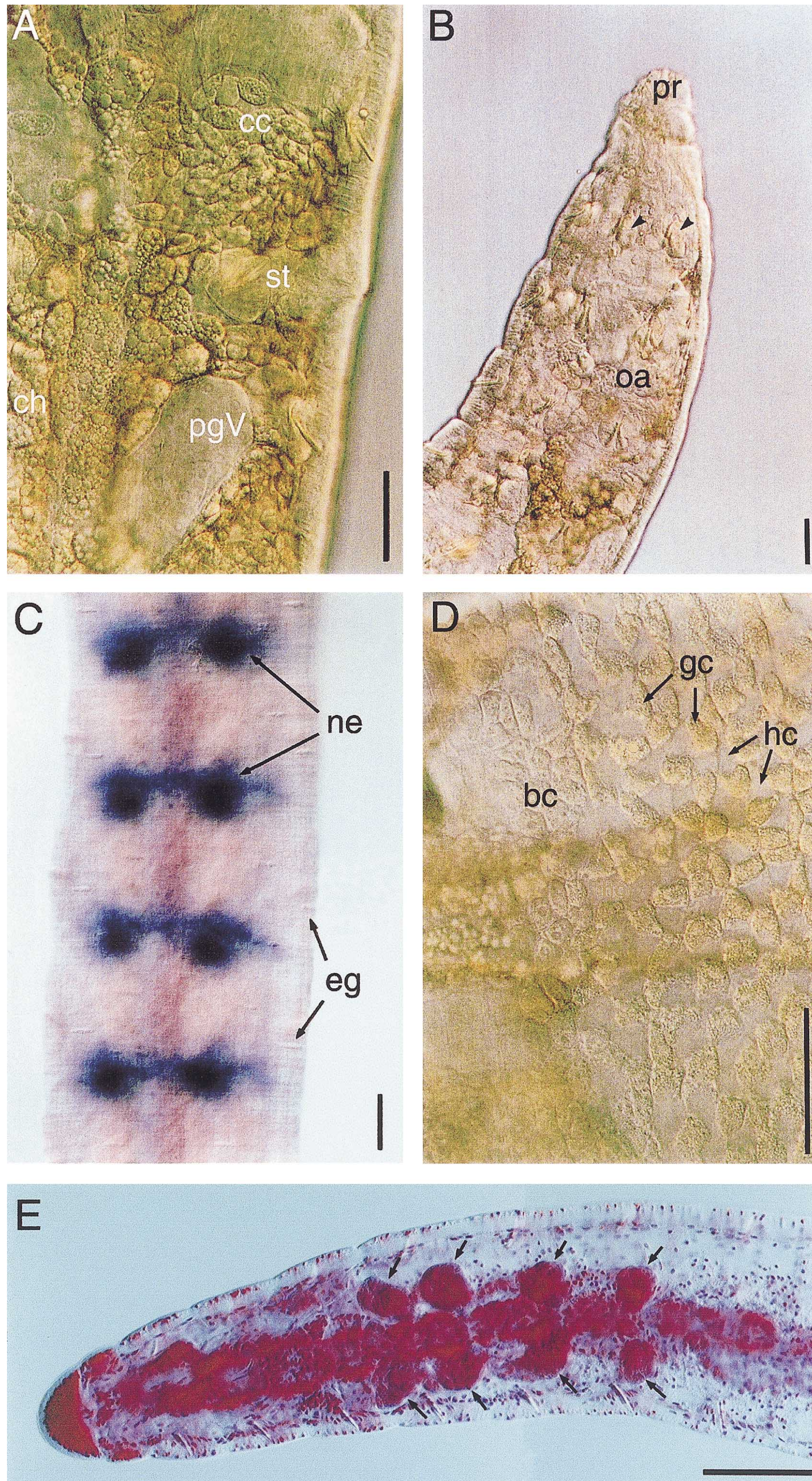

Fig. 2. Microscopical morphology of live (**A**, **B** and **D**) and fixed specimens (**C** and **E**) of *E. japonensis*. (**A**) Spermathecal region. (**B**) Anterior body region, ventral view, with large chaetal follicle cells (arrowheads, compare with Fig. 3B). (**C**) Epidermal gland cells and nephridia in the posterior body region of an asexual specimen stained with orcein and histochemistry for alkaline phosphatase (dark blue). (**D**) Different cell types forming the clitellum. (**E**) Anterior body region, ventral view, of an orcein-stained asexual specimen, showing four pairs of pharyngeal (septal) glands (arrows). **bc**, clitellar border cells; **cc**, coelomocytes; **ch**, chloragocytes; **eg**, epidermal gland cells; **gc**, clitellar granular cells (granulocytes); **hc**, clitellar hyaline cells (globulocytes); **ne**, nephridium; **oa**, oesophageal appendage (peptonephridium); **pgV**, pharyngeal (septal) gland in segment V; **pr**, prostomium; **st**, spermatheca. Bars = 50 µm in **A**–**D**, 100 µm in **E**.

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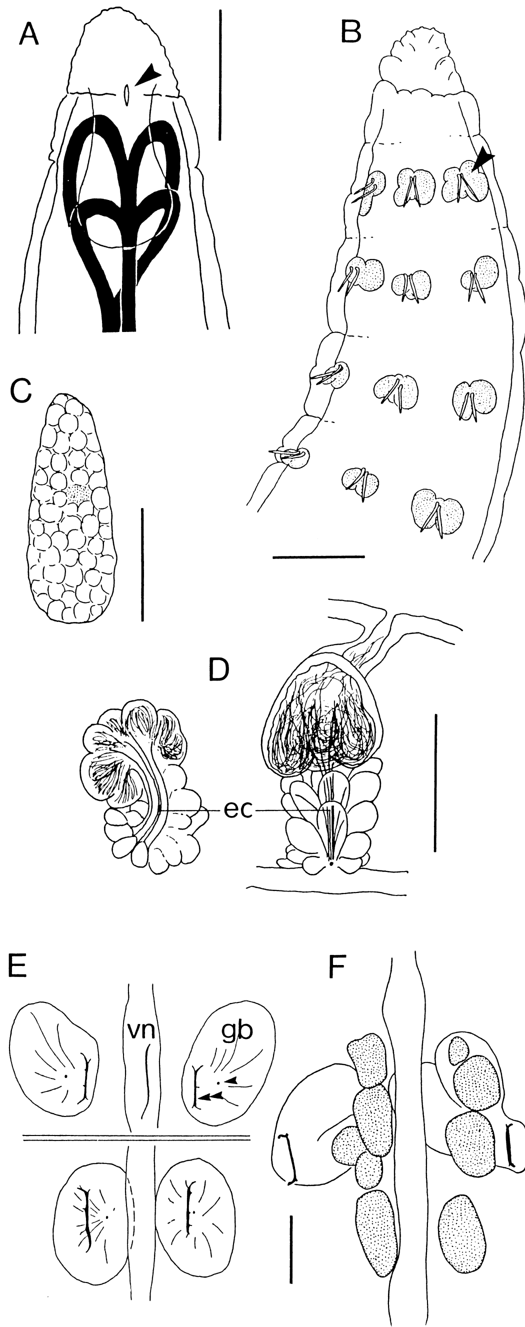

Fig. 3. Schematic drawings of some taxonomical criteria of *E. japonensis* (**A**–**E**) and *E. bigeminus* (**F**). (**A**) Branchings of blood vessels in head region, dorsal view, with dorsal pore (arrowhead) and outline of brain. (**B**) Anterior body region, ventral view, with large chaetal follicle cells (arrowhead, comp. Fig. 2B). (**C**) Coelomocyte. (**D**) Two aspects of the spermatheca. (**E**) Paired male copulatory organs with male pore (single arrowhead), bursal slit (double arrowhead) and the floppy glandular bulb, showing its considerable variability in shape; the bipartite nature of the bulb is not visible in ventral view; top and bottom drawings are from the same object. (**F**) Glandular bodies (dotted) adjacent to male copulatory organ of *E. bigeminus* alongside ventral nerve chord, ventral view. **A**, **B**, **C** and **E** from live observations; **D** from paracarmine stained whole mounts; **F** from orcein-stained specimen. **ec**, ectal duct of spermatheca; **gb**, glandular bulb of male copulatory organ; **vn**, ventral nerve chord. Bars=50 μm in **A**, **D**, **E** and **F**, 100 μm in **B**, 10 μm in **C**.

mic cavity. Most cells are entirely filled with about three dozen droplets arranged in one plane (Fig. 3C). The droplets are spherical, distinct, pale, not refractile, and more or less of equal size. They are best visible in live specimens but do not disappear in dehydrated whole-mounted specimens. The nucleus is best visible in fixed specimens. In some individuals, a larger number of cells is partly or entirely devoid of droplets and the respective cell regions are transparent and hyaline (Fig. 4B, arrowheads).

The anterior-most *nephridia* (Fig. 1B, C, ne) are attached to dissepiment 8/9 in sexual and asexual specimens. In the latter case, the first nephridia are located inside the clitellar region. A pair of nephridia is present in almost all of the following segments (Fig. 2C, ne). The anteseptale consists of the funnel only; the postseptale is a bulged body containing the much folded canal; the body merges gradually posteriorly into the efferent duct. A bladder-like terminal vesicle was not found.

The *dorsal blood vessel* (Fig. 1C, dv) rises in segment XI, XII, or XIII, rarely in X, and passes anteriad. Its origin is roughly dependent on the length of the specimen, and is more anteriorly located in shorter animals and more posteriorly located in longer ones. From the beginning and in the following segments, the dorsal blood vessel is always as densely covered with chloragocytes as is the gut itself, which makes the location of its origin somewhat obscure, particularly in live specimens. Beneath the brain, it divides into two pairs of branches of equal size and diameter (Fig. 3A). Each branch projects antero-laterad for a short distance before it turns posteroventrad. The branches on either side unite ventrally and anteriorly to the final unification into the unpaired ventral blood vessel in segment IV. No further commissural vessels are detectable.

The *digestive tract* can be clearly divided, by using histochemistry for alkaline phosphatase (ALP), into three distinct sections: the oesophagus, the anterior midgut, and the posterior midgut, which show low, high, and no ALP activity, respectively (Myohara *et al.*, 1999). In living worms or in orcein—or paracarmin—stained preparations, these regionalizations are inconspicuous. Here the oesophagus (Fig. 1A, C, ol) is distinguishable from the anterior midgut (Fig. 1A, C, il) only by its smaller diameter. At the transition, beginning two segments anterior to the origin of the dorsal blood vessel, the gut epithelium is thickened and contains small intercellular crevices. In the following two segments, the gut enlarges and reaches its full diameter in the first segment that does not include the dorsal blood vessel.

Reproductive organs

In most enchytraeid species, the clitellum is located in segments XII and XIII, and the testes and ovaries are in segments XI and XII, respectively (Nielsen and Christensen, 1959). However, in *E. japonensis*, as well as in *E. bigeminus*, all the reproductive organs except spermatheca shifted four segments anteriorly. The shift of the position of gonads is common in fragmenting oligochaetes (Christensen, 1994).

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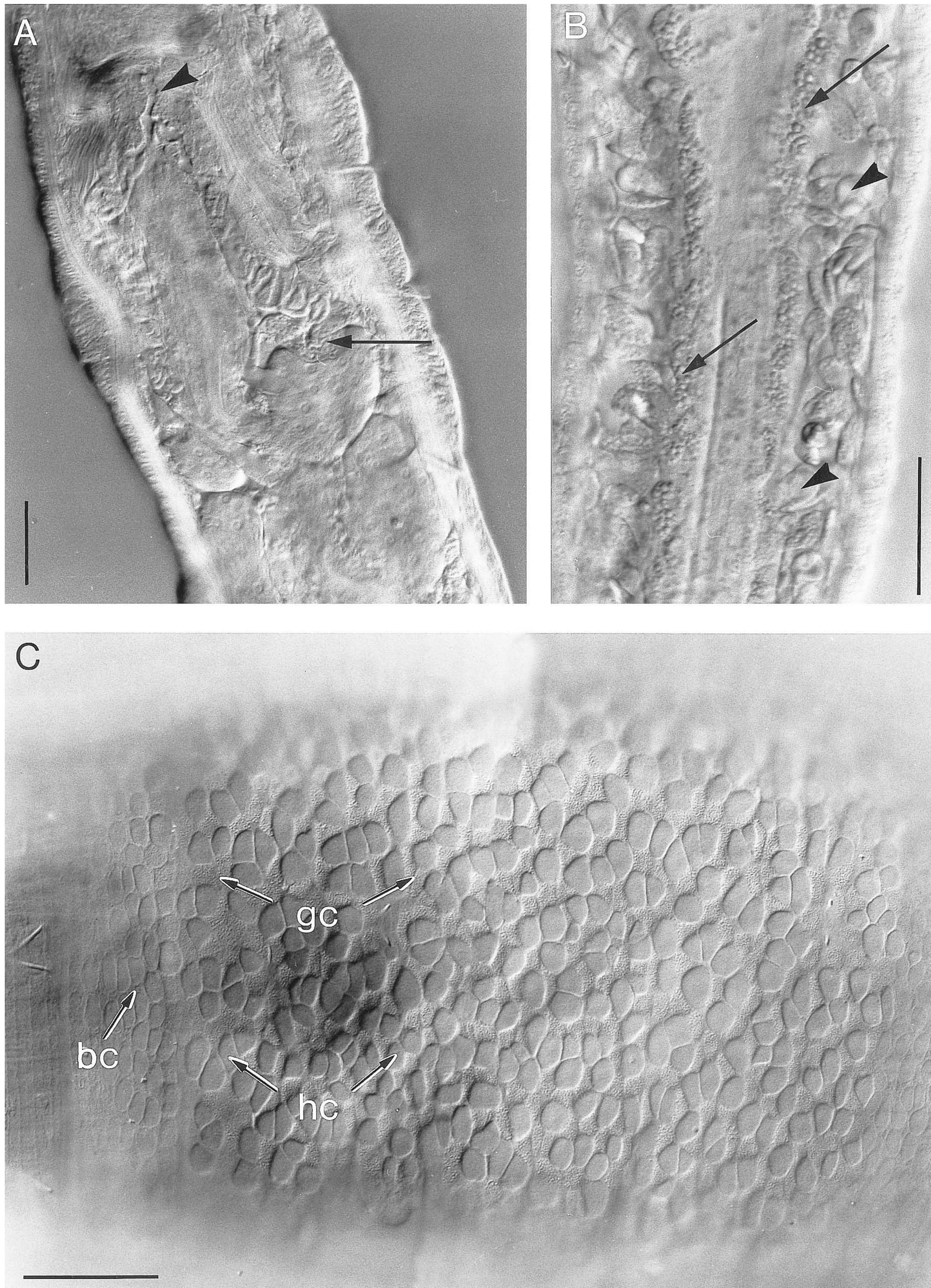

Fig. 4. Microscopical morphology of *E. japonensis*. (**A**) Oesophageal appendages in a living specimen. Note the joint unpaired origin (arrowhead) and the sponge-like distal mass (arrow). (**B**) Posterior body region of a live specimen showing chloragocytes (arrows) and coelomocytes (arrowheads). (**C**) Lateral view of clitellum (head region to the left) from a whole-mounted specimen (compare with Fig. 2D). **bc**, clitellar border cells; **gc**, clitellar granular cells (granulocytes); **hc**, clitellar hyaline cells (globulocytes). All bars = 50 μm.

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The *clitellum* (Figs. 1B, C, cl, 2D, 4C) is located in segments VIII and IX, beginning at the level of dissepiment 7/8 and extending slightly beyond dissepiment 9/10. It is saddle-shaped, i.e. undeveloped or only faintly developed ventrally. The gland cells are elevated up to 20 µm in sexually fully mature specimens (Fig. 1B, C). The cellular arrangement is reticulate rather than in transverse rows. Granular cells (Fig. 2D, 4C, gc) and hyaline cells (Fig. 2D, 4C, hc) are clearly distinguished; the latter are slightly larger (9–15 µm) than the former (6–10 µm), but distinctly larger in fixed specimens (Fig. 4C). There are no hyaline cells ventromedially, and granular cells are also lacking here except for some scattered cells that appear anterior and posterior to the male copulatory organs. There are three to four rows of a third type of clitellar gland cells (Figs. 2D, 4C, bc) at the anterior and posterior border of the clitellum, discernible by their very fine granulations. These cells are almost absent ventrally, though one row is usually continuous.

The paired *spermathecae* (Figs. 1A, C, 2A, st, 3D) are located in segment V. Each spermatheca consists of a glandular ectal duct, ampulla, and ental duct that is proximally attached to the lateral side of the oesophagus. There are no diverticula. The distal pore is in the position typical of the genus and of most species of the family, which is at the intersegmental groove between segments IV and V, slightly ventral to the lateral chaetae, on the lateral line devoid of longitudinal muscles. The pore is sometimes surrounded by a thickened epidermis and a circular collar of secretory terminations of ectal duct gland cells. The ectal duct (Fig. 3D, ec) consists of densely packed lobes of gland cells that surround the central canal. The lobes are apparently multicellular; more than one nucleus per lobe is discernible in stained wholemounts. The glandular lobes are arranged in rosettes when viewed in a cross-section or from the top toward the distal pore. Occasionally, the lobes may appear to form two sets of distinct rosettes lying one on top of the other. All lobes seem to release their secretory metabolites on or near the body surface. The central canal, i.e. the ectal lumen, is somewhat longitudinally striped, ridged, or furrowed in fixed specimens; its borders cannot be well determined. In live specimens, the canal appears as a solid hyaline rod. The canal opens proximally into the spherical or pear-shaped ampulla, which is as wide as or wider than the glandular ectal duct (45–50 µm in fixed specimens). Spermatozoa inside the ampullar lumen are arranged in loose loops. Some of them are attached with their heads to the epithelium of the ampullar base. The ampullar base is often equipped with a number of bulges, too slightly developed to be called diverticula (Fig. 3D, left). The interior margins or indentations that separate the ampullar bulges merge distally into the aforementioned ridges inside the ectal duct canal. The ental duct commences as a tapering of the ampulla. It is usually not longer than the ampulla but can be stretched considerably. By virtue of its attachment to the oesophagus, it connects the ampullar and oesophageal lumina.

The *testes* (Fig. 1B, C, ts) are paired, with a common origin below the ventral nerve chord behind the dissepiment of 6/7. They project laterodorsad on both sides and enlarge into voluminous masses of developing sperm. These masses occupy the majority of the coelom in segment VII, often projecting posteriad and anteriad into adjacent segments, displacing gut and pharyngeal glands. Testes and masses of developing sperm are enclosed by a common, presumably peritoneal, epithelium, together forming the testis sac or sperm sac.

The *sperm funnels* (Fig. 1B, C, sf) are inflated, and of variable shape and size due to compressions and deformations. They are more or less barrel-shaped, with a length:width ratio of about 3:2 (measured lengths 90–110 µm, measured diameters 50–100 µm). The collar is most often smaller (35–65 µm), thick-walled when without sperm (Fig. 1C), but flattened and wider with sperm on top (Fig. 1B). Numerous cilia (Fig. 1C) are present inside the funnel, projecting proximad outside the funnel.

Mature *spermatozoa* (Fig. 1B) are arranged in parallel as a dense brush on top of the sperm funnel. By virtue of their dense packaging, their approximate lengths are measurable by light-microscopy. The spermatozoa are ca. 43 µm long, and the nucleus measures 12 µm.

The ciliated *vasa deferentia* (Fig. 1B, C) are not prominent, are coiled ventrolaterally in VIII, and measure ca. 7 µm in diameter.

The paired *male copulatory organs* (Figs. 1B, C, mo, 3E) consist of a prominent glandular (penial) bulb and an inconspicuous eversible epidermal invagination, the bursa, into which the vas deferens opens proximally, between bulb and bursa. This opening is the male pore (Fig. 3E, single arrowhead). The glandular bulb (Fig. 3E, gb) is large and floppy; in live specimens it is moving about with the flow of the coelomic fluid. It is taller than it is long, longer than it is wide, and covered with a thin muscular sheath that also penetrates the bulb medially down to the eversible bursa, separating the gland into an anterior and a posterior part of identical size (Fig. 1B, C). The parts are closely attached to one another. Strands of secretory vesicles are visible inside the bulb in live specimens, projecting to and concentrating around the male pore. Additional glandular bodies, present in *E. bigeminus* (Fig. 3F) (Bouguenec and Giani, 1987) adjacent to the glandular bulbs, are absent in *E. japonensis*. This is the only morphological character that clearly distinguishes *E. japonensis* from *E. bigeminus*. The shape and size of the bursa itself is difficult to ascertain, but its elongated opening to the exterior, the bursal slit, is conspicuous (Fig. 3E, double arrowhead). In fixed specimens, the bursal slit is shaped like a square bracket, arranged longitudinal to the body axis, with the tips oriented laterad (Fig. 1B, C). The body surface between the exterior openings is always devoid of clitellar gland cells, but a somewhat thickened, rugose epidermal field with a narrow median longitudinal fold is present, visible when the live specimen is not pressed (Fig. 3E, top). In one specimen, one of the two male reproductive systems was found to be rudimentary on one side, with a sperm funnel measuring 30×30 µm, a collar width of 12

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µm, but with mature sperm on top, and with an almost absent glandular bulb.

The *ovaries* (Fig. 1B, C) are paired and have a common origin below the ventral nerve chord behind dissepiment 7/8. They project dorsolaterad and merge into a number of lateral lobes containing the oogonia (Fig. 1B, C, og). There are 2–6 mature eggs (Fig. 1B, C, oc) present at a time, which occupy the majority of the coelom in VIII and IX, often projecting further posteriad, displacing gut and septa.

There are no additional *copulatory* or *subneural glands*.

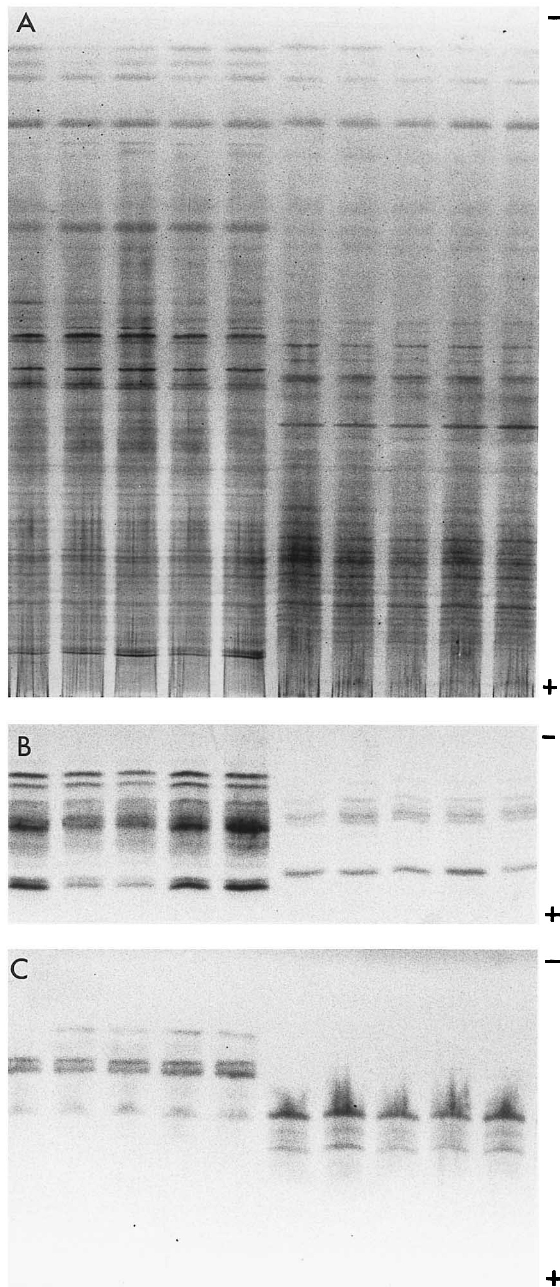

Fig. 5. Comparison of protein patterns (PAGIF on LKB Multiphor II) of *E. japonensis* and *E. bigeminus*, showing five specimens per species, one specimen per column. Left five columns, *E. japonensis*; right five columns, *E. bigeminus*. (**A**) General protein silver staining. (**B**) Esterases (EST). (**C**) Malate dehydrogenase (MDH).

Protein patterns of *E. japonensis* and *E. bigeminus*

All protein patterns of specimens belonging to the same species were identical, except that one cathodic MDH band of *E. japonensis* did not show in some specimens (Fig. 5C, leftmost). On the other hand, the protein patterns were distinctly different between *E. japonensis* and *E. bigeminus*.

General protein: Figure 5A shows the general protein patterns of five specimens each of *E. japonensis* (left) and *E. bigeminus* (right). Of the 53 discernible band positions (some anodic regions were not resolved), 29 (54.7%) were species-diagnostic, 19 for *E. japonensis* (i.e., present in *E. japonensis* and absent in *E. bigeminus*), and 10 for *E. bigeminus* (i.e., present in *E. bigeminus* and absent in *E. japonensis*); 24 band positions were common for both species.

Isozymes: Five of the seven examined enzyme systems showed interspecific differences. Only LDH and HE showed identical patterns in the two species, one band evident for each. The other enzymes exhibited multiple band patterns with high resolving power. A total of 27 bands was observed, of which 25 were species-specific, 13 for *E. japonensis* and 12 for *E. bigeminus*. The EST-patterns (Fig. 5B) were particularly complex, showing 13 detectable bands, all species specific, 7 for *E. japonensis* and 6 for *E. bigeminus*. The patterns of *E. japonensis* showed a wider range of pH than those of *E. bigeminus*. The MDH-patterns (Fig. 5C) were also entirely different between the two species, with 4 bands each. (The weak anodic band in *E. japonensis* and the strong cathodic band in *E. bigeminus* are not in the same position.) Other complex patterns were shown by PGM (3 bands, 2 diagnostic) and GPI (3 bands, 2 diagnostic). The ME pattern revealed no clear bands but showed a smear of staining over a wide pH-range that was in a more cathodic position in *E. japonensis.*

DISCUSSION

Morphological comparison

Enchytraeus japonensis was described as a new species distinct from *E. bigeminus* by one single character, the absence of a fourth pair of pharyngeal glands (Nakamura, 1993). However, in our cultures of *E. japonensis*, descendants of Nakamura's initial culture, specimens with four pairs of pharyngeal glands were more frequent than those with three pairs of these glands, regardless of the state of sexual maturity. This character is apparently as variable in *E. japonensis* as in *E. bigeminus* (Nielsen and Christensen, 1963; Bouguenec and Giani, 1987), and therefore unsuitable to use as a criterion for discriminating the two species.

Another difference might be inferred from Figure 1C of the original description (Nakamura, 1993), in which the oesophageal appendages appear to be widely separated in *E. japonensis*, though our investigation showed that they are proximally jointed as in *E. bigeminus* (Nielsen and Christensen, 1963; Christensen, 1964). Our comparison of live specimens

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of both species ascertained that additional non-sexual characters unknown for *E. bigeminus*, such as the epidermal gland cells and rapid behavior of live specimens, were identical in these two species. The examination was complicated by the extreme variability of many characters within each species. Characters first thought to be species-specific were found to be variable when live specimens from cultures reared under different conditions were compared. For example, partly hyaline coelomocytes were found to occur in specimens of both species. The size of the chaetal follicle cells, presented as a species-specific trait in the original description of *E. bigeminus*, is influenced by the culturing conditions. And the location of the posterior origin of the dorsal blood vessel is dependent on the size of the animal, which in both species varies to extremes not found in other species of the genus. Thus, judging from our investigation, asexual animals of *E. japonensis* and *E. bigeminus* are morphologically indistinguishable from each another.

The only reliable morphological difference lies in the sexual organs. Our information on the sexual characters of *E. bigeminus* is derived from three sources: (1) the description in Bouguenec and Giani (1987), (2) the examination of sexually mature specimens, living and preserved, of our *E. bigeminus* culture, and (3) the study of orcein-stained wholemounts of mature specimens from the original culture. In all three cases, the male copulatory organ is accompanied by a number of associated bodies (Fig. 3F). These are attached to the body wall, mid-ventrally of the male copulatory organs, bordering the ventral nerve chord on both sides. They are apparently anatomically independent of the glandular bulb, as they do not follow its movements. In the orcein-stained specimens, the size, number, and distribution of the bodies were not as regular as was reported by Bouguenec and Giani (1987). Only slightly thicker than the body wall itself, these bodies of unknown function are reminiscent of the "accessory glands" of *E. albidus* (Bell, 1958, Fig. 8) or the outer lobes of the male copulatory organ in *Fridericia isseli* Rota 1994 (Rota, 1995). The absence of these bodies in *E. japonensis* is the only morphological character that discriminates the two species.

Other minor differences in the redescription of *E. bigeminus* (Bouguenec and Giani, 1987) are not present in Christensen's material or in our specimens from the *E. bigeminus* culture; they are apparently intraspecifically variable. These include the following: (1) variations in the location of the male pore (segment VIII or IX); (2) ten rows of epidermal gland cells (3); ventral chaetae present adjacent to the male pore.

Our observations suggest that *E. japonensis* and *E. bigeminus* are closely related, possibly sibling species. Interestingly, many of the characters that these species have in common are not found in other non-fragmenting species of the genus; namely, the frequent occurrence of four pairs of pharyngeal glands, the peculiar pattern of the anterior branching of the dorsal blood vessel, and the bipartite male glandular bulb. This last character is also present in two other fragmenting *Enchytraeus* species, *E. dudichi* Dózsa-Farkas, 1995, described from Iran, and an undescribed species from Amazonian forest soils (Schmelz, unpublished observation). Further morphological and genetical studies will elucidate the possible monophyly of this group within the genus.

Protein patterns

In contrast to the morphological data, inter-specific differences in the protein patterns between these two species are marked and definitive. Twenty-nine (55%) out of 53 distinguishable bands of general proteins and 25 (86%) out of 29 enzyme bands of the seven enzymes examined (EST, MDH, PGM, GPI, LDH, ME, and HE) are species-specific, suggesting that E. japonensis is a species separate from *E. bigeminus*.

A strict numerical interpretation of the data with a calculation of genetic identity, however, is difficult because the number of involved loci cannot be established for the multiple bands in EST, MDH, GPI, and PGM where either a larger number of loci or a larger number of alleles per locus may account for the high variability. *Enchytraeus bigeminus* is a polyploid species and an octoploid biotype with n=144 and a decaploid biotype with n=180 has been identified (Christensen, 1980). The comparable complexity of isozyme band patterns in these species suggests that *E. japonensis* is also polyploid. Moreover, the investigated cultures must be regarded as clones because our culture of *E. japonensis* was derived from one single animal, and the culture of *E. bigeminus*, maintained for more than 10 years, has certainly undergone severe "bottlenecks" that would have eliminated genetic variability. However, a genetic divergence by random fixation of alleles of a genetically highly heterogeneous amphimictic parent population, which was assumed by Christensen (1989) for parthenogenetic strains of *Fridericia striata* (Levinsen, 1884), should be excluded because both species do reproduce sexually, and the easy inducibility of sexual reproduction under laboratory conditions implies that this is not a rare phenomenon under natural conditions. Occasional amphimixis would then reintegrate the genetically divergent strains. Moreover, a recent divergence from an ancestral species should produce more genetic overlap than was observed in our species. We think that reproductive isolation due to geographical separation or due to chromosome incompatibilities are better explanations for the differences in the protein patterns of these species. To confirm this, crossbreeding experiments and karyological studies are planned.

Comments on *E. fragmentosus* Bell, 1959

The results of the present study entail a change in the taxonomic status of a third *Enchytraeus* species, *E. fragmentosus* Bell 1959, which was the first species in the genus described to reproduce by fragmentation (Bell, 1959), and is also highly similar to *E. japonensis* and *E. bigeminus* in morphological terms. Two characters are said to distinguish *E. bigeminus* from that species (Nielsen and Christensen, 1963); the presence of chaetal follicle cells and the absence of branches in the oesophageal appendages additional to the

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proximal bifurcation. The former, however, was shown to be variable in the present investigation. Further, it is not mentioned in the description of *E. fragmentosus*. With regard to the latter, the additional branches that Bell (1959) claimed to have observed in the oesophageal appendages of *E. fragmentosus* are most probably identical with the intricate foldings of the unbranched tubes that we observed in *E. japonensis* and in *E. bigeminus*. Tube foldings are easily taken for ramifications when the organ is contracted. Other reports on the morphology of *E. fragmentosus*, including the sexual organs (Vena and Philpott, 1968; Hess and Vena, 1974) and the ultrastructure of epicuticular particles (Hess and Menzel, 1967), coelomocytes (Hess, 1970), and clitellar gland cells (Hess and Vena 1974) present characteristics either identical with those of *E. bigeminus* and *E. japonensis* or not yet investigated in these species.

Given that no distinguishing character can be found in *E. fragmentosus*, it is inconclusive whether E. fragmentosus is identical with *E. bigeminus* or *E. japonensis*, or whether it is a third species distinct from both. Unfortunately, living cultures of *E. fragmentosus* that would allow a taxonomic analysis including a genetic characterization are no longer maintained (L. H. Harris, Natural History Museum of Los Angeles County, personal communication), and resampling from field sites is impossible because the geographical origin of *E. fragmentosus* is unknown (Bell, 1959). Brockmeyer (1991) compared the general protein and isozyme patterns of *E. bigeminus* (original culture) and *E. fragmentosus*, but the identification of the latter species is doubtful as it was based on the absence of chaetal follicle cells (Dózsa-Farkas, personal communication). Therefore, at the present and to our knowledge, *E. fragmentosus* must be declared as a *species inquirenda*.

ACKNOWLEDGEMENTS

We thank Dr. Y. Nakamura of the Tohoku National Agricultural Experimental Station for providing *E. japonensis*. We are grateful to Prof. B. Christensen of the University of Copenhagen for providing orcein-stained specimens of sexually mature *E. bigeminus* from the original culture, and for his helpful comments on the manuscript. Prof. W. Westheide of the University of Osnabrück kindly supported our investigation. This work was supported in part by a Grant-in Aid (Bio Design Program) from the Ministry of Agriculture, Forestry and Fisheries, Japan. R.M.S. was a recipient of a Research Award for Foreign Specialists in 1997 administrated by the Science and Technology Agency of Japan.

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(Received August 26, 1999 / Accepted December 1, 1999)

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