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Authors: Kuznedelov, Konstantin D., Ishida, Sachiko, and Nishitani, Shin-ichiro

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Genetic Divergence of Japanese Turbellarians, Studied by Comparisons of Partial 18S rRNA Gene Sequences I. On Representatives of Dendrocoelidae (Platyhelminthes: Tricladida: Paludicola)

Konstantin D. Kuznedelov[1], Sachiko Ishida[2]* and Shin-ichiro Nishitani[3]

[1]*Limnological Institute SD RAS, Irkutsk 664033, Russia*
[2]*Department of Biofunctional Science, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan*
[3]*Sakurazuka High School, Osaka 560-0881, Japan*

ABSTRACT—This study obtained 18S rDNA sequence data to investigate the extent of genetic divergence in the Japanese dendrocoelid group, and applied the data to the estimation of phylogenetic relationships and taxonomy. The sequence data, consisting of 558 base positions in an aligned data set, were obtained by using the polymerase chain reaction and direct sequencing. For comparative analysis, the 18S rRNA gene sequence data on dendrocoelids from different distant geographical places (Europe, Lake Baikal in Siberia, Kamchatka) published previously were used. Such analysis showed that: (1) *Bdellocephala* species from Japan, Kamchatka and Lake Baikal are closely related ; (2) Japanese species classified within the genus *Dendrocoelopsis* do not form a single cluster of closely related organisms; (3) *Dendrocoelopsis ichikawai* differs minimally from *Bdellocephala baicalensis* (from Lake Baikal), reliably groups with all *Bdellocephala*, and should therefore be classed with this genus.

Taxonomic conclusions. (1) The genus *Dendrocoelopsis* should be revised after molecular typification of European and American representatives. (2) The current generic status of *D. ichikawai* based on the presence of a well-developed penis papilla should be reconsidered. (3) Sequence divergence amongst representatives of *B. brunnea* indicates that representatives should be closely examined for the presence of morphological characters by which they might be distinguished.

INTRODUCTION

Within the territories of Japan, the family Dendrocoelidae Hallez, 1893, comprises seven valid, all monotypic, species - three of *Bdellocephala* De Man, 1875 and four of *Dendrocoelopsis* Kenk, 1930 : *B. annandalei* Ijima and Kaburaki, 1916; *B. brunnea* Ijima and Kaburaki, 1916; *B. borealis* Kawakatsu, 1978; *D. lactea* Ichikawa and Okugawa, 1958; *D. ezensis* Ichikawa and Okugawa, 1958; *D. ichikawai* Kawakatsu, 1977; *D. kishidai* Kawakatsu, 1978.

In terms of geographical distribution, Japanese dendrocoelids may be roughly classified under two types, widespread or confined to a unique locality. In the former we place three species *D. lactea*, *D. ezensis* and *B. brunnea* ("comparatively wide" and "wide" spread species according to Kawakatsu *et al.*, 1967). The others may be regarded as phylopatric species, exhibiting a tendency to remain in the native locality and showing little capacity to spread or disperse (for more detailed distribution data on the Japanese dendrocoelid species, see Kawakatsu, 1965,1967,1969,1974).

The real number of phylopatric species is likely to be considerably higher. Zoological collections often contain unique immature specimens that have not been recognised or named (not only on the species but the generic level) because they lack sexual characters. We know two published cases that record such specimens: *Dendrocoelopsis*? sp. from Lake Biwa-ko (Kawakatsu, 1966) and a dendrocoelid specimen from Middle Honshu (Nakano *et al.*, 1976).

The discovery of *D. kishidai* provides a dramatic example of a missing, unknown species of rare occurrence. This species was discovered by Dr. Kawakatsu after close re-examination of dendrocoelid material accumulated over 25 years in his laboratory. It had initially been assigned to *B. brunnea* species due to the similarity of its body shape (Kawakatsu *et al.*, 1978).

Other unidentified and undescribed dendrocoelid forms, the taxonomical positions of which have not been clarified, should also be considered as potentially new species, for example: *Bdellocephala* sp. from Lake Biwa-ko (Teshirogi *et*

* Corresponding author: Tel. +81-172-39-3587;
FAX. +81-172-39-3587.
E-mail sachikoi@cc.hirosaki-u.ac.jp

al., 1982; Yagihashi *et al.*, 1995), and two dendrocoelid forms from Shingenji Temple and Sakanoshita in Wakkanai City characterized by unique karyotypes (Nishitani, 1998). On the other hand, *Bdellocephala* sp. from Lake Biwa-ko is also considered as a juvenile form of *B. annandalei* (Oki *et al.*, 1998).

It is therefore apparent that there are some ambiguities in the taxonomic assignment of dendrocoelid specimens, not only to species positions but to generic ones as well. These ambiguities cause unique taxonomic and/or zoogeographical data to be lost or unusable for a long time.

Application of karyology to the identification of unusual specimens does not always result in unambiguous taxonomic assessment (see, for example, Nishitani, 1998).

The present paper examines genetic divergence between Japanese dendrocoelids, based on 18S rDNA sequence data. It evaluates the extent of this genetic divergence in the context of species diversity, phylogenetic relationships, and taxonomy.

MATERIALS AND METHODS

Samples and DNA isolation

Dendrocoelids used in this study are listed in Table 1. Total DNA from 21 living specimens was extracted according to de Vos and Dick (1988).

Amplification of target rDNA

Two primers (Kuznedelov *et al.*, 1996), namely 5'-TACCT GGTTGATCCTGCCAGTA-3' and 5'-ATTACCGCGGCTGCTGGC ACC-3' homologous to nucleotide positions 1 to 22 and 630 to 610, respectively, of the human 18S rRNA, were used for amplification of target rDNA fragments by Polymerase Chain Reaction (PCR). The PCR was performed in a 20-microL mixture containing PCR-buffer, 0.2 mmol/L each dNTP, 1 U Taq DNA polymerase (all from TaKaRa Shuzo Co.,Ltd.), 10 picomoles each primer, and 1–50 ng total DNA. The PCR was carried out with 31 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 70 sec, and extention at 74°C for 2.5 min in Gene Amp PCR System 9600 (PERKIN ELMER).

Sequence determination

PCR-amplified DNA fragments, first purified with Wizard PCR Preps DNA Purification System (Promega), were subjected to sequencing by the dideoxynucleotide-terminated Sanger's method (Sanger *et al.*, 1977) according to Murray (1989). Sequencing reactions were performed with RPN 2440 (or 2444) Thermo Sequenase kits (Vistra systems) according to the protocols "Dye-primer 2-step cycle sequencing" and "Direct loading". DNA fragments were sequenced in both directions with the PCR-primers labelled by Texas Red (Nippon Flour Mills Co.,Ltd.). The products of the sequencing reactions were analysed using a Hitachi SQ-5500 sequencer machine according to the manufacturer's instructions.

Sequence data analysis

Sequences were aligned by eye and identified with published planarian sequences (see below).

Secondary structures were reconstructed according to the models for eukaryotic 16S-like rRNA published by Gutell *et al.* (1985) and used to infer positional homology for ambiguous nucleotide characters in the highly variable regions, especially those containing deletions or insertions.

For comparative analysis, previously published 18S rRNA gene sequence data on the following planarians from Japan, Kamchatka, Lake Baikal (the last two in Russia), and Europe were used: *Bdellocephala brunnea* (from Fukushima Pref., Japan), *B. annandalei* (Lake Biwa-ko, Japan), *B. a. angarensis* (Gerstfeldt, 1958), *B. melanocinerea* (Korotnev, 1912), *B. bathyalis* Timoshkin and Porfirjeva, 1989, *Armilla livanovi* Kenk, 1974, *Protocotylus flavus* Korotnev, 1908 —all from Lake Baikal (Kuznedelov *et al.*, 1996); *B. parva* Zabusova, 1936 from Kamchatka (Kuznedelov *et al.*, 1997), *Dendrocoelum lacteum* (Müller, 1774) from Europe (accession number M58346), *B. baicalensis* (Zabusov, 1903) (Y18763), *Bdellocephala* sp. (Y18764)— the two last from Lake Baikal.

Comparative analysis was carried out with the PHYLIP package (Felsenstein, 1993). Evolutionary distances were calculated by the Kimura "2-parameter" model of nucleotide substitution (1980). An unrooted tree was constructed by the algorithm of Saitou and Nei (1987) from a pairwise distance matrix. A confidence interval on a set of species was measured by the bootstrap resampling method (Felsenstein, 1985) using 1000 bootstrap replications.

In addition to the distance-matrix method, we used maximum -parsimony (MP) and maximum-likelihood (ML) methods, simulated by the computer programs DNAPARS in the PHYLIP package (Felsenstein, 1993) and PUZZLE (Strimmer and von Haeseler, 1997), respectively.

RESULTS

Individuals of five species (Table 1) from the family Dendrocoelidae were used to study genetic divergence in nucleotide sequences in the 5'-end domain of the 18S rRNA. Although sequence data from *B. brunnea* were already available (Kuznedelov *et al.*, 1996), they were based on a single specimen (from Fukushima Pref.). Furthermore, the sequence of *B. brunnea* specimens that we collected from Hirosaki City (Aomori Pref.) was found to differ from that of the specimen from Fukushima Prefecture. To clarify this confusion, we used *B. brunnea* specimens from four localities (Table 1).

Table 1. Dendrocoelid species sampled for this study

Species	Number of specimens	Locality
Bdellocephala		
B. brunnea	3	Hirosaki, Aomori Pref.
	2	Tashirotai Heights, Aomori Pref.
	3	Fukushima Pref.
	3	Matsumoto, Nagano Pref.
B. borealis	3	Hime-numa, Rishiri Is., Hokkaido
Dendrocoelopsis		
D. ichikawai	3	Hime-numa, Rishiri Is., Hokkaido
D. lactea	3	Kuroishi, Aomori Pref.
D. ezensis	1	Asahikawa, Hokkaido

Sequence data

Readable DNA direct sequencing data are shown in Figure 1. These sequences were identified with published planarian 18S rRNA gene sequences. There were variations in the sequence length caused by insertions or deletions within the aligned regions.

Among the five dendrocoelid species, including representatives of four populations of *B. brunnea*, there are five variability types: (I) *D. ezensis*; (II) *D. lactea*; (III) *D. ichikawai*; (IV) *B. brunnea* (Fukushima Pref.); (V) *B. brunnea* (from the

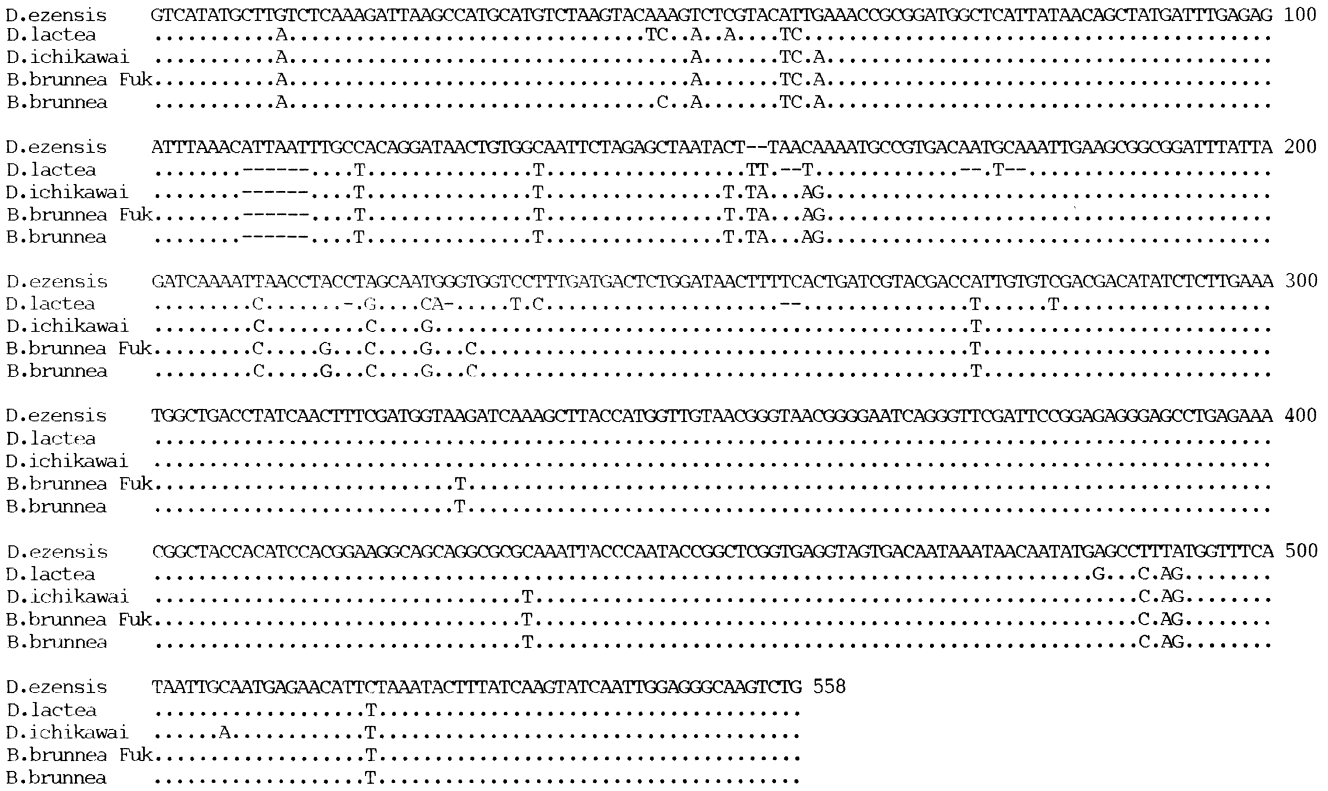

Fig. 1. Alignment of 5 sequences of the 5'-end portion of the 18S rRNA gene from dendrocoelid species listed in Table1. Dots in each base position indicate nucleotides identical to the top one belonging to *D. ezensis.* Dashs mean deletions.

other localities, see Table 1), and *B. borealis*. The taxa belonging to the same variability type have identical sequences. *B. annandalei* from Lake Biwa-ko belongs to type V, having an identical sequence (Kuznedelov *et al*., 1996). The new sequence data on *B. brunnea* specimens from Fukushima Pref. are identical with those obtained earlier from a single specimen (Kuznedelov *et al*., 1996).

Here, it is worth noting that the previously published 18S rRNA sequence data on *D. lactea* (Katayama *et al*., 1996) differ from ours by five base positions, the character states in three of which are in explicit contradiction with the 18S rRNA secondary structure.

Comparative analysis

To more fully establish the relationships between Japanese dendrocoelids, our data were compared with previously published 18S rRNA sequence data on dendrocoelids from different distant geographical places (Europe, Lake Baikal, Kamchatka).

As it was found earlier, the sequence data on *B. parva* from Kamchatka (Russia) are identical to those on *B. annandalei* (Kuznedelov *et al*., 1997). *B. parva*, therefore, also shares the 5th variability type. Thus, in the dendrogram depicting degrees of relationships between dendrocoelids (Fig. 2), the terminal node assigned to *B. brunnea* corresponds also to *B. borealis*, *B. annandelei*, and *B. parva*. The other tip corresponding to Japanese *Bdellocephala* is that assigned to *B. brunnea* Fuk.(a single population from Fukushima Prefecture). Both the OTUs (operational taxonomic units) occurred inside a monophyletic group, consisting of all the *Bdellocephala* species, with high statistical support (100%).

On the other hand, representatives of the genus *Dendrocoelopsis* are scattered across the tree and do not form a single monophyletic cluster. *D. ichikawai* is reliably (100%) placed apart from the other species of *Dendrocoelopsis*, clustering with the *Bdellocephala* group, showing close affinity with Baikalian *B. baicalensis.* For this reason the species can be classed with the genus *Bdellocephala*. As may be seen from the dendrogram, *D. lactea* and *D.ezensis* occur as separated lineages not having a common direct ancestral node. Their ancestral nodes are separated from each other by the 56% bootstrapped internal branch.

The Bootstrap approach used here allowed us to estimate the probability that some species groups of interest represented monophyletic ones, although they were not included in the (resulting) consensus tree. On the basis that it occurs in only 0.1% (less than 5%, Felsenstein, 1985) of the bootstrap estimates, we can hereby reject the monophyly of the Dendrocoelopsis group, consisting of three species (*D. lactea, D. ezensis, D. ichikawai*). Monophyly of the group *D. lactea and D. ezensis* is supported at the 12% level (more than 5%) and thus should not be rejected as an alternative to the corresponding subsets included in the consensus tree.

The MP and ML consensus trees had the same configuration as the distance-matrix one, although the bootstrap estimated differed (see Fig. 2).

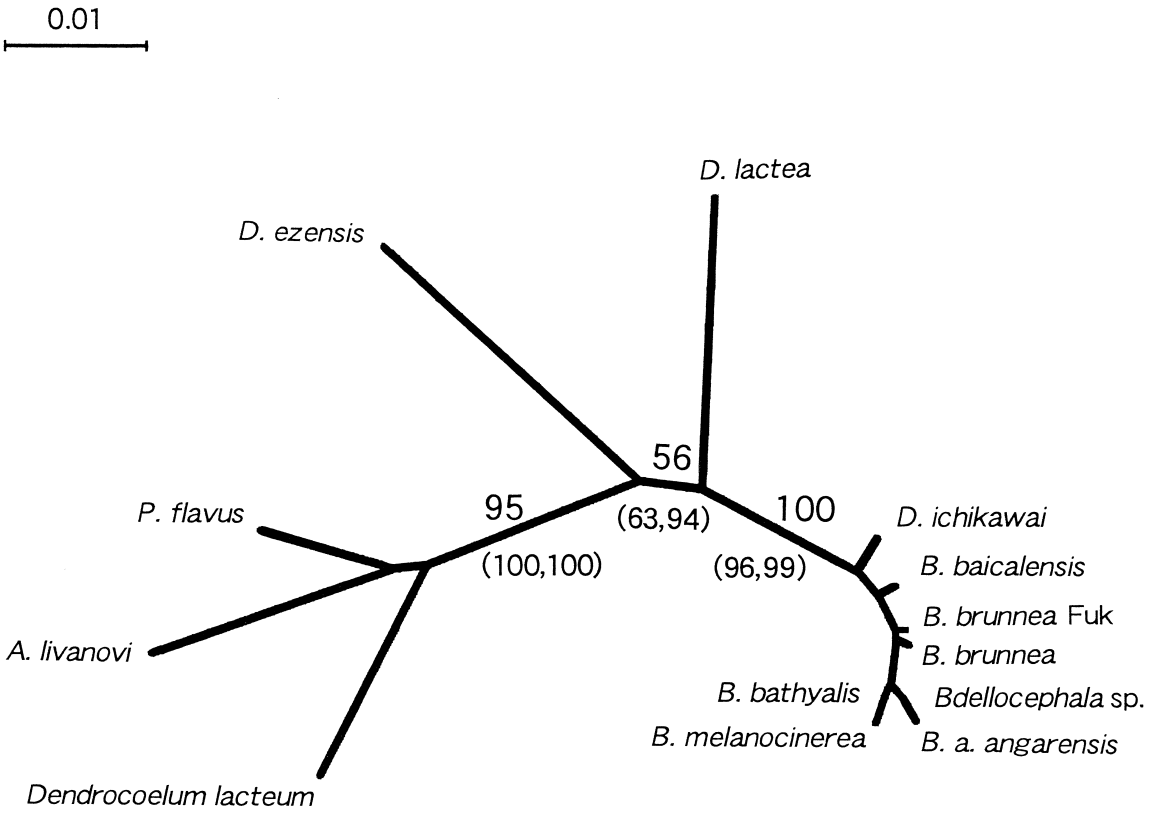

Fig. 2. Unrooted consensus tree with numbers indicating bootstrapping estimates (%). Numbers in parentheses indicate the MP and ML bootstrapping estimates. The scale means that line length equalizes to 0.01 evolutionary distance calculated by Kimura's formula (1980). The branch tree length is corresponded to the evolutionary distances between species by this scale.

DISCUSSION

We obtained sequence data from the 18S rDNA locus of a number of representatives of the Japanese dendrocoelid group, and evaluated the data obtained in relation to some questions of species diversity, phylogenetic relationships, and taxonomy.

Although there are a number of limitations to the application of this DNA fragment for these objectives, we can, nevertheless, draw some conclusions concerning the aspects under consideration.

Species diversity

The use of 18S rRNA sequence data (especially partial) to discriminate species, especially closely related ones, is limited by the evolutionary conservation of this sequence constrained by the ribosome structural and functional requirements. Indeed, all described Japanese *Bdellocephala* species (except *B. brunnea* from Fukushima Pref.), despite clear morphological and ecological differences between them, shared the identical target sequence, i.e. they were unresolved by this sequence. However, precisely because of this degree of conservation, sequence divergence should be interpreted as indicative of speciation. In this context, the genetic heterogeneity of a widespread species from different localities may imply that we are dealing here with divergent species.

Thus, representatives of *B. brunnea* from four localities that clearly split into two sequence variants may belong to two separate species.

Phylogenetic relationships

Inferring phylogenetic relationships from molecular data requires sufficient variation between species under comparison. The extent of genetic divergence within the Japanese dendrocoelids is extreme. The differentiation between *Dendrocoelopsis* species was maximal, while sequence differences between *Bdellocephala* species were absent (except for the *B. brunnea* population from Fukushima Pref.).

Comparative analysis with non-Japanese dendrocoelids (from Europe, Lake Baikal, and Kamchatka) showed three principal things: (1) *Bdellocephala* species from Japan, Kamchatka and Lake Baikal have very close relationships and show well-marked distributional patterns; (2) species from the genus *Dendrocoelopsis* do not form a single cluster of closely related organisms; (3) *Dendrocoelopsis ichikawai*, differs minimally from *Bdellocephala baicalensis* (from Lake Baikal), groups reliably with all *Bdellocephala*, and should, therefore, be classed with this genus.

Taxonomy

The taxonomic status of *Dendrocoelopsis ichikawai* should be changed in the light of the above phylogenetic analy-

sis indicating a position within the genus *Bdellocephala*. The maximal sequence similarity between *D. ichikawai* and *B. baikalensis* makes it appropriate to reconsider their morphological similarities. Actually, Dr. Masaharu Kawakatsu (Kawakatsu *et al*., 1977) stated that the external appearance of *B. baicalensis* was very similar to that of the new Japanese form (i.e. *D. ichikawai*), and that the anatomical structure of the copulatory apparatus of these species revealed some similarity (p. 211). However, based on the presence of a well -developed penis papilla in the new species, he excluded it from the genus *Bdellocephala* (penis papilla is absent) and classified it with *Dendrocoelopsis*. This viewpoint was supported by Kenk on the same basis (Kawakatsu *et al*., 1977).

Another taxonomic point concerning the genus *Bdellocephala* relates to the separation of *B. brunnea* representatives, on the basis of sequence divergence, into two groups: (1) from Aomori (two localities) and Nagano Pref., and (2) from Fukushima Pref. The groups may justify classification into different species. However, this point should be given more attention in terms of morphological examination for a set of specific characters by which they can be distinguished. We will discuss this point further in a separate paper.

The separate taxonomic status of the other species of Japanese *Dendrocoelopsis* (*D. ezensis* and *D. lactea*) seems to be justified, because they exhibit a high level of sequence divergence from one another. This level is approximately twice that found between one European and two Baikalian dendrocoelids (all from different genera).

On the other hand, Kenk's (1930) diagnosis of the genus *Dendrocoelopsis* Kenk, 1930, was developed to separate *D. spinosipenis* (Kenk, 1925) from the genus *Dendrocoelum* Örsted, 1844, and contained the following points: no adenodactyl; well-developed penis papilla; penis bulb of ordinary structure; oviducts unite without embracing bursa stalk; testicular zone extending to behind the copulatory apparatus; anterior end with subterminal true sucker; eyes not multiple. Later, the diagnosis was amended by omitting the three last-named points in which some new species (described under a new genus – *Amyadenium* de Beauchamp, 1931) classed with *Dendrocoelopsis* by Kenk (1953, pp. 177–178) showed differences from *D. spinosipenis* de Beauchamp (1931) recognized the close relationship of *Amyadenium* to *Dendrocoelopsis*, but separated it from the latter, based on the absence of a highly complex grasping organ, or true sucker (i.e., an adhesive organ separated from the surrounding mesenchyme by a muscle layer).

Within the genus *Dendrocoelopsis* there have been observed two species groups closely related to the Japanese forms. The three pigmented and two-eyed species with an adhesive organ, namely, *D. ezensis*, *D. piriformis* Kenk, 1953, from Alaska, and *D. vaginata* Hyman, 1935, from the northwestern part of the United States resemble each other rather closely in their external appearance (Ichikawa and Okugawa, 1958; Kenk, 1973), and show similar external appearance to the representatives of the genus *Bdellocephala*, particularly in the head region (Kenk, 1953; Ichikawa and Okugawa, 1958). Apart from the lack of a grasping or adhesive organ, *D. lactea* bears some resemblances to *D. alaskensis* in color, shape and size of the body, the supernumerary eyes and the ventral position of the testes (Ichikawa and Okugawa, 1958). In view of the distributional patterns of the four species (*D. ezensis, D. lactea, D. piriformis* and *D. alaskensis*) on each side of the North Pacific and their morphological features, it seems possible to consider each of the groups as a natural phyletic unit. On the other hand, it is doubtful whether these groups should be classified within the same genus, considering the high level of the genetic divergence between *D. ezensis* and *D. lactea*. Hence, the genus *Dendrocoelopsis* should be revised, based on the molecular typification of the genus representatives.

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