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A new species in the *Daphnia curvirostris* (Crustacea: Cladocera) complex from the eastern Palearctic with molecular phylogenetic evidence for the independent origin of neckteeth

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Little is known of the biology and diversity of the environmental model genus Daphnia beyond the Nearctic and western Palearctic. Here, we describe Daphnia sinevi sp. nov., a species superficially similar to Daphnia curvirostris Eylmann, 1878, from the Far East of Russia. We estimated its phylogenetic position in the subgenus Daphnia s. str. with a rapidly evolving mitochondrial protein coding gene [NADH-2 (ND2)] and a nuclear protein-coding gene [heat shock protein 90 (HSP90)]. Daphnia curvirostris, D. sinevi sp. nov., Daphnia tanakai and D. sp. from Ootori-Ike, Japan, (which, probably, is D. morsei Ishikawa, 1895) formed a monophyletic clade modestly supported by ND2 and strongly supported by HSP90. Our results provide evidence of hidden species diversity in eastern Palearctic Daphnia, independent origins of defensive neckteeth and phylogenetic informativeness of nuclear protein-coding genes for zooplankton genera.

INTRODUCTION

Biogeographic and phylogeographic studies have revealed pronounced regionalism in freshwater zooplankton that is often associated with glacial or orogenic vicariance (Haney and Taylor, 2003). Still, the diversity and biology of freshwater zooplankton is poorly understood outside of the Nearctic and western Palearctic (Benzie, 2005). In the eastern Palearctic, for example, there is marked disagreement about the diversity of the zooplankton model genus *Daphnia* O. F. Müller, 1785 (Crustacea: Cladocera). Glagolev (Glagolev, 1995) reported no endemic eastern Russian species or lineages, despite the existence of well-established glacial refugia. Ishikawa (Ishikawa, 1895a, 1895b, 1896) and Uéno (Uéno, 1972), however, described several endemic *Daphnia* species from Japan. Using a combined morphological and genetic approach, Ishida *et al.* (Ishida *et al.*, 2006) provided evidence that populations of a *Daphnia curvirostris*-like species from the Hida Mountains in Japan belonged to a separate divergent species (*Daphnia tanakai* Ishida, Kotov et Taylor, 2006). The results suggested that further geographic sampling in the eastern Palearctic with a combined morphological and genetical assessment of the diversity might reveal new lineages.

Daphnia curvirostris-like species possess a mixture of several diagnostic morphological features from the commonly recognized subgenera of *Daphnia*. As such, an understanding of the diversity and evolution of this anomalous group is critical for understanding morphological innovations and adaptative radiation of the entire genus *Daphnia*. For example, the functional significance

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of the subgenera-defining second pecten on the postabdominal claw is unknown. Ishida *et al.*'s (Ishida *et al.*, 2006) evidence that a *curvirostris*-like species can possess the extreme phenotypes from a row of fine setules (*long-ispina* type) to a group of strong teeth (*pulex* type) makes *D. tanakai* a valuable subject to understand the functional significance of the pecten. Unfortunately, despite the mtDNA sequence analysis of Ishida *et al.* (Ishida *et al.*, 2006), the phylogenetic position of *D. tanakai* within *Daphnia* remains unresolved.

In a survey of 80 samples from the Far East of Russia (Primorski Krai, Khabarovsk Area and Sakhalin Area), we found a *curvirostris*-like population (Fig. 1) with yet another suite of mixed morphological characters. The specimens possessed neckteeth, defensive structures previously recorded from species closely related to *Daphnia pulex* and to *Daphnia dentifera* but unrecorded from *D. curvirostris*-like specimens (Benzie, 2005). This structure, which generally appears in the smaller instars, is believed to be a low-cost adaptation against invertebrate predation (Tollrian, 1995; Colbourne *et al.*, 1997). Beaton and Hebert (Beaton and Hebert, 1997) found that polyploid nuclei were associated with the region of necktooth growth and suggested that a common developmental mechanism may be present in the distantly related *Daphnia* that express neckteeth.

Here, we aimed to (i) address the phylogenetic positions of *D. curvirostris*-like specimens from the eastern Palearctic by using both nuclear and mtDNA protein-coding genes, (ii) describe a new species from the *curvirostris* complex, *Daphnia sinevi* sp. nov. and (iii) assess the number of evolutionary origins of neckteeth in *Daphnia*.

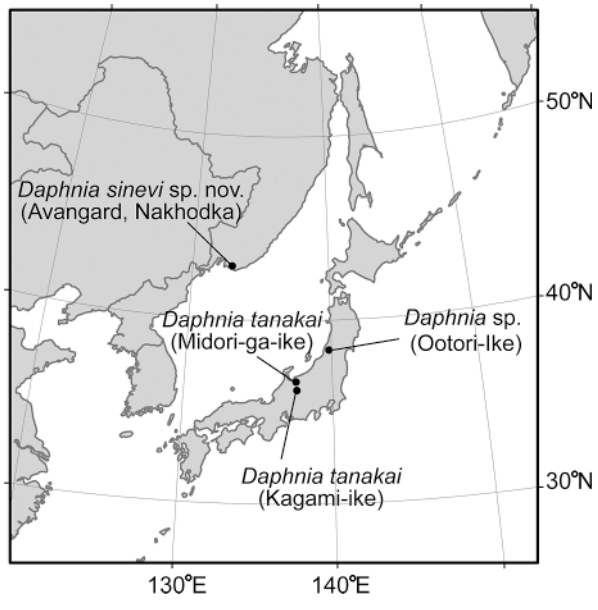

Fig. 1. A map of localities in Japan and the Far East of Russia, where *Daphnia curvirostris*-like species were collected for this study.

METHOD

Genetic analyses were performed using the sequences of a nuclear protein-coding gene [heat shock protein 90 (*HSP90*)] and a mitochondrial protein-coding gene [NADH-2 (*ND2*)] for *D. sinevi* sp. nov. and 16 other species of *Daphnia* including an undescribed species from Ootori-Ike (Table I). The *ND2* sequences of seven species were obtained from GenBank (DQ132610, DQ132613–DQ132620 and DQ132627). Multiple specimens (2–10) of each *curvirostris*-like species (*D. curvirostris*, *D. tanakai*, *D. sinevi* and *D.* sp.) were used for sequence analyses. We extracted genomic DNA using QuickExtract (Epicentre) as described in Ishida *et al.* (Ishida *et al.*, 2006). Each 50 μL PCR reaction consisted of 5 μL of extracted DNA, 10× PCR buffer [50 mM KCl, 1.5 mg $MgCl_2$, 10 mM Tris–HCl pH 8.3, 0.01% (w/v) gelatin], 2 mM each dNTPs, 1 μM each primer and 1 U of *Taq* DNA polymerase. The primers and the PCR thermal cycling parameters for the mitochondrial *ND2* gene (∼1000 bp) were described in Ishida *et al.* (Ishida *et al.*, 2006). We initially used the degenerate *HSP90* primers (listed as hsp82) of Welch and Meselsen (Welch and Meselsen, 2000) and obtained an ∼760 bp PCR product from *Daphnia dubia*, *Daphnia laevis* and *Daphnia magna*. Cloning was performed for these PCR products using the TOPO TA Cloning Kit (Invitrogen), and the colonies were sequenced. We then developed specific primers for *Daphnia* (∼700 bp fragment of *HSP90* with two introns): 5′-TTACGAGTCCA-GATGGGCTT-3′ and 5′-ATCCGTTATGAATCC-CTGACTGA-3′. PCR thermal cycling parameters for these specific primers were 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min with Peltier thermal cycler (Bio-Rad). The *HSP90* PCR products of *Daphnia pulicaria* possessed numerous sequence ambiguities and were cloned before sequencing. Direct sequencing was performed for the other *HSP90* PCR products. Sequencing was performed by Genaissance Pharmaceuticals (New Haven, USA) or the Roswell Park Cancer Institute (Buffalo, USA).

Intron boundaries of the *HSP90* sequences were identified by comparing arthropod *HSP90* mRNA sequences (e.g. AY528900 and AY423488) and by examining intron-splicing signature sequences. The *HSP90* exon sequences (621 bp) and the *ND2* sequences (962 bp) were aligned manually. These nucleotide alignments were used for neighbor joining (NJ), maximum

Table I: Daphnia specimens subjected to DNA sequencing

Taxon	Sampled location	Latitude	Longitude	Number of specimens		Gene accession number	
				HSP	*ND2*	*HSP*	*ND2*
Daphnia sinevi sp. nov.	Avangard, Nakhodka Area, Primorski Krai, Russia	42°48′N	132°53′E	3	2	DQ845251	DQ845269
Daphnia sp.	Ootori-Ike, Yamagata, Japan	38°22′N	139°50′E	2	4	DQ845252	DQ845270
Daphnia tanakai Ishida, Kotov et Taylor, 2006	Kagami-ike (K), Gifu, Japan	36°20′N	137°36′E	1	5	DQ845253	DQ132618
Daphnia tanakai Ishida, Kotov et Taylor, 2006	Midori-ga-ike (M-1), Toyama, Japan	36°35′N	137°36′E	1	4	DQ845254	DQ132616
Daphnia tanakai Ishida, Kotov et Taylor, 2006	Midori-ga-ike (M-2), Toyama, Japan	36°35′N	137°36′E	0	1	n/a	DQ132617
Daphnia curvirostris Eylmann, 1887	Pilgrim Hotspring, AK, USA	65°5′N	164°55′W	3	1	DQ845255	DQ132619
Daphnia curvirostris Eylmann, 1887	Somotor, Southeastern Slovakia	48°23′N	21°49′E	0	1	n/a	DQ132620
Daphnia longispina O. F. Müller, 1785	Unnamed pond south of Muono, Lapin Laani, Finland	67°37′N	23°33′E	1	1	DQ845256	DQ132610
Daphnia dentifera Forbes, 1893	Crossman's Pond, NY, USA	43°2′N	77°28′W	1	1	DQ845257	DQ845271
Daphnia cucullata Sars, 1862	Hancza, Poland	54°15′N	22°48′E	1	1	DQ845258	DQ845272
Daphnia galeata Sars, 1864	Ikeda-Ko, Kagoshima, Japan	31°14′N	130°34′E	1	1	DQ845259	DQ845273
Daphnia laevis Birge, 1897	Cape Cod, MA, USA	42°4′N	70°12′W	1	1	DQ845260	DQ132614
Daphnia dubia Herrick, 1883 emend. Herrick, 1885	Pond in the South of Cornerbrook, Newfoundland, Canada	n/a	n/a	1	1	DQ845261	DQ132615
Daphnia cristata Sars, 1862	Puruvesi, Ita-Suomen Laani, Finland	61°46′N	29°21′E	1	1	DQ845262	DQ132613
Daphnia longiremis Sars, 1861	Deer Lake, Newfoundland, Canada	49°11′N	57°27′W	1	1	DQ845263	DQ845274
Daphnia pulicaria Forbes, 1893	Honeoye Lake, NY, USA	42°45′N	77°31′W	1	1	DQ845264	DQ845275
Daphnia ambigua Scourfield, 1947	Fresh Pond, MA, USA	41°41′N	70°9′W	1	1	DQ845265	DQ845276
Daphnia ephemeralis Schwartz et Hebert, 1985	Pond in Amherst, NY, USA	43°2′N	78°47′W	1	1	DQ845266	DQ845277
Daphnia lumholtzi Sars, 1885	Burn Lake, NM, USA	32°18′N	106°48′W	1	1	DQ845267	DQ845278
Daphnia magna Straus, 1820	Clone from WARD'S Natural Science, USA	n/a	n/a	1	1	DQ845268	DQ132627

HSP90, heat shock protein 90; *ND2*, NADH-2.

The columns of HSP90 and ND2 indicate the number of specimens used for the phylogenies of each gene. The specimens of *Daphnia tanakai* indicated as M-1 and M-2 were sampled from the same location but have different haplotypes of ND2. Gene accession numbers are from GenBank.

parsimony (MP), maximum likelihood (ML) and the Bayesian inference (BI) (Huelsenbeck and Ronquist, 2001; Swofford, 2002). Likelihood ratio tests selected the TrN + I + G model for the *HSP90* exon sequences and the GTR + I + G model for the *ND2* sequences (Posada and Crandall, 1998). NJ analysis used distance based on the best ML model and had 1000 NJ bootstrap replicates. MP analysis used a heuristic search with Tree bisection and reconnection (TBR) branch swapping, and support was estimated by 1000 bootstrap replicates with TBR branch swapping. ML analysis was based on the best ML model and used a heuristic search with TBR branch swapping and support estimated by 1000 bootstrap replicates with nearest neighbor interchange (NNI) branch swapping. BI analysis partitioned the three codon positions and sampled 1 000 000 generations of Markov chain Monte Carlo (MCMC). We discarded the trees during a 'burn-in period' (the initial 10% trees after inspection for convergence) and made a consensus tree from the remaining set of Bayesian trees. Statistical tests of topological differences were carried out in PAUP using the SH test with RELL bootstrapping (1000 replicates) (Shimodaira and Hasegawa, 1999). We used the uncorrected *p* distance and the corrected ML distance (based on the best ML model) to assess the genetic distance among taxa.

For morphological study, animals were picked from the sample, placed on slides (in a drop of a glycerol–formaldehyde mixture) and studied under an optical microscope *in toto*. Then, five adult and two juvenile females and five adult males were dissected for the analysis of appendages. We applied a system of seta enumeration initially proposed for chydorids (Kotov, 2000) and recently applied to *Daphnia* (Ishida *et al.*, 2006).

RESULTS

Genetics

The 621 bp *HSP90* nucleotide sequences from 16 species had 194 bp variable sites and 131 bp parsimony informative sites. Three best MP trees were found that had 385 steps. One best ML tree was found that had a likelihood score of –ln L = 2652.6450. All *HSP90* trees of BI, NJ, MP and ML were concordant with the NJ bootstrap consensus tree (Fig. 2A). The 962 bp *ND2* nucleotide sequences from 16 species had 696 bp variable sites and 652 bp parsimony informative sites. One best MP tree was found that had 3278 steps. One best ML tree was found that had a likelihood score of –ln L = 12 713.2836. All *ND2* trees of NJ, MP, ML and BI were concordant with the NJ bootstrap consensus tree (Fig. 2B).

Trees of NJ, MP, ML and BI for *HSP90* and *ND2* sequences showed a divergent lineage of *D. sinevi* sp. nov. (Fig. 2A and B) with *D.* sp. from Ootori-Ike (Japan) as a sister species (*HSP90* NJ: 89%, MP: 83%, ML: 87%, BI: 95%; *ND2* NJ: 100%, MP: 100%, ML: 100%, BI: 100%). The *D. sinevi* Ootori-Ike clade grouped with *D. tanakai* with strong branch support both in *HSP90* (NJ: 100%, MP: 85%, ML: 86%, BI: 100%) and in *ND2* (NJ: 89%, MP: 79%, ML: 65%, BI: 100%). The average genetic distances among *D. sinevi* sp. nov., Ootori-Ike *D.* sp and *D. tanakai* were greater than the largest distances within well-established ancient species clades: *Daphnia longispina*/*Daphnia cucullata*/*D. dentifera*/*Daphnia galeata* and *D. laevis*/*D. dubia* (Table II). The analyses also supported a monophyletic clade of the *curvirostris* species complex (*D. curvirostris*, *D. tanakai*, *D. sinevi* sp. nov. and *D.* sp. from Ootori-Ike) with strong branch support from *HSP90* (NJ: 98%, MP: 96%, ML: 96%, BI: 100%) and variable branch support in *ND2* trees (NJ, MP: not supported, ML: 55%, BI: 99%). The SH test for *HSP90* sequences also supported the monophyly of the *D. curvirostris* clade ($P < 0.05$). The unconstrained ML score was –ln L = 2652.645 and the ML score of trees constrained so that the *D. curvirostris* complex is not monophyletic was –ln L = 2565.886. The *ND2* and *HSP90* trees (Fig. 2A and B) agreed on the nodes with strong support and differed only where one gene possessed an unresolved node. Thus, there was no well-supported conflict between the nuclear and mtDNA gene trees. Each gene tree supported the polyphyly of necktooth production (Fig. 2A and B).

Taxonomy

Daphnia (Daphnia) sinevi sp. nov.

Etymology: This species is dedicated to our colleague Dr A. Yu. Sinev, who collected this species.

Type locality: A pond about 10 m in diameter in Avangard, Nakhodka Area (42°48′N, 132°53′E), Primorski Krai, Russia. The type series was collected on 25 September 2004 by A. Yu. Sinev.

Holotype: A parthenogenetic female, deposited at the collection of the Zoological Museum of Moscow State University, MGU Ml 46. Label of the holotype: '*Daphnia sinevi* sp. nov., 1 parth. ♂ from a pond in Avangard, Nakhodka Area, Russia, coll. in 25.ix.2004 by A. Yu. Sinev, HOLOTYPE'.

Allotype: Adult male, MGU Ml 47.

Paratypes: Twenty parthenogenetic females, MGU Ml 48; three ephippial females, MGU Ml 49, 12 juvenile and adult males, MGU Ml 50; 12 females in personal collection of AAK, AAK 2005–195. All samples are preserved in 90% alcohol.

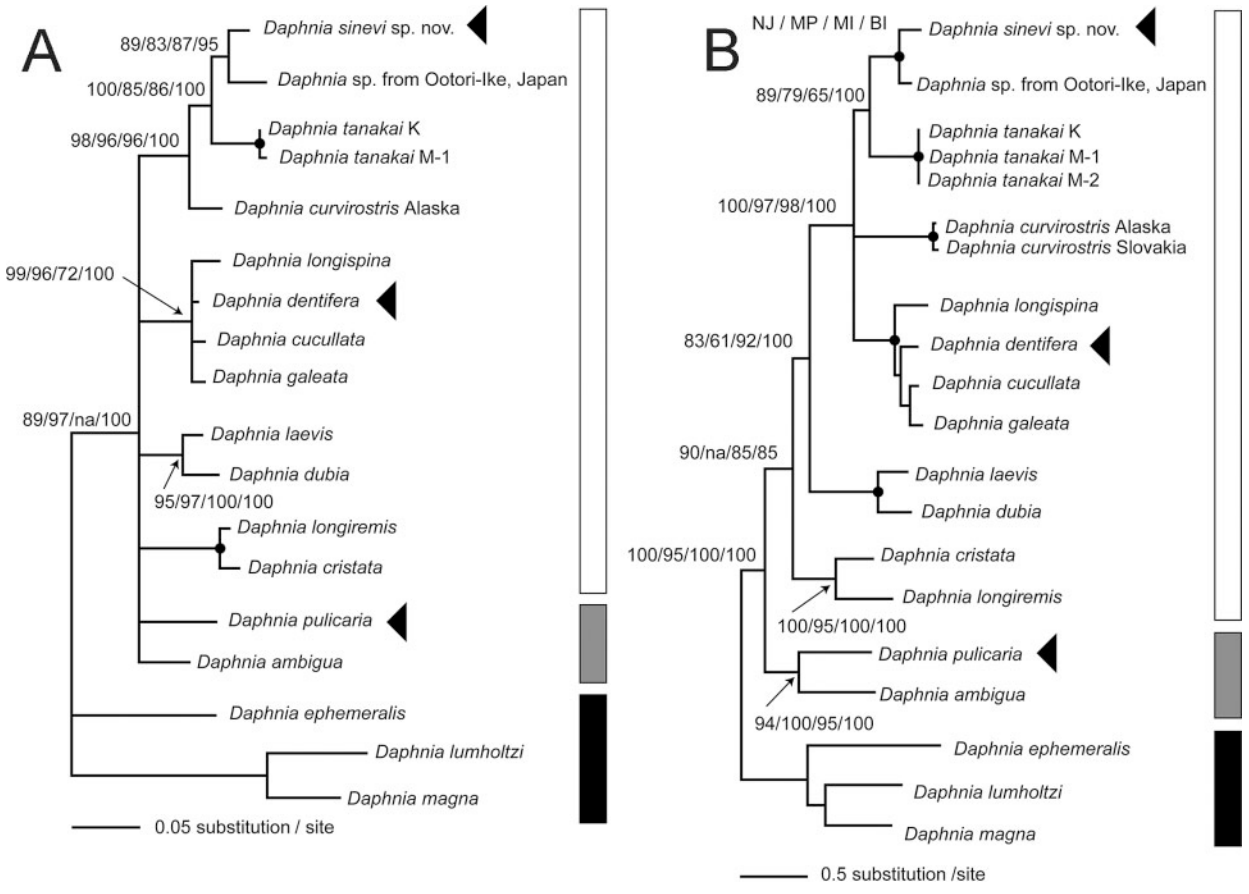

Fig. 2. Neighbor-joining (NJ) bootstrap consensus trees of *Daphnia* species based on (**A**) nuclear [heat shock protein 90 (*HSP90*)] sequences and (**B**) mitochondrial [NADH-2 (*ND2*)] sequences. The black triangles indicate species known to produce neckteeth. The shaded rectangles indicate subgenus *Ctenodaphnia* (black), *Daphnia pulex* group (gray) and *Daphnia longispina* group (white). Each branch with a black filled circle has 100% support from Bayesian inference (BI), NJ bootstrap, maximum parsimony (MP) bootstrap and maximum likelihood (ML) bootstrap. Other branches have the support values of BI, NJ bootstrap, MP bootstrap and ML bootstrap as indicated. The support value of 'not available (na)' means that the particular analysis failed to support the branch.

Short diagnosis: Parthenogenetic female. Body subovoid, caudal spine very short to completely reduced. Rostrum of moderate length, its tip not bent and subdividing into two lobes by a 'line' of prerostral fold. Posterior margin of head with a strong prominence proximally to antenna I. Spinules present on dorsal and ventral margin only near caudal spine, or completely absent when spine reduced. First abdominal process moderately long, bent anteriorly, second process relatively short, third process low, massive, all processes covered with fine setules. Postabdominal claw long, the proximal pecten consisting of 9–13 slender teeth, the medial (second) pecten consisting of six to seven large teeth, a gap between two pectens distinct, the distal pecten with fine setules. Body of antenna I as a low mound, a rudimentary antennular sensory seta arising immediately from head surface, aesthetascs protruding ventrally, their tips somewhat projected beyond the tip of rostrum. Limbs very similar to those in *D. curvirostris*, but limb I with longer seta 3; limb II with a short anterior seta 1 and setae of filter plate of gnathobase significantly varying in number (11–13), limb III with shorter seta 3 and 56–62 filtering setae, limb IV with 48–53 filtering setae and limb V with more projected inner distal portion.

Ephippium includes postero-dorsal portion of valves with caudal spine (if present).

Adult male: Head with well-developed rostrum and straight posterior margin. Abdomen without processes on two distal segments, two basal most segments with small processes. Postabdomen shape and armature in general as in female, gonopore opens subdistally, without a genital papilla. Antenna I relatively short, among aesthetascs three members longer than the rest, antennular sensory seta small, flagellum on top of a conical, postaesthetasc process, its distal segment with a hooked

Table II: The list of genetic distances among species of Daphnia based on HSP90 and ND2 sequences

Taxa	*HSP90*		*ND2*	
	ML distance	*p* distance	ML distance	*p* distance
Among *sinevi, tanakai* and Ootori	0.0626	0.0495	0.5884	0.2519
Between *sinevi* and *tanakai*	0.0711	0.055	0.7909	0.3004
Between *sinevi* and Ootori	0.0434	0.0371	0.2508	0.1694
Among *longispina, cucullata, dentifera* and *galeata*	0.0221	0.02	0.3333	0.2015
Between *laevis* and *dubia*	0.0418	0.0354	0.4757	0.2419
Between *longiremis* and *cristata*	0.0217	0.0193	0.7164	0.2921
Between *pulicaria* and *ambigua*	0.0982	0.0696	1.1521	0.3577

HSP90, heat shock protein 90; ML, maximum likelihood; *ND2*, NADH-2.
ML distance shows corrected genetic distance based on the best maximum likelihood model; *p* distance shows uncorrected genetic distance.

tip, setulated distally. Inner distal lobe (IDL) of limb I with a bent copulatory hook, and two setae of different size; setae 2 and 2′ long, seta 3 large, seta 4 somewhat larger than in female. On distal most endite of limb II, anterior seta 1 short, hook-like, setulated distally.

Juvenile males and females of first instar with reduced rostrum and a single neck tooth.

Size up to 1.73 mm.

Description

Adult parthenogenetic female

Body subovoid in lateral view, maximum height in middle of valves (Fig. 3A). Dorsal margin of valves slightly elevated above head, slightly and regularly convex, a shallow depression between head and rest of body. Postero-dorsal angle usually lacking of a caudal spine, or with a rudimentary spine, ventral margin regularly convex.

Head with a moderate rostrum, its tip not bent, in lateral view, the tip subdividing into two lobes by a 'line' of prerostral fold (Fig. 3B–D); posterior margin of head with a strong prominence proximally to antenna I; ventral margin of head remarkably concave. No crest or large helmet on head, compound eye large, ocellus small and located far from base of antenna I. Labrum with a fleshy main body and a large, setulated distal labral plate (Fig. 3B).

Carapace subovoid, spinules present on dorsal and ventral margins only near caudal spine (Fig. 3E) or completely absent when spine reduced (Fig. 3A). No setae at ventral margin, in posterior portion of valve (on its inner face) only a row of delicate, setulated setae, rows of minute setules between them (Fig. 3F).

Abdomen relatively short, consisting of four segments. The first (basal most) abdominal segment with a moderately long (as long as postabdominal claw) process bent anteriorly; the second segment with a short, bulb-like process and the third segment with a low, mound-like process; on all processes, there are transverse rows of minute setules (Fig. 3G–H). The fourth segment lacking of a process, with slightly convex dorsal margin.

Postabdomen elongated, tapering distally, with ventral margin straight, lacking of setules. Preanal margin long (longer than anal plus postanal portions of postabdomen), slightly concave, with series of minute setules. Preanal angle distinct, postanal angle not expressed. Ten to fourteen paired spines on postanal and anal portions, their size continuously increasing distally. Postabdominal seta longer than preanal margin, its distal segment shorter than basal one. Postabdominal claw regularly bent, with a pointed tip (Fig. 3I). On the outer side, three successive pectens along the dorsal margin: the first (proximal) pecten consisting of 9–13 thin teeth; the second (medial) pecten consisting of six to seven large teeth (as long as claw diameter ad base) and the third pecten consisting of numerous setules, approximately two times shorter than those in the second pecten, not reaching the tip of claw. Rows of fine setules at ventral margin of the claw on the level of distal end of the second pecten and at the level of the middle of the distal pecten.

Antenna I as a small mound, with nine aesthetascs of different length terminally, their tips somewhat projected beyond tip of rostrum, antennular sensory seta fine, arise immediately from head surface instead of mound of the antenna I. Antenna II with coxal part possessing two short sensory setae of different length (Fig. 4A). Basal segment elongated, a well-developed (remarkably longer than the basal segment of exopod) distal sensory seta on its posterior face (Fig. 4B), minute distal spine at its anterior face (Fig. 4C). Antennal branches longer than basal segment, four-segmented exopod slightly shorter

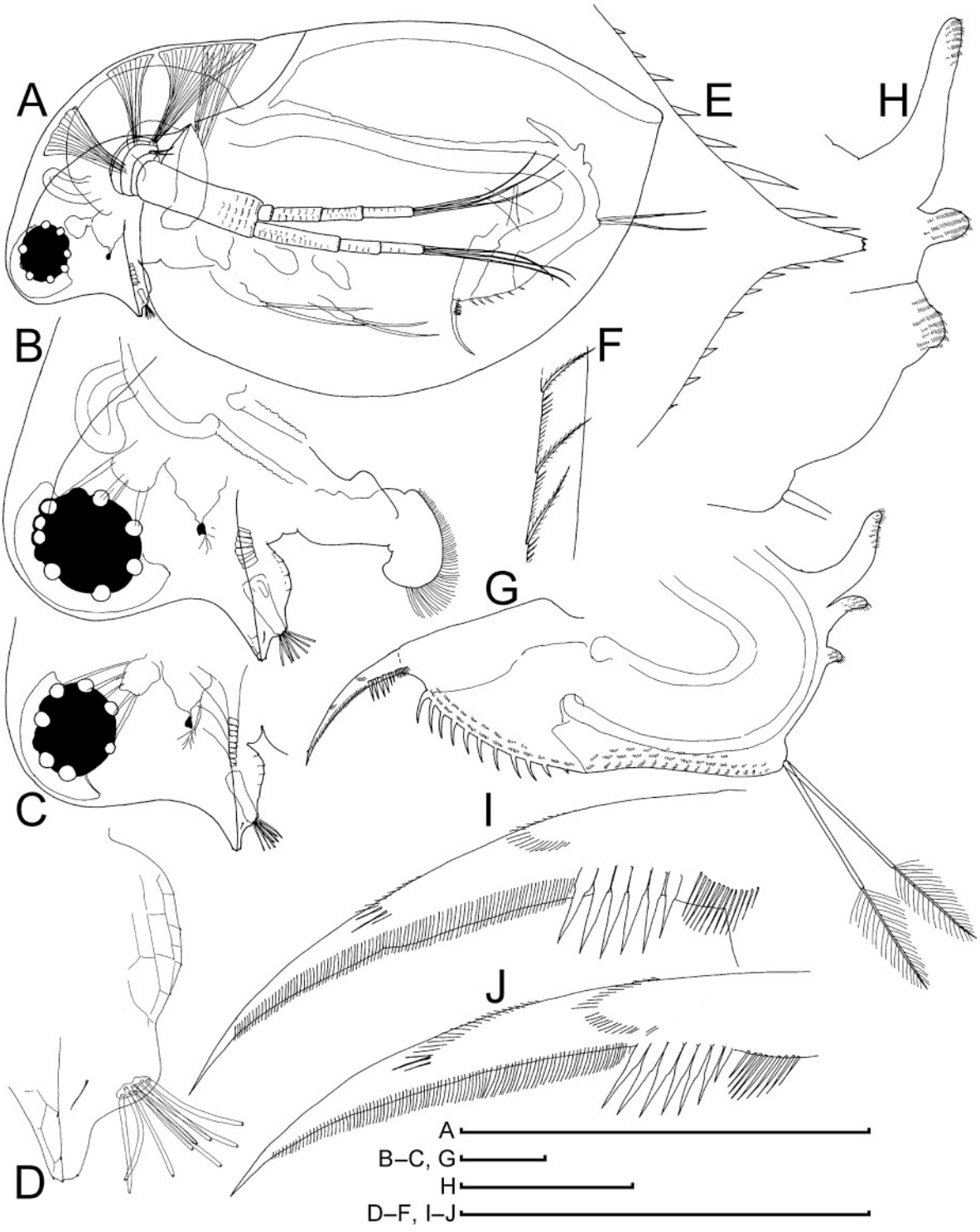

Fig. 3. *Daphnia sinevi* sp. nov., parthenogenetic female from a pond in Avangard, Nakhodka region, Far East of Russia. (**A**) Holotype, lateral view. (**B**, **C**) Head. (**D**) Rostrum and antenna I. (**E**) Postero-dorsal portion of valve. (**F**) Armature of postero-dorsal portion of valve. (**G**) Postabdomen. (**H**) Abdominal projections. (**I**, **J**) Postabdominal claw. Scale bars: A, 1 mm and B–J, 0.1 mm.

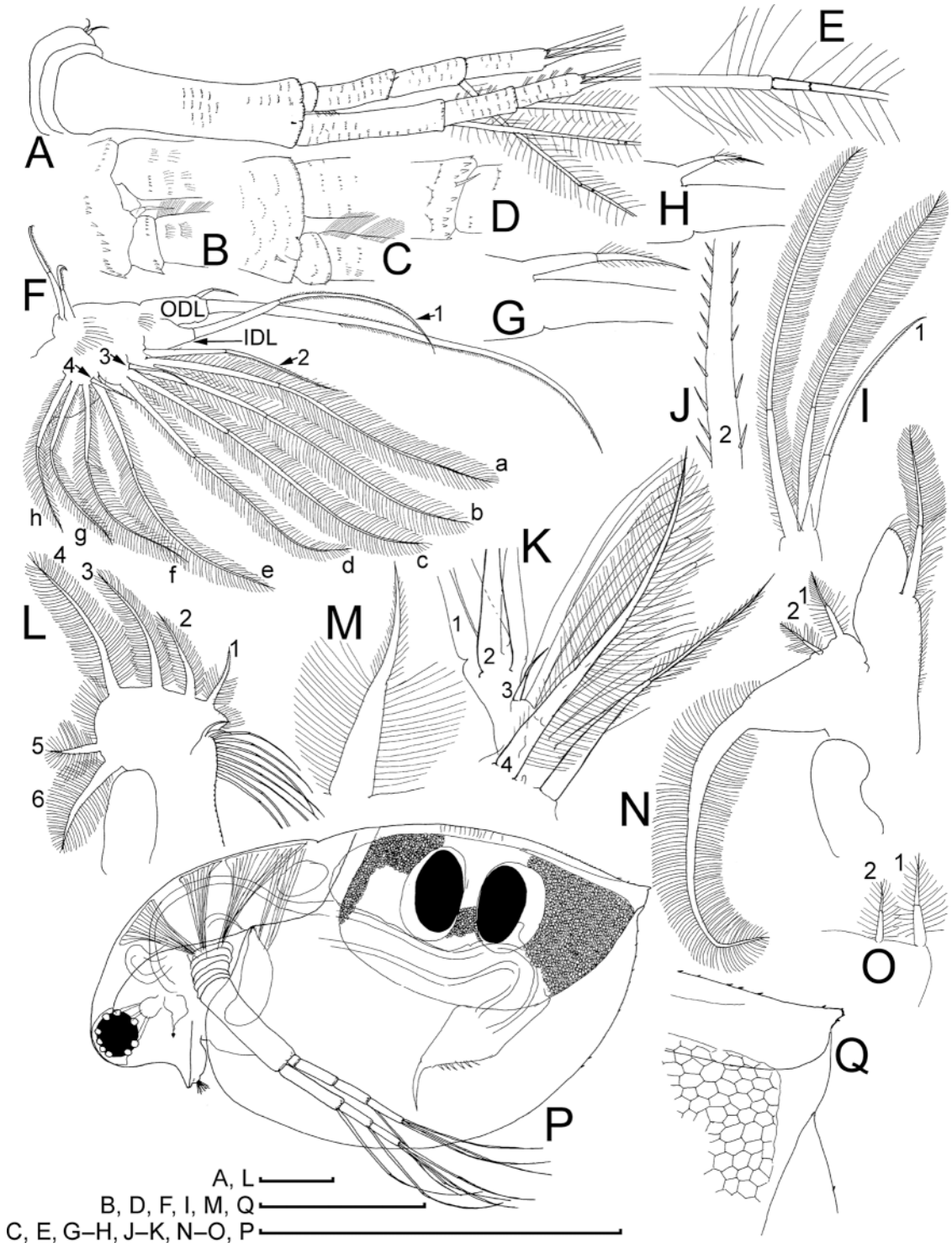

Fig. 4. *Daphnia sinevi* sp. nov. from a pond in Avangard, Far East of Russia. (**A**) Antenna II. (**B**, **C**) Distal portion of basal segment, posterior and anterior view. (**D**) Spine on second segment of exopod. (**E**) Apical swimming seta. (**F**) Limb I. (**G**, **H**). Outer distal lobe (ODL) of two different females. (**I**) Inner distal portion of limb II. (**J**) Seta 2 of exopod III. (**K**) Inner distal portion of limb III. (**L**) Limb IV. (**M**) Seta 1 of limb IV. (**N**) Limb V. (**O**) Setae 1–2 of exopod V. (**P**, **Q**) Ephippial female and its postero-dorsal angle. Scale bars: A–O, Q, 0.1 mm; P, 1 mm.

than three-segmented endopod, all with series of minute denticles. Spines on apical segments rudimentary, spines on the second segment of exopod small (its length less than half of diameter of third segment) and thin (Fig. 4D). Antennal formula: setae 0–0–1–3/1–1–3. Each swimming seta with basal and distal segments bilaterally setulated, a chitinous insertion within distal segment near joint with basal segment (Fig. 4E).

Limb I without accessory seta; outer distal lobe (ODL, Fig. 4F) with a long seta unilaterally armed distally with short setules and a thin, short (length about 1.5–2 diameters of ODL) seta bilaterally setulated distally (Fig. 4G and H); IDL (Fig. 4F), or endite 4, with a single, long anterior seta (Fig. 4F: 1), bearing short setules distally. Endite 3 with a long anterior seta 2, armed with minute setules and two posterior setae (a and b). Endite 2 with a short and thin anterior seta 3, which is however relatively longer than in *D. curvirostris*, and two posterior setae (c and d). Endite 1 with a small anterior seta 4 and four posterior setae (e–h). Two ejector hooks of different length. Limb II as in earlier described *D. curvirostris* or *tanakai* (see Ishida *et al.*, 2006), a stiff, anterior seta 1 about 2/3 length of other setae on distal most endite, unilaterally setulated distally with minute setules (Fig. 4I), setae of filter plate of gnathobase II significantly varying in number (11–13). Limb III as in earlier described *D. curvirostris* or *tanakai*, longest seta of exopod III (seta 2) distally with short denticles (shorter that seta width) (Fig. 4J), inner distal portion of limb III with seta 3 relatively long (reaching base of seta 2) and large seta 4 (Fig. 4K), 56–62 filtering setae in gnathobase III. Limb IV with as in *D. curvirostris*, but seta 2 of exopod with short setules distally (Fig. 4L and M), 48–53 filtering setae in gnathobase IV. Exopod of limb V with seta 2 shorter than seta 1 and long inner distal projection (Fig. 4N and O).

Ephippial female

Dorsal margin of valves slightly convex (Fig. 4P), dorsal wall of carapace additionally chitinized, forming a dorsal plate, bearing fine spinules in its posterior most portion (Fig. 4Q). Ephippium with two resting eggs, axes of which perpendicular to its dorsal margin, egg chambers well separated from each other, most part of ephippium additionally darkly pigmented and covered with sculpturing of polygonal cells, postero-dorsal portion of valves with caudal spine incorporated into ephippium.

Juvenile female and male

Head without rostrum, with well-developed necktooth and a round dorsal organ in its posterior portion, dorsal margin of carapace almost straight, well-developed caudal spine and spinules covered 1/3–1/2 of ventral margin (Fig. 5A).

Adult male

Body low, dorsal margin of valves almost straight, not elevated above head, depression between head and valves almost absent, postero-dorsal angle distinct, with a short caudal spine (Fig. 5B). Head with a well-developed rostrum, region of antenna I joint with a special depression (Fig. 5C, arrow) and straight posterior margin. Anterior most extremity occupied with optic vesicle, a shallow supra-occular depression posteriorly to it. Eye large, ocellus small. Valve with antero-ventral angle distinctly prominent ventrally, all ventral margins with long, numerous setae submarginally on inner face of valve (Fig. 5D and E). Postero-ventral portion of valve with small marginal denticles, armature of inner face of valve posterior margin as in female.

Abdomen with reduced processes, only small mound present on each first and second segment (counting from basal end) (Fig. 5F, arrow). Postabdomen shape and armature in general as in female, but preanal margin shorter and postanal angle expressed. Eight-eleven paired teeth large (longer than claw diameter) and strongly increasing in size distally. Gonopore opens subdistally, without a genital papilla. On the outer surface of postabdominal claws, a basal pecten of fine setules, second pecten of five to seven teeth increasing in size distally, third pecten consisting of fine, numerous setules (Fig. 5G).

Antenna I long, almost straight (Fig. 5C and H); antennular seta small (length ~1.5 diameter of antenna I), located far from the distal end of antenna I body (Fig. 5I, arrow); aesthetascs of different length, among them, three members longer than the rest, largest aesthetasc 1.5–2 times longer than antenna I maximum diameter. Male seta (flagellum) on top of a conical, distal (postaesthetasc) process. This seta long, bisegmented, its distal segment setulated, with a hooked tip (Fig. 5J).

Limb I: ODL large, cylindrical (Fig. 5K: ODL), bearing a rudimentary seta and a very large seta supplied with minute setules distally; IDL with a bent copulatory hook and two setae of different size (Fig. 5K: 1 and 1′); in contrast to female, endite 3 with four setae (additional seta marked as 2′), both setae 2 and 2′ long, seta 3 remarkably larger than in female, seta 4 somewhat larger than in female. Limb II: distal most endite with a modified, hook-like anterior seta 1, setulated distally (Fig. 5L and M).

Size

Holotype 1.67 mm, parthenogenetic females 0.69–1.73 mm, minimal size of reproduction 1.39 mm, ephippial females 1.36–1.60 mm, juvenile males 0.71–1.02 mm, adult males 1.01–1.22 mm, allotype 1.22 mm.

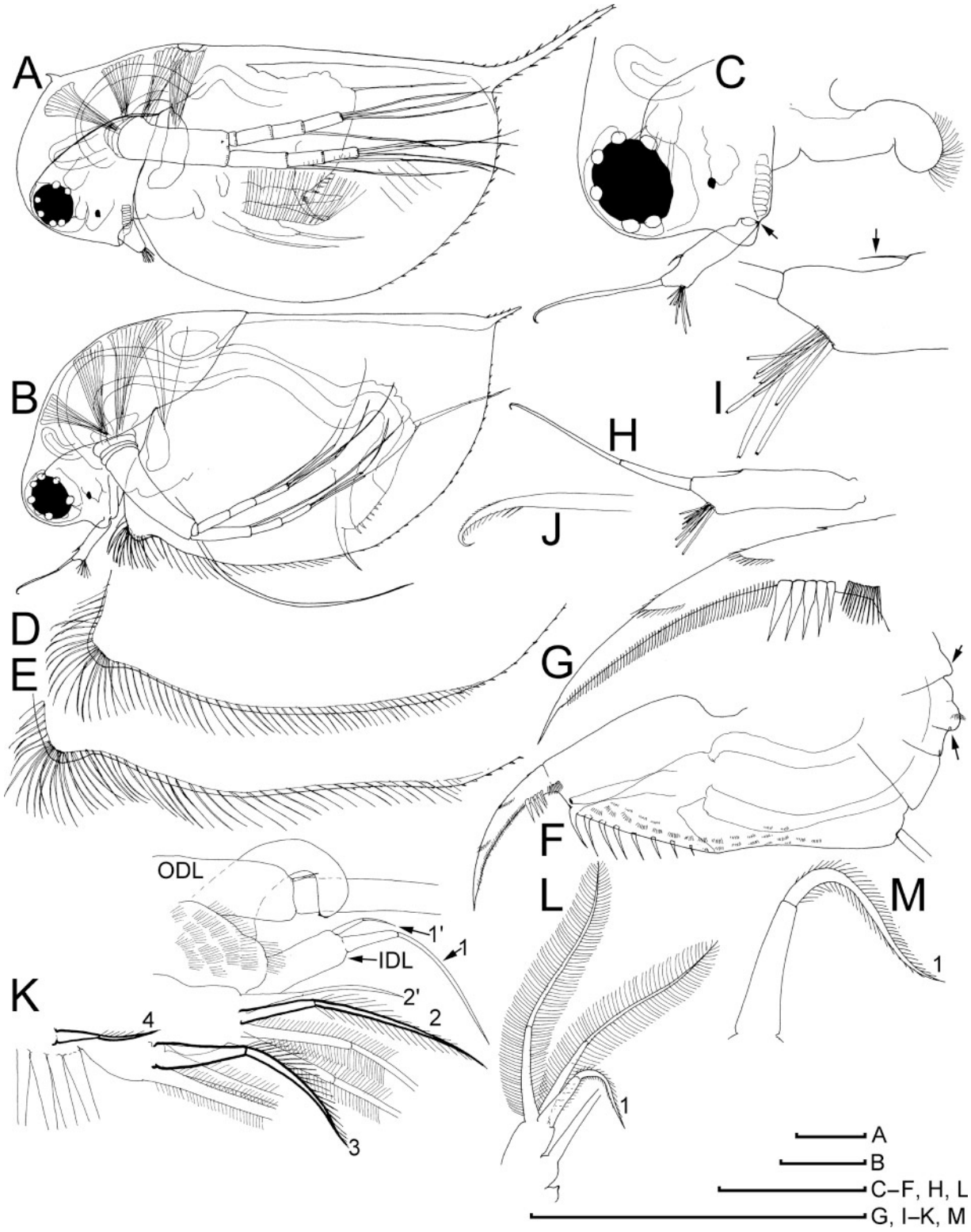

Fig. 5. *Daphnia sinevi* sp. nov., males from a pond in Avangard, Far East of Russia. (**A**) Juvenile male of instar I. (**B**) Adult male. (**C**) Head, lateral view. (**D**, **E**) Ventral margin of valve. (**F**, **G**) Postabdomen and postabdominal claw. (**H**–**J**) Antenna I, its middle portion and tip of male seta (flagellum). (**K**) Limb I. (**L**, **M**) Inner distal portion of limb II and its stiff seta. Scale bars: 0.1 mm.

Differential diagnosis

See Table III. The poorly studied *D. morsei* Ishikawa, 1895, is likely the closest congener of *D. sinevi* sp. nov., but the former differs from the latter in having a reduced rostrum and a strongly incurved preanal margin of the postabdomen in males. These characters are species specific in *Daphnia* and strongly support the distinctness of *D. sinevi* sp. nov. from *D. morsei*. Presently, the discrimination of these two species based solely on parthenogenetic females is difficult because *D. morsei* needs a detailed redescription.

Except for the congeners listed in the Table III, only a single *curvirostris*-like species has been described from Asian Pacific Coast, *Daphnia whitmani* Ishikawa, 1895. *Daphnia whitmani* differs from *D. sinevi* in that (i) the female has no projection basally to antenna I, (ii) the ephippium does not include the posteriormost portion of valves and (iii) the male bears a large sensory seta, reaching the distal end of the distal projection on antenna I.

Distribution

The known distribution of *D. sinevi* is the type locality [Avangard, Nakhodka Area, Russia (42°48′N, 132°53′E)].

DISCUSSION

We found nuclear and mtDNA evidence that the *D. curvirostris* complex is monophyletic and that at least four lineages exist in the eastern Palearctic: *D. curvirostris*, *D. tanakai*, *D. sinevi* sp. nov. and *D.* sp. from Ootori-Ike (Honshu, Japan). The species from Ootori-Ike is similar to *D. morsei* Ishikawa, 1895, but we have deferred name assignment and descriptions until specimens from the type locality can be examined.

Additional Palearctic species that might be confused with the *D. curvirostris* complex are *D. mitsukuri* Ishikawa, 1896, and *D. whitmani* Ishikawa, 1895. However, *D. mitsukuri* is clearly part of the *Daphnia obtusa* complex, as it possesses setae on the ventral margin of its valves, whereas *D. whitmani* is *curvirostris*-like but remains an *incerta sedis*. *Daphnia whitmani* has been regarded as a junior synonym of *D. obtusa* (Uéno, 1927) and of *D. pulex* (Tanaka, 1997). The high diversity of the *D. curvirostris* complex in the eastern Palearctic may be, in part, due to the lack of glaciation in this region. Ancient lineages may be preserved and recolonize adjacent areas from such refugia. Still, records of *D. curvirostris* s. str. from the Far East need morphological and genetic verification; the closest confirmed localities with *D. curvirostris* are from the vicinity of Lake Baikal (Glagolev, 1995) and from northwest Alaska (Ishida *et al.*, 2006).

We found that, despite being nested within the traditional *longispina* group, the *D. curvirostris* clade contains a surprising new suite of *pulex*- and *longispina*-like characters. *Daphnia sinevi* sp. nov., for example, possesses an emergent body of antenna I (a condition previously known only from the *pulex* group). Also, *D. sinevi* possesses a well-developed male rostrum as in *D. curvirostris*. The neckteeth in *D. sinevi* are similar to those in *D. dentifera*,

Table III: Morphological differences between Daphnia curvirostris, Daphnia tanakai, Daphnia sinevi sp. nov. and Daphnia morsei

Character	*D. curvirostris*	*D. tanakai*	*D. sinevi* sp. nov.	*D. morsei* (after Ishikawa, 1895a)
Female				
Rostrum	Long, with bent tip	Medium-sized	Medium-sized	Medium-sized
Rostrum subdividing into two lobes by a 'line' of prerostral fold	+	–	+	+
Posterior margin of head with a prominence proximally to antenna I	Moderate	Absent	Strong	Moderate
First and second abdominal projections very long	+	–	–	+
Second pecten consisting of teeth	10–14 large teeth	Variable	6–7 large teeth	Variable
Body of antenna I completely reduced	+	+	–	–
Aesthetascs projected behind tip of rostrum	–	–	+	–
Postero-dorsal angle of valves incorporated into ephippium	+	–	+	+
Male				
Rostrum (well-defined)	+	–	+	–
Postabdomen with strongly concave preanal margin	–	–	–	+
Antenna I, sensory seta reaches the tip of postaesthetasc projection	+	–	–	–
Limb I, setae 2 and 2′ large	–	+	+	?
Limb I, seta 3 large	–	+	+	?

whereas other *curvirostris*-like species lack neckteeth. Could the unusual combination of morphological characters in *D. sinevi* indicate hybrid ancestry? The hybridization hypothesis predicts that the mtDNA should match the maternal parent and that the nuclear alleles should group with each hybrid parent lineage. However, the mtDNA (*ND2*) of *D. sinevi* clearly is an ancient separate lineage with no closely related sequences. The nuclear DNA also lacks a heterozygous hybrid signature. Instead, the nuclear *D. sinevi* sequences are unique and group together, not with separate putative parent lineages. Moreover, to obtain the suite of morphological characters in *D. sinevi*, hybridization would have to occur between two of the deepest lineages in *Daphnia* (*pulex* and *dentifera*), which possess different chromosome complements. Nonetheless, we cannot rule out that *D. sinevi* resulted from an ancient introgression event involving extinct species or lineages not sampled in our analysis. The concordance of unique male morphological characters, divergent nuclear DNA and divergent mtDNA (i.e. at the subgeneric level of divergence from *D. curvirostris*) indicates that *D. sinevi* is likely a unique species.

As neckteeth are present only in distantly related nonsister species (*D. pulicaria*, *D. dentifera* and *D. sinevi*) (Fig. 2), they likely originated at least three times in *Daphnia*. Beaton and Hebert (Beaton and Hebert, 1997) proposed developmental homology for the *pulex* and the *longispina* types (*D. dentifera*) of roseate neckteeth, it is unclear whether the *curvirostris* type of neckteeth (which lack the roseate morphology) is developmentally homologous. The results show that even unusual defensive structures (i.e. not helmets) can arise independently in *Daphnia* and provide further caution in using defensive structures for systematic characters.

Another variable character in the *D. curvirostris* complex that has confused relationships is the postabdominal claw pecten. For example, Ishikawa (Ishikawa, 1895a: 139) noted that, in *D. morsei*, the basal portion of the postabdominal claw is 'provided with two sets of closely set teeth, whose number varies greatly according to different individuals'. Ishida *et al.* (Ishida *et al.*, 2006) reported the same variability for *D. tanakai*. Because the claw pectens have been considered as a definitive taxonomic character, several populations of *D. tanakai* and *D. morsei* may have been erroneously assigned to *D. pulex* or *D. longispina*. For example, some of specimens schematically pictured from Kurile Islands (Uéno, 1938: Fig. 8) and North China (Uéno, 1940: Figs 40–42) are similar to *D. tanakai* and *D. morsei*. Variable claw pecten morphologies have been linked to habitat types in *Daphnia* (Ishida *et al.*, 2006) and to nutrition in *Ceriodaphnia* (Berner, 1986). However, controlled experiments are needed to determine the environmental basis of pecten morphology. The claw pecten character also has been used as the major distinction for main groups of *Daphnia* s. str., *pulex* and *longispina* (Wagler, 1936; Glagolev, 1995). But, early DNA sequence data placed *D. curvirostris* with large teeth in the second pecten among the *longispina* group (although with weak statistical support) (Lehman *et al.*, 1995; Colbourne and Hebert, 1996). The finding of *D. tanakai* (Tanaka and Tominaga, 1986; Ishida *et al.*, 2006) with variable morphology of the second pecten makes the morphological discrimination of the *pulex* and the *longispina* groups intractable.

Because weakly supported branches are a large source of phylogenetic incongruence in empirical data (Taylor and Piel, 2004), efforts should be made to obtain data that provide strong support for the questions of interest. Although *ND2* data dramatically improved support for many of the deeper clades in *Daphnia* over 12S rRNA and mitochondrial cytochrome oxidase subunit I (COI) (Ishida *et al.*, 2006), *ND2* failed to resolve the *curvirostris* complex. Here, we found that only Bayesian analysis (PP = 99) provided robust support for the monophyletic placement of *D. curvirostris* with *ND2*. In contrast, all analyses of the *HSP90* gene yielded strong resolution for the *D. curvirostris* clade. The poor performance of ML/MP for *ND2* compared with Bayesian analyses may indicate either a better fitting model when codons are partitioned into three sites or the demonstrated reduction in 'false' weak support for Bayesian analysis (Taylor and Piel, 2004). With both the *HSP90* and *ND2* data, there are at least five divergent species groups in *Daphnia* (excluding the subgenus *Ctenodaphnia*). Some of these clades (*Daphnia longiremis*/*Daphnia cristata*, *D. laevis* and *D. curvirostris*) possess similar within-clade divergences to the most divergent members of the subgenus *Daphnia sensu* Colbourne and Hebert (1996) (*pulicaria*/*ambigua*). So, the present subgeneric distinctions likely reflect neither morphological evolution nor the major evolutionary lineages within *Daphnia*. More genetic information and fossil evidence would help improve the understanding of the rates of molecular divergence and age of the major clades of *Daphnia*. We conclude that, even when the rates of amino acid change are conserved (as in *HSP90*), nuclear protein-coding genes will be informative and complementary for estimating an evolutionary framework of the major clades and morphological traits of *Daphnia*.

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