The 18S rDNA sequences support polyphyly of the Hypsibiidae (Eutardigrada)

Ernst KIEHL, Hieronymus DASTYCH1), Jochen D’HAELSE and Hartmut GREVEN*

Institut für Zoomorphologie und Zellbiologie, Universität Düsseldorf, Universitätsstr,1, D-40225 Düsseldorf, Germany
1)Biozentrum Grindel und Zoologisches Museum. Universität Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany
*e-mail corresponding author: grevenh@uni-duesseldorf.de

ABSTRACT

To extend data on 18S rDNA gene phylogeny within the Eutardigrada and to provide additional information on unclear taxonomic status of a glacier tardigrade Hypsibius klebelsbergi, gene sequences from seven tardigrade species of the family Hypsibiidae (Hypsibius klebelsbergi, Hypsibius cf. convergens 1, Hypsibius cf. convergens 2, Hypsibius scabropygus, Hebesuncus conjungens, Isohypsibius cambrensis, Isohypsibius granulifer) were analysed together with previously published sequences from ten further eutardigrade species or species groups. Three distinctly separated clades within the Hypsibiidae, 1) the Ramazzottius - Hebesuncus clade, 2) the Isohypsibius clade (Isohypsibius, Halobiotus, Thulinii), and 3) the Hypsibius clade (Hypsibius spp.) have been obtained in each of four phylogenetic trees recovered by Maximum Parsimony, Neighbour Joining, Minimum Evolution and UPBMA. Hypsibius klebelsbergi has been located always within the Hypsibius clade. The detailed sister group relationship was not resolved adequately, but there is robust support for a sister group relationship between the Hypsibius clade and the remaining clades. We cannot exclude that the Ramazzottius - Hebesuncus clade is a sister group of the Macrobiotus clade. Our findings suggest polyphyly of the Hypsibiidae, and thus multiple evolutions of some structures currently applied as diagnostic characters (e.g., claws, buccal apophyses).

Key words: Tardigrada, Hypsibiidae, polyphyly, 18S rRNA, morphological characters

1. INTRODUCTION

Previous studies using 18S rDNA gene sequences have suggested that Tardigrada 1) belong to the Ecdysozoa and 2) are the sister group of the Arthropoda (Garey et al. 1996; Giribet et al. 1996; Moon & Kim 1996; Aguinaldo et al. 1997; Garey 2001). These suggestions are largely congruent with morphological data (e.g. arthropod relationships were discussed in detail already by Marcus 1929) and elaborated with additional details in numerous subsequent articles (e.g., Dewel & Dewel 1997; for review see Schmidt-Rhaesa et al. 1998). Within the Tardigrada the classes Heterotardigrada and Eutardigrada are well established by morphological characters (for review of the older literature see Marcus 1929) and the monophyly of both classes as well as the ancestral Apochela and the derived Parachela (see Schuster et al. 1980) among the Eutardigrada appears to be supported by molecular data (Garey et al. 1999; Jørgensen & Kristensen 2004; Nichols et al. 2006). Currently, relationships within and between the various tardigrade families, based mainly on morphological characters and cladistic analyses, exist for the Echiniscidae (Kristensen 1987; Jørgensen 2000) and Macrobiotidae (Guidetti & Bertolani 2001). More recently Nichols et al. (2006) studied morphological data and 18S rDNA to evaluate congruence of morphological and molecular data at the family level and Guidetti et al. (2005) within the Macrobiotidae.

In a study on the biology and diagnostic morphological characters of the unique glacier dwelling eutardigrade Hypsibius klebelsbergi Mihelčič, 1959, it was suggested that some structural traits in this species (e.g. slightly asymmetrical, concave and flattened apophyses of the mouth tube, claws in shape between Isohypsibius- and Hypsibius-type with shortened main branches and their flattened accessory spines) actually did not allow a clear assignment to the genus Hypsibius (see Dastych et al. 2003).

This prompted us to analyse 18S rDNA gene sequences of some Hypsibiidae including H. klebelsbergi to prove the above suggestion regarding relationships of this species within the family and the Hypsibiidae in general, and to extend the taxa sampling for improvements of future molecular analyses of Eutardigrada. In contrast to Jørgensen & Kristensen (2004), our molecular data indicate that the Hypsibiidae might be polyphyletic.

2. METHODS

Specimens of Isohypsibius cambrensis Morgan, 1976 (status nov.: in preparation), Isohypsibius granulifer Thulin, 1928, Hebesuncus conjungens (Thulin, 1911), Hypsibius scabropygus Cuènot, 1929, Hypsibius cf. convergens 1, Hypsibius cf. convergens 2 and H. klebelsbergi were collected at several localities in Germany and Austria (see Tab. 1). The species colonizing each sample were firstly diagnosed on permanent mounted specimens at maximum magnification.
Tab. 1. List of tardigrade species collected for the study and their origin.

| Species               | Substrate                                                                 | Locality / date                                | m (a.s.l.) | Geogr. Coordinates |
|-----------------------|---------------------------------------------------------------------------|-----------------------------------------------|------------|--------------------|
| Isohypsibius cambrensis| Mosses on a low, concrete fence near the Zoological Museum               | Germany, Hamburg / 17.2.2005                  | 8          | 9°58’37’’ E, 53°34’02’’ N |
| Isohypsibius granulifer| Algae from pond in a garden, coll. M. Preuß                              | Germany, Henstedt-Ulzburg / 27.3.2005         | 37         | 9°59’21’’ E, 53°45’18’’ N |
| Hypsibius scabropygus  | Bryophytes from silicate rocks                                            | Austria, the Ötzal Alps, Mt. Festkogel / 20.6.2003 | 3020       | 11°03’03’’ E, 46°51’’ 08’’ N |
| Hypsibius cf. convergens 1 | Edge of oak forest, wet mosses on soil, near a brook            | Germany, Siebenichen / 21.2.2005              | 12         | 10°37’36’’ E, 53°30’36’’ N |
| Hypsibius cf. convergens 2 | Mosses on a low, concrete wall at the Zoological Museum           | Germany, Hamburg / 14.3.2006                   | 8          | 9°58’37’’ E, 53°34’02’’ N |
| Hypsibius klebelsbergi | Cryoconite holes                                                          | Austria, the Ötzal Alps, glacier Langtöllerfer / 29.8.2005 | 2580       | 11°00’26’’ E, 46°48’02’’ N |
| Hebesuncus conjugens   | Bryophytes from silicate rocks                                            | Austria, the Ötzal Alps, slope of Mt. Hangerer, NN ridge, alpine meadow / 31.8.2004 | 2620       | 11°00’57’’ E, 46°50’07’’ N |

(1200×). For molecular analysis living specimens from another sub-sample have been identified individually at 400× magnification (up to 2-3 individuals on each slide, under cover glass) and transferred with micro-pipette into 100% ethanol.

Five to 10 specimens were used. DNA was prepared according to manufacturer’s specifications with the DNeasy tissue kit (Quiagen) with an over night incubation step with proteinase K. 18S rDNA was amplified with the primer pairs SSU_F_04 (CATTCTTGGCAAATGCTTTCG); the resulting amplification was carried out for 15 min initial denaturing at 94 °C followed by 35 cycles with 30 sec denaturing at 94 °C, 60 sec annealing at 50 °C, and 180 sec extension at 72 °C. The program ended with 20 min incubation at 72 °C. The sample volume was 25 µl (sample concentrations of 0.5 mM each dNTP, 0.5 µM each primer, 6 mM Mg**, 1.25U Taq (Biomaster), 1 µl template-DNA in 1x PCR-buffer (Biomaster)). Sequencing was carried out in both directions. PCR-products were sequenced by Seqlab (Göttingen).

The sequences of the 18S rDNA of the following species were taken from Genbank data. Echiniscus viridissimus Péterfi, 1956 (AF056024) (Garey et al. 1996), Halobiotus stenostomus (Richters, 1908) (AY582121) (Jørgensen & Kristensen 2004), Macrobiotus hufelandi group (X81442) (Giribet et al. 1996), Macrobiotus areolatus group (U32393) (Jørgensen et al. 1996, named Macrobiotus tonolli Ramazzotti, 1956) Macrobiotus echinogenitus Richters, 1904 in the figures in Nichols et al. 2006), Milnesium tardigradum (Doyère, 1840)(AY582120) (Aguinaldo et al. 1997), Ramazzottius oberaeae (Doyère, 1840), (AY582122) (Jørgensen & Kristensen 2004), Richtersius coronifer (Richers, 1903) (AYS821239) (Jørgensen & Kristensen 2004), and Thulinius stephanieae (Pilato, 1974) (AF056023) (Garey et al. 1996; Thulina renamed Thulinius by Bertolani 2003).

Sequences were aligned with Clustalw (Thompson et al. 1994) and phylogenetic trees were calculated using Maximum Parsimony (MP), Neighbour Joining (NJ), Minimum Evolution (ME) and Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) analyses of corresponding regions (ranging from 755 to 849 bps) of the first half of the 18S rDNA were performed using MEGA2 (Kumar et al. 2001). Confidence levels were evaluated by bootstrap analysis with 500 and 1000 bootstrap replicates. The heterotardigrade Echiniscus viridissimus Péterfi, 1956 was used as out-group.

GenBank accession numbers of the partial 18S rDNA genes obtained in the present investigation are: Hebesuncus conjugens (AM 500646), Hypsibius cf. convergens 1 (AM 500647), Hypsibius klebelsbergi (AM 500648), Hypsibius scabropygus (AM 500649), Hypsibius cf. convergens 2 (AM 500650), Isohypsibius granulifer (AM 500651), Isohypsibius cambrensis (AM 500652).

3. RESULTS

Cladograms recovered from Maximum Parsimony, Neighbour Joining, Minimum Evolution and Unweighted Pair-Group Method using Arithmetic Averages revealed four clades among the Parachela identical in all trees, but partly with unresolved relationships (Figs 1a and 1e). Within the Eutardigrada monophyly of the Parachela is well supported (bootstrapping values 96-100%).

With the exception of the outgroup and the Apochela (Milnesium tardigradum), the four clades identified are 1) the Isohypsibius clade (Isohypsibius, Halobiotus, and Thulinius), 2) the Macrobiotus clade (Macrobiotus, Richtersius), 3) the Ramazzottius – Hebesuncus clade, and 4) the Hypsibius clade (all Hypsibius species examined). Bootstrap support of the four clades varied from 73% to 100% (Fig. 1).

All trees show a high bootstrap value (96-100%) supporting a sister group relationship between the Hyp-
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'sibius clade and the remaining clades identified in the Parachela. Relationships between the Ramazzottius - Hebesuncus clade, the Macrobiotus and the Isohypsi-
bius clade were not resolved by Neighbour Joining (Fig.
1b), Maximum Parsimony (Fig. 1c) and Minimum
Evolution (Fig. 1b). In the UPGMA-tree the Isohypsi-
bius clade appears to be the sister group of a Macro-
biotus – Ramazzottius – Hebesuncus clade (bootstrap
value 83%) and the Ramazzottius - Hebesuncus clade a
sister group of the Macrobiotus clade (bootstrap value
99%) (Fig. 1a).

4. DISCUSSION

Analyses of the 18S rDNA gene sequences taken
from Genbank data correspond (as expected) with those
presented by Garey *et al.* (1999), Jørgensen & Kris-
tensen (2004) and Nichols *et al.* (2006), i.e. support of
the monophyly of Hetero- and Eutardigrada and sister
group relationships of Parachela and Apochela. Analysis
of the broader taxa sampling, however, that covers
various previously analysed Hypsibiidae (*H. stenosto-
tomus*, *T. stephaniae*, *R. oberhaeuseri*) and those
examined newly herein (*I. cambrensis*, *I. granulifer*, *H. con-
jungens* and four *Hypsibius* species: *H. klebelsbergi*, *H.
cf. convergens* 1, *H. cf. convergens* 2 and *H. scabropy-
gus*) strongly suggests polyphyly of the Hypsibiidae.
Jørgensen & Kristensen (2004) suggested monophyly of
this eutardigrade family, based on the analysis of Thu-
linius and Halobiotus species only; an undetermined
Hypsibius sp. also considered by these authors very

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**Fig. 1.** a-c: Phylogenetic trees of Eutardigrada based on the comparison of corresponding pieces of 18S rDNA gene sequences recovered for UPGMA (a), Neighbour Joining and Minimum Evolution (b), and Maximum Parsimony (c). In b are the numbers above each fork the bootstrap values for Minimum Evolution, below are the numbers for Neighbour Joining. Branches are not to
scale. Numbers are bootstrap values. For further explanation see text.
probably is Thulinius (see Garey et al. 1999). Further, they inserted R. oberhaeuseri in the Macrobiotidae without any comment (see i.e., page 667, Tab. 1), where this species forms a clade with the Macrobiotidae with bootstrap values of 86 and 96% (see Jorgensen & Kristensen 2004, Fig. 2). Our results obtained by UPGMA analysis are in congruence with this finding. Independently from the procedure of analysis we always identified three distinctly separated clades currently included in the Hypsibiidae, i.e., the Ramazzottii - Hebesuncis clade (perhaps a sister group of the Macrobiotus clade: see above), the Isohypsibiidae clade and the Hypsibiidae clade, including H. klebelsbergii. The close relationship between Macrobiotus and Ramazzottii (Hebesuncis) is inconsistent from the morphological point of view, as some characters of the bucco-pharyngeal apparatus and claws suggest stronger affiliation between Macrobiotus and Isohypsibiidae (e.g. Pilato 1969).

Jorgensen & Kristensen (2004) analysed 185 rDNA of two Hypsibiidae (Thulinius and Halobiotus species; regarding Ramazzottii see the comments given above), which have been inserted in the Isohypsibiidae clade due to the new dataset presented herein. Interestingly, the close relationship of Thulinius and Isohypsibiidae is corroborated by similar analyses using the sequences of translational elongation factor EF2 and Pol II (RNA-Polymerase II) (Regier et al. 2004).

In a few articles the most important morphological characters of tardigrade taxa were mapped onto the gene tree (see Garey et al. 1999; Nichols et al. 2006) showing a considerable congruence of morphological and molecular data even at the family level (see Nichols et al. 2006). However, the Hypsibiidae were represented there (l.c.) only by T. stephaniae and Hypsibius dujardini (Doyère, 1840).

Claw structure and features of the buccal apparatus have been used for a long time to classify Eutardigrada (e.g., Thulin 1928; Marcus 1929; Ramazzotti 1972; Pilato 1969, 1982; Schuster et al. 1980; Bertolani & Kristensen 1987; Guidetti & Bertolani 2001; Guidetti et al. 2005). If the putative polyphyly of the Hypsibiidae should be confirmed by further molecular (and morphological) studies including further genera, e.g. Doryphoribius, the family would need to be re-diagnosed. We do not want to discuss this problem in detail herein, but, currently used key characters, including those at the generic level, show problems with clear application of these features. The difficulties are partly reflected in two recent general identification keys for tardigrades by Nelson (2001) and Nelson & McInnes (2002). The Macrobiotidae and Murrayidae recently separated from the Macrobiotidae (Guidetti et al. 2005) are clearly distinguished by their claws; the two double claws per leg are of similar size and shape and the sequence of the branches is secondary, primary, primary, secondary (2112). The Hypsibiidae share asymmetrical claws (sequence of the claw branches is alternate with the sequence: secondary, primary, secondary, primary (2121)) with the Calohypsibiidae, Eohypsibiidae and Microhypsibiidae. However, current taxonomy and our gene trees indicate multiple evolutions of asymmetrical claws. Within the Hypsibiidae an Isohypsibiidae-claw (present in the members of our Isohypsibiidae clade), i.e., where the secondary branch of the claw forms a right angle (often difficult to define precisely) with its base and a Hypsibiidae-claw, where the secondary claw is continuous with its base forming (more or less distinctly) an arc, are distinguished. This type is present in our Hypsibiidae clade and the Ramazzottius – Hebesuncis clade. However, within the Hypsibiidae-claw, two subtypes are differentiated, (see e.g., Bind & Pilato 1986; Nelson 2001; Nelson & McInnes 2002). In view of our findings these subtypes deserve further attention. Other diagnostic features such as peribucal lamellae and the buccal apparatus (symmetry and shape of anterior apophyses of the mouth tube) are currently not suitable to distinguish our clades. Interestingly, Ramazzottii - Hebesuncis (as well as the Macrobiotidae) lay ornamented eggs freely on the substrate, whereas "Hypsibiidae" (from the Hypsibiidae and Isohypsibiidae clade) deposit smooth eggs mostly in their exuviae (H. klebelsbergii seems to lay eggs freely or in the exuviae and Acutuncus antarcticus (Richters, 1904) lays free, partly ornamented eggs).

We have presented herein data derived from the 18S rDNA. However, the suitability of this genetic marker is limited to relatively ancient relationships (e.g. Abouheif et al. 1998). Therefore, more independent data from several molecules are required to ascertain phylogenetic trees. Studies on the Internal Transcribed Spacer 1 and 2 are in progress to include more rapidly evolving sequences.

REFERENCES
Abouheif, E., R. Zardoya & A. Meyer. 1998. Limitations of metazoan 18S rRNA sequence data: Implication for reconstructing a phylogeny of the animal kingdom and inferring the reality of the Cambrian explosion. J. Mol. Evol., 47: 394-405.
Aguinaldo, A.M., J.M. Tubeville, L.S. Linford, M.C. Rivera, J.R. Garey, R.A. Raff & J.A. Lake. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. Nature, 387: 489-493.
Bertolani, R. 2003. Thulinius, new generic name substituting for Thulini Bertolani, 1981 (Tardigrada, Eutardigrada). Zootaxa, 314: 1-4.
Bertolani, R. & R. Kristensen. 1987. New records of Eohypsibiidae nadiniae Kristensen, 1982, and revision of the taxonomic position of two genera of Eutardigrada (Tardigrada). In: R. Bertolani (Ed.), Biology of Tardigrades. Selected Symposia and Monographs U.Z.I., 1. Mučchi Editore, Modena, Italy: 339-372.
Binda, M.G. & G. Pilato. 1986. Ramazzottii, nuovo genere di Eutardigrada (Hypsibiidae). Animalia, 13: 159-166.
Dustych, H., H.-J. Kraus & K. Thaler. 2003. Redescription and notes on the biology of the glacier tardigrade Hypsibius klebelsbergii Mihelčič, 1959 (Tardigrada), based on material from the Otztal Alps, Austria. Mitt. hamb. zool. Mus. Inst., 100: 73-100.
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Dewel, R.A. & W.C. Dewel. 1997. The place of tardigrades in arthropod evolution. In: R. Fortey & R.H. Thomas (Eds), Arthropod relationships. The Systematic Association, 55. Chapman & Hall, New York: 109-125.

Garey, J.R. 2001. Ecdysozoa: The relationship between Cycloneuralia and Panarthropoda. Zool. Anz., 240, 321-330.

Garey, J.R., M. Krotec, D.R. Nelson & J. Brooks. 1996. Molecular analysis supports a tardigrade-arthropod association. Invertebr. Biol., 115: 79-88.

Garey, J.R., D.R. Nelson, L.M. Mackey & J. Li. 1999. Tardigrade phylogeny: congruence of morphological and molecular evidence. Zool. Anz., 238: 205-210.

Giribet, G., S. Carranza, J. Baguna, M. Riutort & C. Ribeira. 1996. First molecular evidence for the existence of a tardigrada+arthropoda clade. Mol. Biol. Evol., 13: 76-84.

Guidetti, R. & R. Bertolani. 2001. Phylogenetic relationships in the Macrobiotidae (Tardigrada: Eutardigrada: Parachela). Zool. Anz., 240: 321-330.

Garey, J.R., M. Krotec, D.R. Nelson & J. Brooks. 1996. Molecular analysis supports a tardigrade-arthropod association. Invertebr. Biol., 115: 79-88.

Guidetti, R., A. Gandolfi, V. Rossi & R. Bertolani. 2005. Phylogenetic analysis of Macrobiotidae (Eutardigrada, parachela): a combined morphological and molecular approach. Zool. Scripta, 34: 235-244.

Jørgensen, A. 2000. Cladistic analysis of the Echiniscidae (Tardigrada). Mem. Ist. Idrobiol., 14: 1-732.

Ramazzotti, G. 1969. Il phylum Tardigrada (2 ed.). Mem. Ist. Idrobiol., 14: 1-732.

Regier, J.C., J.W. Shultz, R.E. Kambic & D.R. Nelson. 2004. Robust support for tardigrade clades and their ages from three protein-coding nuclear genes. Invertebr. Biol., 123: 93-100.

Schuster, R.O., D.R. Nelson, A.A. Grigarick & D. Christenberry. 1980. Systematic criteria of the Eutardigrada. Trans. Am. Microsc. Soc., 99: 284-303.

Thulin, G. 1928. Über die Phylogenie und das System der Eutardigraden. Hereditas, 11: 207-266.