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FONDAZIONE EDMUND MACH

Cryptic diversity of *Keratella cochlearis* – genetical, morphological and demographic aspects

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Summary

Biodiversity is under threat in recent decades, with many natural habitats irreversibly disappearing due to global warming and human activity. Our perception of species loss highly depends on an accurate species estimate. However, occurrence of cryptic species (i.e. distinct species that are impossible or difficult to distinguish based on their morphology) hinders a correct assessment of biodiversity. Cryptic species have been described for rotifers of the class Bdelloidea and Monogononta. Rotifers of the class Monogononta are widespread in freshwater lakes all over the world and can serve as model organisms for speciation and adaptation.

The main aim of this thesis was to investigate and describe the genetic diversity of one of the most common freshwater rotifer - *Keratella cochlearis* - in relation to its morphological variability. Beside the assessment of genetic diversity, a detailed study of *K. cochlearis* life cycle and reproductive strategy was performed.

The results of the first study demonstrated that based on the cytochrome c oxidase subunit 1 (COI) gene different putative evolutionary significant units (ESU; a.k.a. cryptic species) can be delimited in *Keratella cochlearis* (I). Based on morphology, two ESUs can be delimited from the other six ESUs found. We also reported on co-occurrence of different putative ESUs of *K. cochlearis* in the same lakes, and presented the first SEM pictures of *K. cochlearis* females showing some detailed morphological characteristics.

Life histories and demographic parameters differences between various haplotypes of *K. cochlearis* were determined in the second study (II). Several differences between life history traits and demographic parameters of haplotypes were found corroborating their status as cryptic species and demonstrating that genetic diversity of *K. cochlearis* is also reflected in demographic diversity. Additionally, morphologically deformed females occurring during the life table experiment were documented and photographed for the first time in this species. Moreover, the first case of an amphoteric female (producing both males and females) in *K. cochlearis* was reported.

In the third study (III), mitonuclear discordance in three rotifer species complexes was assessed. Mitonuclear discordance hinders the assessment of species delimitation based on only one gene (single-locus). Discordance between mitochondrial and nuclear phylogenies was reported for three rotifer species complexes (*K. cochlearis*, *Polyarthra dolichoptera*, *Synchaeta pectinata*) with different levels of discordance between the mitochondrial COI and the nuclear ITS gene. The results corroborated the previous description of two ESUs in *K. cochlearis*.

During our studies on *K. cochlearis* males, we developed a method to film zooplankton in general and rotifers specifically (IV). We connected a commercial single-lens reflex camera to a microscope and presented an affordable system with widely available components for filming. In filming male-female interactions of *Brachionus angularis*, our film showed a thread-like structure linking male and female. However, the purpose of this structure remained unclear.

In conclusion, this PhD provided evidence for a high genetic and morphological diversity of *K. cochlearis*. Existence of a species complex of *K. cochlearis* was corroborated by mitochondrial and nuclear genetic information. This high genetic diversity in *K. cochlearis* was reflected to some extent in life histories and morphology. First videos of *K. cochlearis* males and of *B. angularis* males interaction with females were presented.

Zusammenfassung

Die biologische Vielfalt ist in den letzten Jahrzehnten bedroht, da viele natürliche Lebensräume durch die Erderwärmung und die menschliche Aktivität irreversibel verschwinden. Unsere Wahrnehmung dieses Rückganges hängt stark von einer genauen Schätzung der Artenvielfalt ab. Das Auftreten kryptischer Arten (d. h. verschiedener Arten, die aufgrund ihrer Morphologie unmöglich oder schwierig zu unterscheiden sind) verhindert jedoch eine genaue Abschätzung der biologischen Vielfalt. Kryptische Arten wurden für Rädertierchen der Klasse Bdelloidea und Monogononta beschrieben. Rädertierchen der Klasse Monogononta sind in Süßwasserseen auf der ganzen Welt verbreitet und können als Modellorganismen für Artbildung und Anpassung dienen.

Das Hauptziel dieser Arbeit war es die genetische Diversität eines der häufigsten Süßwassertierchen - *Keratella cochlearis* – zusammen mit dessen morphologischer Vielfalt zu untersuchen und zu beschreiben. Neben der Bewertung der genetischen Variabilität wurde eine detaillierte Studie des Lebenszyklus und der Reproduktionsstrategie von *K. cochlearis* durchgeführt.

Die Ergebnisse der ersten Studie zeigten, dass basierend auf dem Cytochrom c-Oxidase-Untereinheit-1 (COI) -Gen verschiedene mutmaßliche evolutionär signifikante Einheiten (ESU; a.k.a. kryptische Spezies) in *Keratella cochlearis* (I) unterschieden werden können. Zwei ESUs können von den anderen sechs gefundenen ESUs aufgrund ihrer Morphologie abgegrenzt werden. Wir berichteten auch über das gemeinsame Auftreten verschiedener mutmaßlicher ESUs von *K. cochlearis* in denselben Seen und präsentierten die ersten SEM-Bilder von *K. cochlearis* Weibchen mit morphologischen Details.

Die Unterschiede im Lebenszeit und demographische Parameter zwischen verschiedenen Haplotypen von *K. cochlearis* wurden in der zweiten Studie (II) bestimmt. Mehrere Unterschiede zwischen den Merkmalen der Lebenszeit und den demographischen Parametern von Haplotypen bestätigten ihren Status als kryptische Arten und zeigten, dass sich die genetische Vielfalt von *K. cochlearis* auch in der demografischen Vielfalt widerspiegelt. Zusätzlich wurden morphologisch deformierte Weibchen erstmals dokumentiert und fotografiert, die während des Lebenszeit-Experimentes vorkamen. Darüber hinaus wurde der erste Fall eines amphoteren Weibchens (welches sowohl Männchen als auch Weibchen produziert) in *K. cochlearis* berichtet.

In der dritten Studie (III) wurde die mitonukleare Diskordanz in drei Rotatorenkomplexen untersucht. Die mitonukleare Diskordanz erschwert die Abgrenzung der Arten auf der Basis von nur einem Gen (single-locus). Für drei Rotatorenkomplexe (*K. cochlearis*, *Polyarthra dolichoptera, Synchaeta pectinata*) wurde eine Dissonanz zwischen mitochondrialen und nuklearen Phylogenien gefunden. Die Ergebnisse bestätigten die vorherige Beschreibung von zwei ESUs in *K. cochlearis*.

Während unserer Untersuchungen an *K. cochlearis*-Männchen entwickelten wir eine Methode, um Zooplankton im Allgemeinen und Rädertierchen im spezifischen zu filmen (IV).

Wir verbanden eine handelsübliche Spiegelreflexkamera mit einem Mikroskop und stellten ein erschwingliches System mit weit verbreiteten Komponenten für das Filmen vor. Beim Filmen der Interaktion zwischen Männchen und Weibchen von *Brachionus angularis* zeigte unser Film eine fadenförmige Struktur zwischen Männchen und Weibchen. Der Zweck dieser Struktur blieb jedoch unklar.

Zusammenfassend konnte mit dieser Doktorarbeit eine hohe genetische und morphologische Diversität von *K. cochlearis* nachgewiesen werden. Die Existenz eines Spezieskomplexes von *K. cochlearis* wurde durch mitochondriale und nukleare genetische Information bestätigt. Diese hohe genetische Diversität in *K. cochlearis* spiegelte sich zum Teil im Lebensverlauf und der Morphologie wider. Erste Videos von *K. cochlearis* Männchen und der Interaktion von *B. angularis* Männchen mit Weibchen wurden vorgestellt.

List of original articles with specified contribution to the articles

The PhD thesis includes a summary of the four articles on which this thesis is based. Articles are indicated by Roman numbers (I-IV) in the text.

I. Cieplinski, A., Weisse, T. and Obertegger, U. (2017). **High diversity in *Keratella cochlearis* (Rotifera, Monogononta): morphological and genetic evidence**. *Hydrobiologia, 796*(1), 145-159. doi: 10.1007/s10750-016-2781-z

Concept and experimental design were developed by U. Obertegger. Samples were collected by U. Obertegger and A. Cieplinski. Sample extraction, processing and creating morphology base was performed by A. Cieplinski. Data analyses were performed by U. Obertegger and A. Cieplinski. The article was written by A. Cieplinski together with T. Weisse and U. Obertegger.

II. Cieplinski, A., Obertegger, U. and Weisse, T. (2018)**. Life history traits and demographic parameters in the *Keratella cochlearis* (Rotifera, Monogononta) species complex**. *Hydrobiologia, 811*(1), 325-338. doi: 10.1007/s10750-017-3499-2

Concept and experimental design were developed by A. Cieplinski, T. Weisse, and U. Obertegger. Experiments were performed by A. Cieplinski. Data were analyzed by A. Cieplinski, U. Obertegger and T. Weisse. The article was written by A. Cieplinski together with T. Weisse and U. Obertegger.

III. Obertegger, U., Cieplinski, A., Fontaneto, D. and Papakostas, S. (2018). **Mitonuclear discordance as a confounding factor in the DNA taxonomy of monogonont rotifers**. *Zoologica Scripta 47*(1), 122-132. doi: 10.1111/zsc.12264

Concept and experimental design were developed by U. Obertegger. Data were collected by U. Obertegger and A. Cieplinski. Sequences were prepared by U. Obertegger and A. Cieplinski. Data analyses were performed by U. Obertegger, S. Papakostas and D. Fontaneto. The article was written by U. Obertegger together with S. Papakostas, D. Fontaneto and A. Cieplinski.

IV. Colangeli, P., Cieplinski, A**.** and Obertegger, U. (2016)**. Filming of zooplankton: a case study of rotifer males and *Daphnia magna***. *Journal of Limnology 75*(1), 204-209. doi: 10.4081/jlimnol.2015.1306

Concept and experimental design were developed by P. Colangeli and U. Obertegger. Rotifers were provided by A. Cieplinski. Constructing experimental set-up and recording videos was performed by P. Colangeli. The article was written by P. Colangeli together with U. Obertegger and A. Cieplinski.

1. Introduction

1.1 Species diversity in aquatic communities

In the past decades, the destruction of many natural ecosystems by human activity or by climate change resulted in rapid loss of many plant and animal species (Brooks et al., 2002; Brook et al., 2006). Knowledge on species diversity is much wider regarding well-studied and easily accessible areas of the world and in particular for macroscopic animals. However, species diversity studies are much less detailed in case of remote areas and in case of small, microscopic animals such as freshwater zooplankton. Small planktonic animals differ significantly from macroscopic animals in many ways; for example they usually have much shorter life span, higher fecundity, asexual reproduction and recurring periods of dormancy, which in some cases may lead to completely different diversity patterns than macroscopic animals (Fontaneto et al., 2009). The known number of environmental niches in freshwater environments could be much higher than on land due to the spatial heterogeneity (known as the paradox of the plankton; Hutchinson, 1961). Furthermore, dispersal between freshwater habitats (even on long distances) by wind or waterfowl (Maguire, 1959, 1963; Malone, 1965) is facilitated due to production of resting eggs by many microscopic animals (Bohonak & Jenkins, 2003). The easiness of long-distance dispersal may lead to a situation where all species are found everywhere, known as the "Everything is everywhere but the environment selects" hypothesis (Baas-Becking, 1934). Because for many zooplankton organisms distances between water reservoirs are not real barriers, lack of geographical isolation may greatly influence the process of evolutionary speciation, with environment of water bodies being often the shaping factor for population compositions and dynamics (Segers & Smet, 2008). However, it appears that migrations from one water body to another are not necessarily the main factor shaping species assemblages of rotifers. Gómez et al. (2002) described for several species of the *Brachionus plicatilis* complex low levels of gene flow, which is surprising given their high degree of sympatry and dispersal possibilities. This indicates that it is in fact the environment that is the main shaping factor for zooplankton communities and not necessarily the difficulties of reaching new environments. However, according to the "persistent founder effect" hypothesis (Boileau et al., 1992) zooplankton communities may be influenced to the great extend not only by the variety of ecological niches but also by the first species that had colonized the water bodies. According to the "Monopolization hypothesis" (De Meester et al., 2002) genetic variation is mainly shaped by colonization events, especially if the colonizer has three characteristics: fast reproduction rate, resting propagules (or resting eggs), and easiness to adapt to new environment. These three characteristics will result in a well-adapted species which monopolizes the new environment, and this long lasting founder effect makes it difficult for newly arriving species to compete.

Another peculiar characteristic of zooplankton communities is the ubiquity of parthenogenetic reproduction. The lack of necessity to find a mate may lead to co-existence of various species with similar environmental requirements in a relatively small area (Montero-Pau & Serra, 2011).

All those particular features yield diversity patterns of aquatic microscopic animals that are different from macroscopic terrestrial animals.

Biodiversity analyses and, consequently, conservation actions rely on accurate identification of species, and considering the large number of still insufficiently described species, efforts to catalogue biodiversity should be a priority. However, our estimates of species richness are often biased by morphological similarities, our misunderstanding of the evolutionary processes that shape biodiversity or by the methods used to identify species. During recent years, our perception of species loss has been also altered by new species discovered in remote and previously hard-to-access areas. Moreover, species diversity estimates based on traditional morphology-based taxonomy may be biased as indicated by advancements in molecular-based phylogenetics and our increased understanding of relatedness (Clément, 1993). Estimation of species richness for microscopic organisms is probably much less exact than for macroscopic animals, and because microscopic organisms play a crucial role in aquatic food web habitats (Schmid-Araya et al., 2002; Shurin et al., 2006), we can assume that these ecosystems are not completely understood.

1.2 Rotifers and their role in ecosystems

Rotifers (from Latin Rotifera) have been known since van Leeuwenhoek who observed them together with other microscopic animals through his primitive microscope for the first time in the 17th century. He named them *"animalculum binis rolulis"* (from Latin: animalcule with two wheels) because moving ciliae appeared to be "rotating" around the mouth of rotifers (Dobell, 1958). This description was the basis for the modern taxonomic term "Rotifera" meaning "wheel bearers" (from Latin "rota" = wheel and "ferre" = to bear/ carry). The typical rotifer anatomy consist of a three distinct features: 1) a head with the corona composed of ciliae used for locomotion, 2) food gathering jaws - throphi - which are located in a muscular pharynx called the mastax, and 3) an often thickened body wall, the lorica. Rotifers are a part of microscopic and near-microscopic zooplankton with body length range from 50-2000 µm (Wallace et al., 2006).

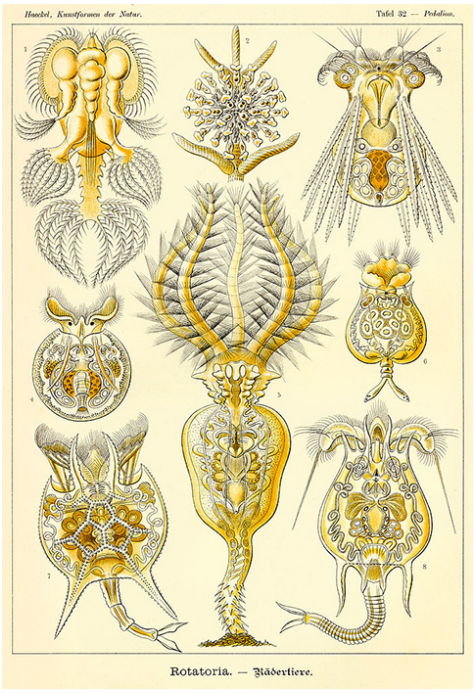

Fig. 1. "Rotatoria" – drawing by Ernst Haeckel (Haeckel, 1899-1904)

Due to their peculiar appearance (Fig. 1), ubiquity and interesting life cycle, rotifers have become important organisms used for studies on evolution, reproduction and biodiversity. Today the phylum Rotifera comprises over 2,000 validly described species, which are traditionally divided into three groups: Monogononta (~ 1,600 species), Bdelloidea (~ 460 species), and Seisonacea (2 species) (Fontaneto & De Smet, 2015). The exact taxonomic position of these three groups and their evolutionary relationships are still unclear. The three groups differ significantly in their reproduction and in the habitat they occupy. Seisonacea reproduce only via sexual reproduction and live exclusively as epibionts of the crustacean *Nebalia*. Bdelloidea are obligate parthenogens, which inhabit wet and moist habitats on land. When exposed to unfavourable conditions they can undergo a process of dormancy through desiccation (Wallace et al., 2006). During this dormant state they show very high resistance to starvation, high pressure, very low temperature and ionising radiation (Ricci & Fontaneto, 2009). One of the possible explanation for their resistance can be DNA-repairing mechanisms (Gladyshev & Meselson, 2008). This resistance to extreme conditions makes Bdelloidae a perfect model organism to study survival in extreme environments. Bdelloidae are also ones of the few multicellular organisms that reproduce only via asexual reproduction (Welch et al., 2004a; Welch et al., 2004b). Due to their purely asexual reproduction and their long evolutionary persistence, Bdelloidea were called an "evolutionary scandal" and became important organisms in the study of sexual reproduction and recombination (Maynard Smith, 1986). However, recent research indicates that, although there is only one sex in Bdelloidae, there exist multiple forms of genetic exchange between individuals probably compensating for lack of sex (Boschetti et al., 2012; Golczyk et al., 2014; Signorovitch et al., 2015; Debortoli et al., 2016). Monogononta are free-living in fresh and marine waters and reproduce by cyclical parthenogenesis (Wallace et al., 2006). Although rotifers are a relatively small group, they are widely distributed in almost all freshwater ecosystems reaching sometimes very high densities, contributing as much as 30% or more to the total plankton biomass (Haberman, 1995; Obertegger et al., 2007). They form an important part of both the 'classical' and the microbial food web (Wallace et al., 2006). Many of Bdelloidae and some Monononota are also abundant in wet environments on land such as forests, or peat bogs (Wallace et al., 2006). Due to their high abundance they also play an important role in peat bog waters (Błędzki & Ellison, 2002) and in nutrient cycling in soils (Sohlenius, 1982; Anderson et al., 1984; Błędzki & Ellison, 2002). Rotifers' role in ecosystem, reproductive patterns and interesting evolution make them particularly fascinating and suitable objects for the study of ecology and evolution (Fussmann, 2011).

1.3 Cryptic species and their importance for biodiversity

Since prehistory people were describing plants and animals which were useful to them as often possessing such information was necessary for survival. Accumulation of knowledge increased the necessity of classification. The first known classification of species was presented by Aristotle and was based on "attributes" (morphological or physiological features) (Leroi, 2014). The first holistic attempt of a hierarchical classification of plant and animal species was done by Carl Linnaeus in the XVIII century (aka Linnean system). His classifications were, however, based purely on shared physical characteristics, and therefore are nowadays often

considered erroneous. Understanding of the roles of environment and natural selection in appearing of new species became only possible after the publication of Darwin's "On The Origin of Species by means of natural selection" in 1859.

One of the first scientists investigating the links between species diversification and their genetics was Theodosius Dobzhansky; in his book "Genetics and the Origin of Species" (1937) he pinpointed the role of genetic mutations for the development of species.

The modern definition of species as the "populations of organisms that can reproduce with one another and are reproductively isolated from other populations" is based on the "Biological Species Concept" first proposed by Ernst Mayer in 1942. However, the Biological Species Concept has some weaknesses. First of all it is more applicable to sexually-reproducing organisms as it is difficult to define reproductive barriers in asexual organisms. Moreover, the Biological Species Concept is also weakened when boundaries between species become hard to define (e.g., with bacteria). The problems with the Biological Species Concept's applicability to various cases led to the development of other concepts of species boundaries with the current number of concepts around 24 (Mayden, 1997). Phenomena that undermine the Biological species concept are asexual reproduction, species hybridization, horizontal gene transfer (HGT) and the occurrence of cryptic species complexes.

A cryptic species complex is a group of closely related (also called cryptic/sibling) species that are impossible or difficult to distinguish based on their morphology. Since the Biological Species Concept was published (Mayr, 1942), many sibling species have been described (Knowlton, 1993. However, only when advanced genetic methods have been applied, the differences between species within cryptic complexes became more apparent (Gomez & Snell, 1996).

Most often cryptic species are identified by using DNA-based species delimitation techniques. In DNA-taxonomy, one or more "barcoding" genes are selected and sequenced, and the genetic information is used to infer a phylogenetic tree or haplotype networks (Fontaneto et al., 2015). The choice of a suitable barcoding gene is crucial because markers that are too specific or have an unpredictable pace of evolution may lead to unpredictable and biased results. Therefore, delimitation of species based on few barcoding genes (so called "multilocus" approach) yields usually more reliable results (Fontaneto et al., 2015) and is currently becoming increasingly favored over single-locus based methods. Especially for non-monophyletic species which can show gene tree discordance, incomplete lineage sorting, and/or gene flow after divergence a multilocus approach is advisable to increase the resolution of delimitation (Camargo et al., 2012; Fujita et al., 2012). Different computational approaches that are tree-based or gene-based are applied to delineate species such as the automatic barcode discovery method (ABGD; Puillandre et al., 2012), K/θ (Birky et al., 2006; Birky, 2013), generalized mixed Yule–coalescent-based approaches (GMYC; Pons et al., 2006; Fujisawa & Barraclough, 2013), Poisson tree process model (PTP; Zhang et al., 2013) or Haplowebs (Doyle, 1995; Flot et al., 2010).

Many new initiatives such as the Consortium for the Barcode of Life (www.barcodeoflife.org) aim at cataloguing genetic biodiversity of the animal kingdom using genetic markers. Without including cryptic species these catalogues would be incomplete. Especially in the last twenty years the number of articles related to cryptic species increased almost exponentially (Fig. 2) (Bickford et al., 2007), mainly due to the increased availability (including reduced costs) of DNA sequencing technologies.

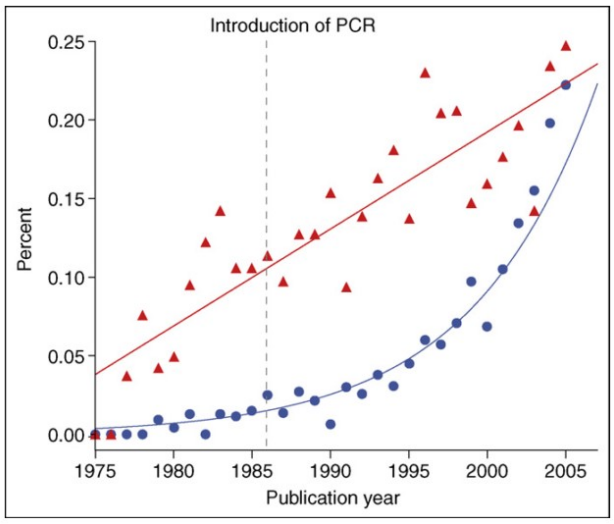

Fig. 2. Cryptic species publications. Increasing percent of peer-reviewed publications in Zoological Record Plus (CSA) that mention 'cryptic species' (circles) or 'sibling species' (triangles) in the title, abstract, or keywords. Figure from: Bickford et al. (2007).

Moreover, cryptic species are almost evenly distributed among major metazoan taxa and biogeographical regions (Pfenninger & Schwenk, 2007). Therefore, the distribution and occurrence of cryptic species may have substantial consequences for biodiversity assessments, biogeography, conservation management, and evolutionary theory (Bickford et al., 2007). Biodiversity assessments as well as conservation work is incomplete without including cryptic species (Esteban & Finlay, 2010). Due to the ease of culturing and collecting genetic material, cryptic species have been mainly described in small animal groups such as protists (Foissner, 2006), ants (Fournier et al., 2012), harvestmen (Arthofer et al., 2013), and rotifers (Gómez & Snell, 1996).

1.4 Cryptic species in rotifers

Since the beginning of rotifers studies, researchers described various morphological forms of rotifers. Called “lifeforms”, “morphoforms” or “sibling species” (Mayr, 1942) those forms were often meticulously depicted and named separately (Fig. 3).

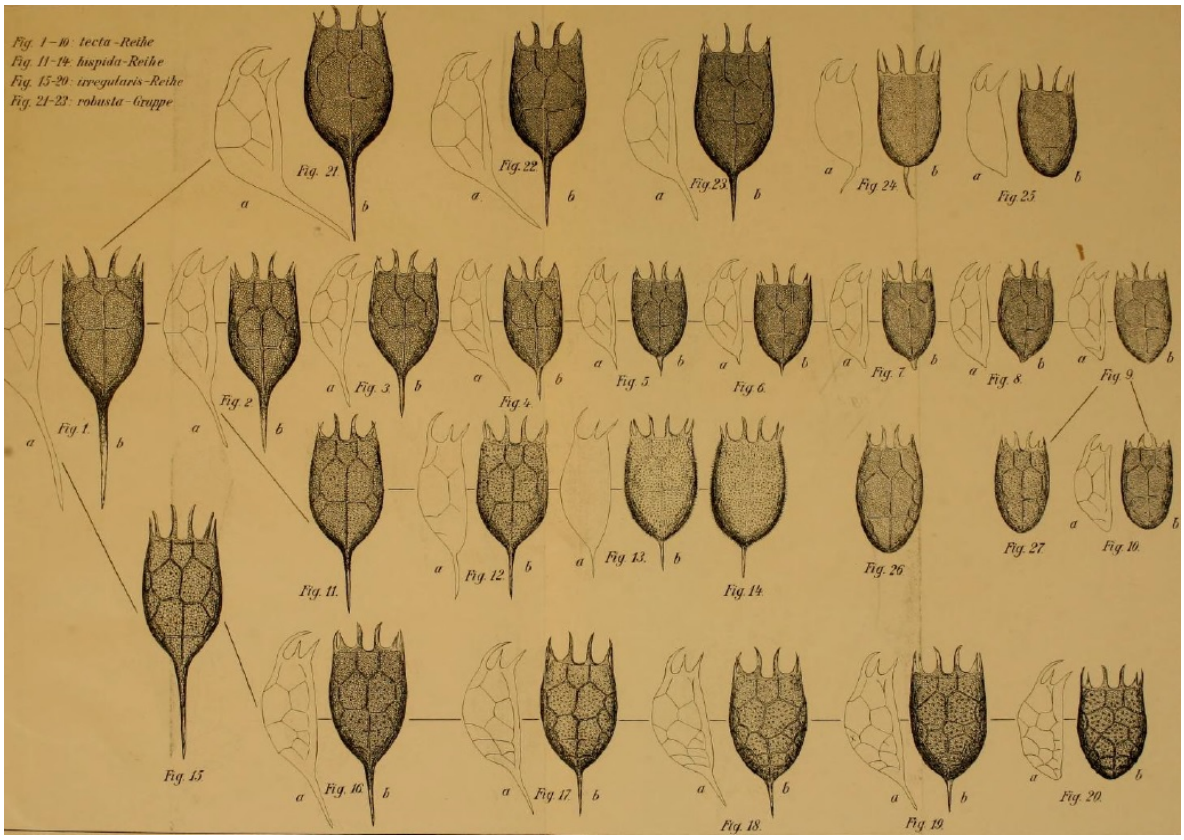

Fig. 3. “Life forms” of *K. cochlearis*. Three series are visible: 1-10 macracantha–typica–tecta; 11-14 hispida; 15-20 irregularis and separated group of robusta (21-23). Figure from: Lauterborn, 1900.

With advances in molecular biology it became obvious that some of the morphological forms differ not only morphologically but also genetically (Segers, 1995; Gomez & Snell, 1996). Such complexes of “cryptic species” (i.e. several or many species that are morphologically difficult to discriminate but are nonetheless genetically different) were described for diverse groups of animals such as *Daphnia* (Herbert & Crease, 1980), protists (Foissner, 2006), ants (Fournier et al., 2012), harvestmen (Arthofer et al., 2013), rotifers (Gomez & Snell, 1996), frogs (Elmer et al., 2007) and giraffes (Brown et al., 2007). In rotifers cryptic species complexes have been described in many species of the class Monogononta with the most famous being *Brachionus plicatilis* (Gómez et al., 2002), *Epiphanes senta* (Schröder & Walsh, 2007), *Synchaeta pectinata*, *Polyarthra dolichoptera* (Obertegger et al., 2012, 2014), and *Testudinella clypeata* (Leasi et al., 2013). The existence of cryptic species has re-shaped our understanding of biodiversity and species richness and undermined the general belief that zooplankton ‘species’ occupy different niches (Hebert & Crease, 1980). Studies on cryptic species in rotifers also contributed to understand local adaptation, genetic population

divergence, and cryptic speciation (Suatoni et al., 2006; Campillo et al., 2011; Fontaneto et al., 2008). For example, some species within one cryptic species complex may exhibit different environmental or food preferences. This is for example the case for *B. plicatilis,* where adaptations to different levels of salinity have been demonstrated for single cryptic species from this complex (Gómez et al., 1997; Ortelles et al., 2003). Another example of differences in preferences in cryptic complex is *Synchaeta* spp., where various ESUs showed different preferences for total phosphorus (and most probably for different algal food or/and concentrations) (Obertegger et al., 2012). Understanding the occurrence of species and their ecological preferences is one of the fundamental aspects in ecology (Gaston, 2000). Moreover, understanding what influences cryptic species speciation is important to understand the influence of the environment on species diversity. Cryptic diversity includes many organisms, which contribute to the functioning of ecosystems but is usually not part of conservation surveys (Esteban & Finlay, 2010). Furthermore, occurrence and richness of cryptic species complexes makes rotifers particularly fascinating and suitable objects for the study of ecology, evolution, and biodiversity (Fussmann, 2011). Because cryptic species are impossible to distinguish through morphology, the most commonly used way to distinguish them is to apply DNA-taxonomy techniques by investigating the variability of barcoding genes; for animals, the mitochondrial gene coding mitochondrial cytochrome oxidase c subunit I is the most often used (Fontaneto et al., 2015).

1.5 Mitonuclear discordance

Despite widespread practice, using only a single mitochondrial gene for species delimitation can be problematic and lead to biased results. For instance, Song et al. (2008) concluded that mitonuclear pseudogenes (nonfunctional copies of mitochondrial DNA in the nucleus) can interfere with standard sequencing methods, and thus result in an incorrect inference of unique species. Moreover, particular markers can show different variability for various groups of animals. This is for example the case for cnidarians where the COI marker is not variable enough for phylogenetic use (Shearer & Coffroth, 2008). Apart from using one gene (single-locus approach) for species delimitation, using multiple markers (multilocus approach) is becoming an increasingly recognized approach. Especially after mitonuclear discordance has been shown to be a widespread phenomenon in rotifers (Papakostas et al., 2016). Mitonuclear discordance occurs when phylogenetic patterns obtained from mitochondrial markers differ from the ones obtained from nuclear markers. Mitonuclear discordance may be caused by several factors such as: introgressive hybridization, horizontal gene transfer, androgenesis, incomplete lineage sorting, and unresolved phylogenetic polytomies. Introgressive hybridization (introgression) is a gene flow from one species into a gene pool of another species usually by hybridization or by repeated backcrossing (Harrison & Larson, 2014). Such processes have been described for many taxa (Toews & Brelsford, 2012), and they may lead to phylogenetic conflict and mislead species identification. Horizontal gene transfer is the movement of genetic material between different taxa, which often involves bacteriophages as vectors. Androgenesis is not a very widespread mechanism in which the maternal nuclear genome fails to participate in forming a zygote and offspring develop as a pure paternal clones instead (Pigneur et al., 2012). Both horizontal gene transfer and androgenesis are believed to be limited to a small number of taxa (Keeling &

Palmer, 2008; Hedtke & Hillis, 2010). Horizontal gene transfer is much more common in Procariota than in Eucariota (Choi & Kim, 2007), and androgenesis has only been described in few species of invertebrates and plants (Hedtke & Hillis, 2010; Pigneur et al., 2012). Another possible source of mitononuclear discordance is incomplete lineage sorting that appears when offspring lack some of the allele present in parents (Toews & Brelsford, 2012). All those processes can alone or in combination lead to mitonuclear discordance or obscure the phylogenetic signal. Therefore, using more than one marker (so called multilocus analysis) and possibly also other data than genetics (integrative taxonomy approach) makes the species delimitation process much more robust.

1.6 Integrative taxonomy

With different variability of various barcoding genes and genetic problems such as mitonuclear discordance, more researchers are inclining towards a more holistic approach of integrative taxonomy (Dayrat, 2005). Integrative taxonomy combines data from various disciplines such as morphology, mitochondrial DNA, nuclear DNA, ecology, behavior, reproductive compatibility, life histories, cytogenetics, chemistry, and whole genome scans (Schlick-Steiner et al., 2010) (Fig. 4). Even though this multidisciplinary approach is in its exploratory stage, it is usually considered more reliable than taxonomy based only on morphology or genetics (Schlick-Steiner et al., 2014). Applying integrative taxonomy sometimes can help solving taxonomical conflicts that can arise for example from DNA hybridization or morphological similarity of closely related species (Andújar et al., 2014). With difficulties that alpha taxonomy (taxonomy describing species) faces, especially with increasing number of described cryptic complexes, combining various data following integrative taxonomy approach can result in more rigorous species delimitation and improve our biodiversity inventory.

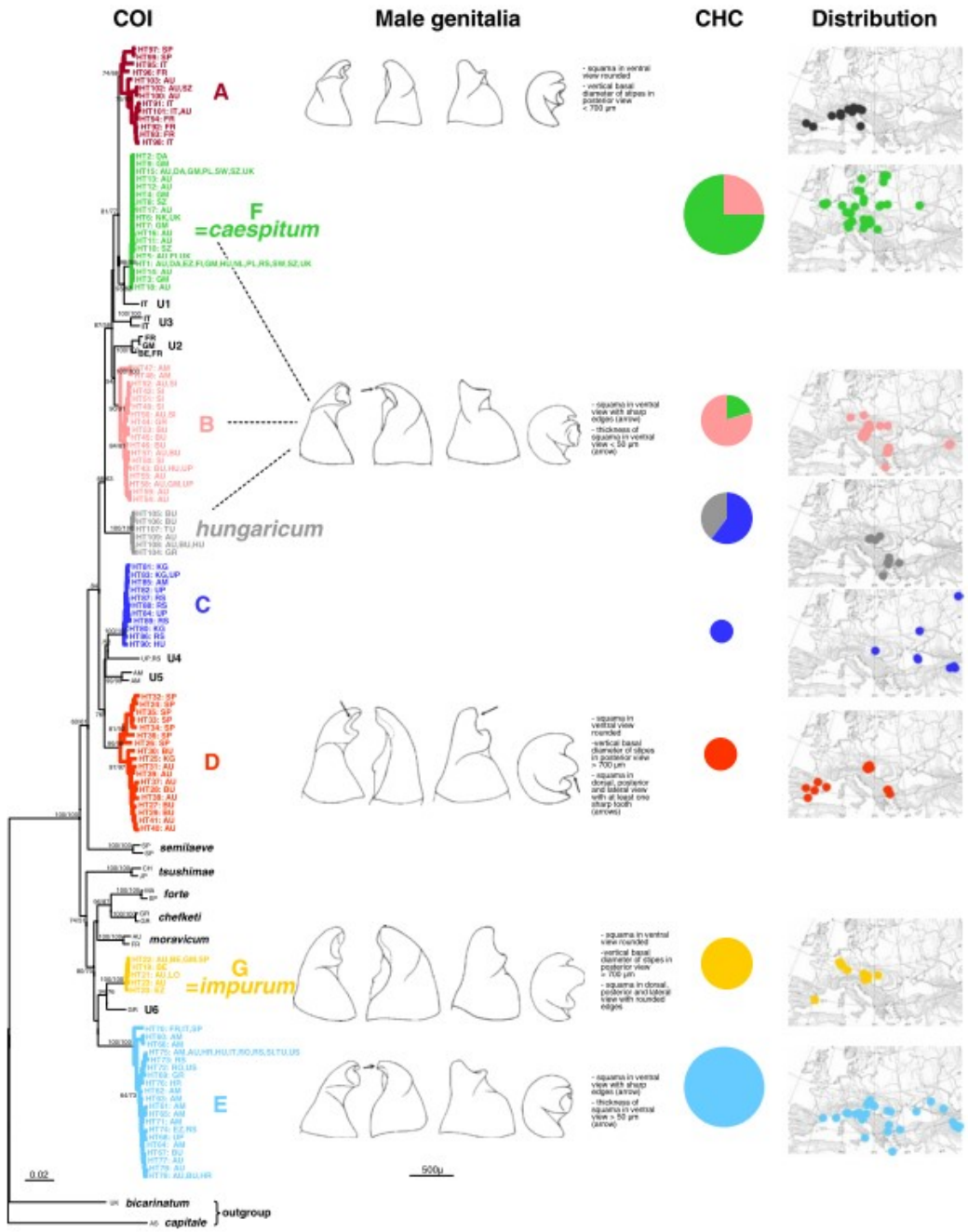

Fig. 4. An example of the integrative taxonomy approach for species delimitation of *Tetramorium* ants. In this case, molecular phylogeny ("COI"), morphometry (capital letters next to clades), morphology ("male genitalia"), chemistry ("CHC" - cuticular hydrocarbons), and biogeography ("Distribution") were used. Figure from: Schlick-Steiner et al., 2006.

1.7 *Keratella cochlearis* – study organism

Keratella cochlearis Gosse, 1851 is one of the most widespread freshwater rotifers in the world (Green, 1987). The whole genus *Keratella* is considered euthrophic, euthermic and cosmopolitan as its species can be found in various lakes and ponds, including lowland and high-mountain lakes (Segers & De Smet, 2008). *Keratella cochlearis* has even been found in extreme habitats such as cryoconite holes (water-filled holes forming on glaciers) on Spitzbergen (De Smet & Van Rompu, 1994) and mine impoundments (Żurek, 2006). *Keratella cochlearis* belongs to the group Monogononta and is, as most of the rotifers of this group, obligate parthenogenic (heterogonic). Obligate parthenogenesis means that most of the time monogonont rotifers reproduce asexually (amictic reproduction) by parthenogenesis. However, when environmental conditions deteriorate and the environmental trigger appears, females start to produce mictic females that produce haploid males, which are then responsible for sexual reproduction (so called mixis) (Fig. 5). In the genus *Brachionus* the switch to mictic production is mediated by the accumulation of a mixis-inducing protein, which is produced by amictic females in response to crowding (Snell et al., 2006). Resting eggs, which are produced through sexual reproduction, have often a thick shell and are resistant to desiccation allowing the population to survive adverse and deteriorating environmental conditions. Environmental cues that trigger sexual reproduction have been studied only for a few species; for *Asplanchna* species, it is dietary α-tocopherol (vitamin E) (Gilbert, 1980, 1981), for *Notommata* it is a change in photoperiod (Pourriot & Clement, 1981), and for *Brachionus* species it is increasing population density (crowding) (Gilbert, 1963).

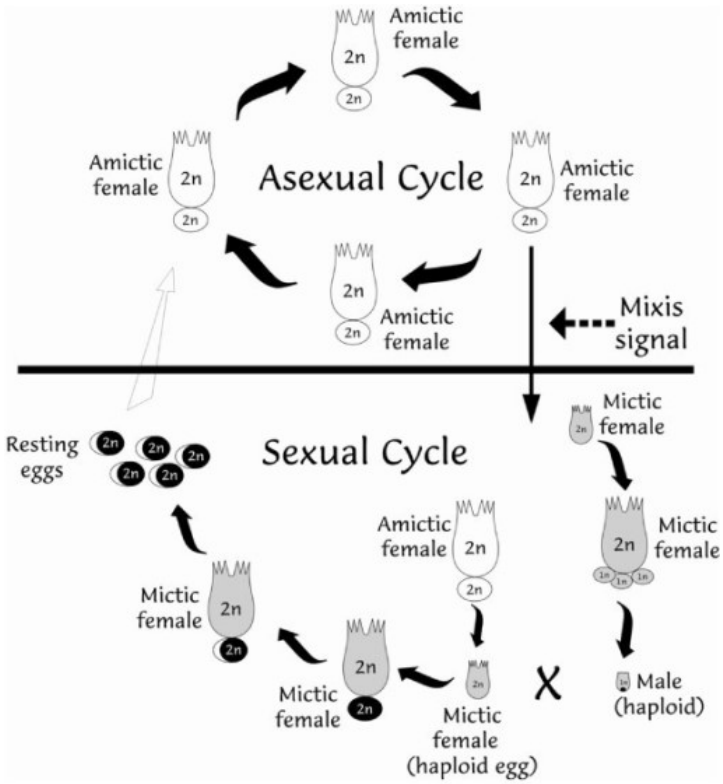

Fig. 5. Reproduction in Monogononta rotifers. Parthenogenic (asexual) and sexual cycles are depicted. Figure from: *www.devbio.biology.gatech.edu*

Most data on sexual reproduction originate from the genus *Brachionus,* while there are no reports of sexual reproduction for *K. cochlearis*. There are, however, very few descriptions of *K. cochlearis* males indicating that sexual reproduction occurs. Wesenberg-Lund (1923) gave the first description of a *K. cochlearis* male. He described it as heaving a broad conical body with a thick lorica without any spines and a corona with ciliae. Even though Wesenberg-Lund (1923) described *K. cochlearis* male lorica as thick and difficult to see through, he nonetheless gave some description of male anatomy: lack of alimentary canal, large testis, two lateral canals, eyespot, prostate gland, and oil globules (Fig. 6).

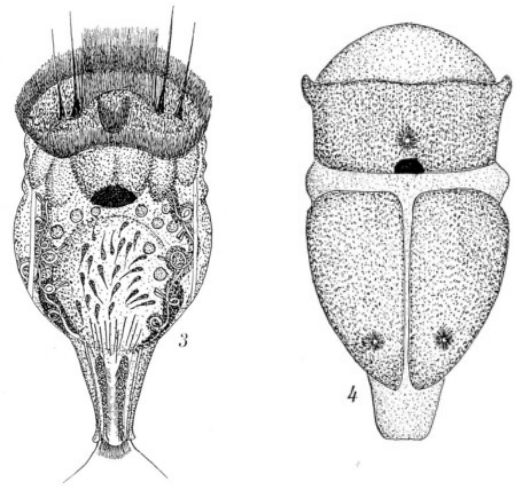

Fig. 6. ***K. cochlearis*** **male.** 3 shows anatomical details 4 a contracted body. Figure from: Wesenberg-Lund, 1923.

Another interesting morphological aspect of *K. cochlearis* is its body – the lorica. Since the early studies on *K. cochlearis*, researchers were faced with a large variety of forms and shapes of the lorica. Some of these morphological forms were considered cyclomorphic as specimens clearly showed seasonal changes (Hofmann, 1980). Lauterborn (1900) produced one of the most detailed drawings of *K. cochlearis* "morphotypes". These drawings became the basis for further taxonomical work on this species (e.g. Ahlstrom, 1943; Ruttner- Kolisko, 1974; Koste, 1978). The morphotypes that Lauterborn (1900) described can be divided into three series (1) macracantha–typica–tecta, 2) hispida, 3) irregularis) and the separated group of robusta (Fig. 3). These series vary in lorica length, spines length, the presence of spinelets (small bumps on the lorica; called ''Pusteln'' after Lauterborn, 1900 and ''spinelets'' after Ahlstrom, 1943), and the angle between the lorica and the long posterior spine. One of the most pronounced feature described by Lauterborn (1900) is the long posterior spine. One of the forms (tecta) described by him exist only without a spine, whereas only some individuals from the irregularis series only occasionally have a spine. Another pronounced feature are small spines, which are present on the lorica of only the hispida series. The complexity and unambiguity of *K. cochlearis* morphology led Lauterborn (1900) to hypothesize subspecies status of some types of his series. Moreover, some other researchers had difficulties in observing exactly the same characteristics as Lauterborn; for instance, Hofmann (1983) questioned Lauterborn's *K. cochlearis* series. Further research indicated that the division in "series" may not be unequivocal. More research led to even more confusion as characteristics of each series appeared to be more flexible than previously thought indicating a very high phenotypic plasticity. Green (1981, 2005) and Bielańska-Grajner (1995)

described that the lorica and posterior spine lengths change with water temperature. Furthermore, Conde-Porcuna et al. (1993), Green (2005) and Stemberger & Gilbert (1984) showed that the posterior spine length changes also when *K. cochlearis* is exposed to predators. However, some research indicated that there are in fact differences between at least some of the morphotypes, but that these differences are not necessarily morphological. Bērziņš & Pejler (1989a) showed that different morphotypes have different tolerance to temperature, trophic state (Bērziņš & Pejler, 1989b), and conductivity (Bērziņš & Pejler, 1989c). Derry et al. (2003) found that spiny and spineless *K. cochlearis* differ by 4.4 % in their COI gene. This was the first indication based on genetic data that there may exist a cryptic species complex in *K. cochlearis*.

1.8 Aims of the thesis

The main objective of this thesis was to assess the morphological and genetic variety of the rotifer *K. cochlearis* collected from various Alpine lakes, as well as investigating if any observed differences are reflected in changes of demography or reproductive strategies of the cryptic entities. Thus, this thesis investigated, in the context of integrative taxonomy, the cryptic species status of *K. cochlearis*. The specific aims were:

1) To assess the presence of a cryptic species complex in *K. cochlearis*.

2) To investigate if genetic differences are reflected in morphological differences between cryptic entities.

3) To investigate if genetically different strains of *K. cochlearis* show differences in their life histories and demographic parameters.

4) To investigate mitonuclear discordance as a confounding factor in *K. cochlearis* species delimitation.

5) To observe and record males of *K. cochlearis* for their mating behavior.

2. Sampling sites and methods

This part gives an overview on the sampling sites, data collecting and experimental set-ups. For more details regarding particular experiments and analytical methods used, I refer the reader to the respective articles.

2.1 Sampling sites

All rotifers used for experiments were collected in the region of Trentino-South Tyrol, Northern Italy. This region is known for its variety of lakes of a wide spectrum of trophic state, depth, and other physical parameters. Most of the lakes in Trentino-South Tyrol were formed after the last Ice Age, i.e. around 10000 years ago. This makes all the sampling sites relatively young from a geological point of view.

Six lakes (in the following text called the "core lakes", Tab. 1, Fig. 7) were sampled monthly from March to November 2014 (article I). For the same article, additionally 11 lakes from Trentino-South Tyrol were sampled irregularly during summer and winter 2010 (sampled by U. Obertegger) 2013 and 2015 (sampled by the author together with U. Obertegger) to increase the geographical coverage (Fig. 7) .

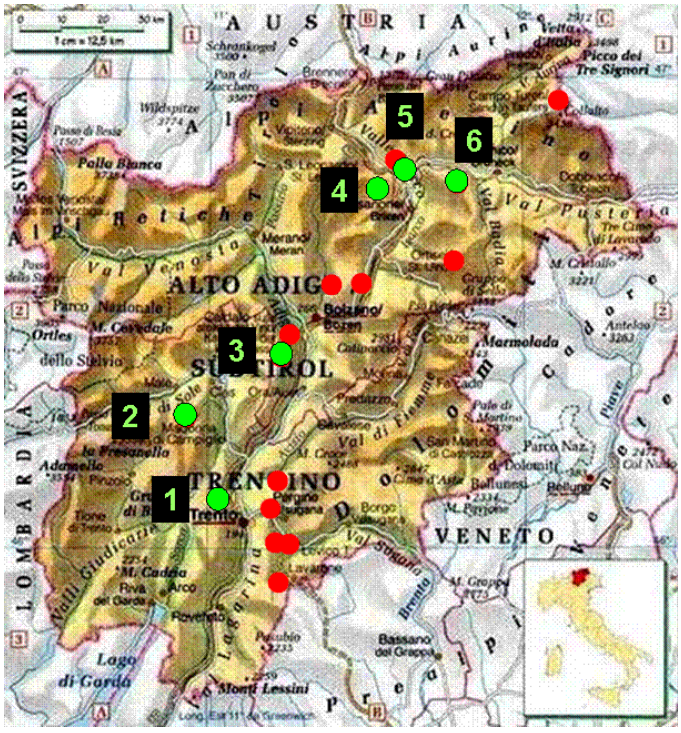

Fig. 7. Map of Trentino-South Tyrol with sampled lakes. All 17 sampled lakes marked as dots (green dots are core lakes, red are the additional lakes). Core lakes: 1 – Terlago; 2 – Tovel; 3 – Kalterer; 4 – Radl; 5 – Vahrn; 6 – Glittner. Map was modified from: *www.centrometeoitaliano.it*

Core lakes were selected according to their parameters to cover a wide range of environmental conditions (Tab. 1).

Lake	Altitude (m.a.s.l.)	Depth (m)	Surface (ha)	Trophic state
Terlago	414	16	11.9	eutrophic
Tovel	1178	38	38.2	oligotrophic
Kalterer	215	5.6	131	mesotrophic
Radl	2258	8	0.8	mesotrophic
Vahrn	678	3.5	1.5	mesotrophic
Glittner	2151	1	0.05	eutrophic

Tab. 1. Core lakes characteristics.

For article II, *K. cochlearis* collected from Lake Terlago on September 23, 2014, on 28 January 2014 from Lake Tovel and on March 2, 2015 from Lake Kaltern were used. For article III, the same specimens of *K. cochlearis* as for article I were used together with *Polyarthra dolichoptera* (Idelson, 1925) sampled from 35 Trentino-South Tyrol lakes described in Obertegger et al. (2014) and with *Synchaeta pectinata* (Ehrenberg, 1832) sampled from 17 Trentino-South Tyrol lakes described in Obertegger et al. (2012). For the article IV, *K. cochlearis* and *Brachionus angularis* (Gosse, 1851) isolated from Lake Tovel on March 2, 2015 were used.

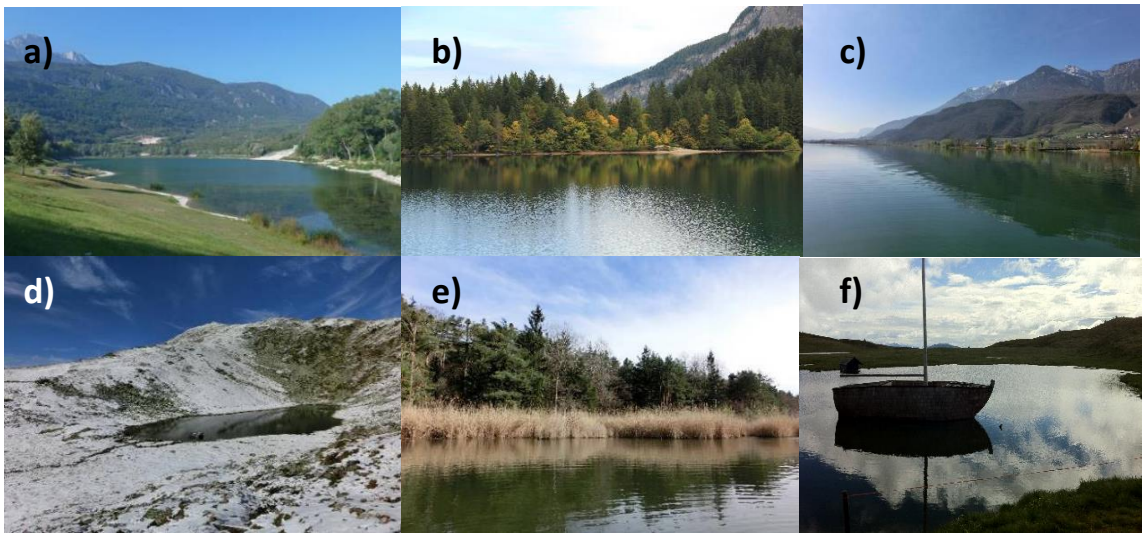

Fig. 8. The core lakes: Terlago (a), Tovel (b), Kalterer (c), Radl (d), Vahrn (e), Glittner (f). Figure (a) from: *www.tr3ntino.it*

Terlago (Fig. 8a) is a small euthrophic and shallow lake (Tab. 1), situated 7 km North-West from the city of Trento, close to the Terlago village. The lake is easily accessible and is a popular fishing and sunbathing spot.

Tovel (Fig. 8b) is a middle-sized oligotrophic lake located in the Adamello Brenta National Park (Trentino Province). Even though it is situated only at 1178 m.a.s.l. (Tab. 1), it is considered an alpine lake due to its environmental characteristics (Obertegger & Flaim, 2015). The lake generally freezes over from December to April (Borsato & Ferretti, 2006) and is easily accessible only during summer months. There are few summer houses around the lake.

Lake Kalterer (Fig. 8c) is a middle-sized mesotrophic alluvial lake (Table 1) situated 3 km south from the village of Kaltern an der Weinstraße (IT: Caldaro sulla Strada del Vino). Its relatively shallow depth (maximum 5.6 m), large surface area and location at low altitude in open plains contribute to a generally warmer water temperature than many other lakes in the neighborhood. Lake Kalterer is surrounded by camp grounds, beaches and is very popular among tourists attracted by camping sites and many water sport possibilities during summer.

Lake Radl (Fig. 8d) is a small, high-altitude mesotrophic alpine lake (2258 m.a.s.l.), situated 21.5 km west from the town of Brixen (IT: Bressanone) in a remote area accessible only by mountain paths. The catchment consists of bare rocks and alpine vegetation. At the lake there is a mountain lodge (Radlseehütte) where guests are hosted during summer months. This lodge is the only near settlement.

Lake Vahrn (Fig. 8e) is s small (0.015 km^2) and shallow lake (3.5 m depth) situated on 678 m.a.s.l. 6.15 km north from the town of Brixen, next to the village of Vahrn. The lake is a popular tourist destination, easily accessible by car with a large grass beach area and walking path around. Part of the lake belongs to the protected area “Vahrner Seemoor”, with vegetation and fauna typical for swamps.

Lake Glittner (Fig. 8f) is a very small and shallow (1 m depth) lake situated on 2151 m.a.s.l. 13 km east from the town of Brixen. The lake is euthrophic, which can be partly due to cattle grazing around the lake during summer months. The catchment consists of alpine grassland.

The core lakes were sampled monthly from March to November 2014 with some additional samplings during 2015 and 2016. Environmental parameters of lakes were based on published data (IASMA, 1996–2000). Water samples were collected over the deepest point of the lakes with a 20 µm (Apstein) or 50 µm (Wisconsin) plankton net depending on lake depth. Both mesh sizes were small enough to catch even small specimens of *K. cochlearis.*

2.2 Specimen measurements – morphological data

Whenever possible, samples were brought immediately to the laboratory and processed; otherwise, samples were fixed with HistoChoice Tissue Fixative (Sigma Aldrich, Saint Louis, USA). For the core lakes, single specimens of *K. cochlearis* were isolated under a microscope and photographed from different angles. Morphological measurements were based on the studies of Green (1981) and Stemberger & Gilbert (1984). In addition to the usually measured posterior spine length (PSL) and total length (TL) (Fig. 9a), the following measurements were added: LW - lorica width at its widest part, HW - head width at the mouth opening, ALS - anterolateral dorsal spine length, AIS - anterointermediate dorsal spine length, AMS - anteromedian dorsal spine length, PSA - and posterior spine angle. PSA was described by Lauterborn (1900) as an important feature for discrimination of the robusta group.

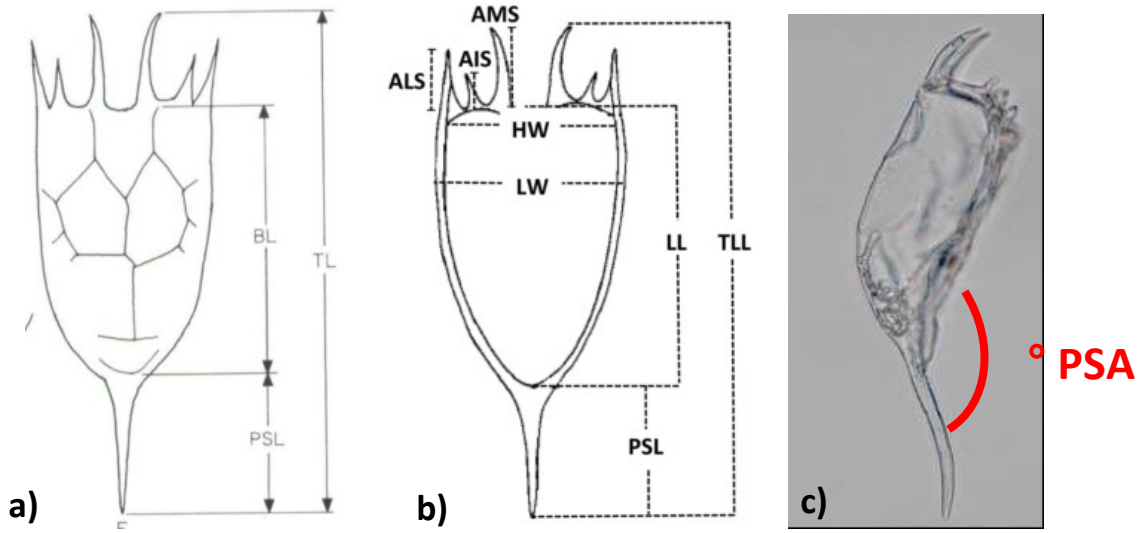

Fig. 9. *K. cochlearis* measurements: a) lorica measurements from Stemberger & Gilbert (1984), b) *K. cochlearis* lorica drawing with measured parameters from the article I, c) posterior spine angle measurements used for the article I.

The Leica IM1000 (Leica Microsystems, Heerbrugg, Switzerland) program was used to obtain rotifer measurements from pictures (Fig. 10).

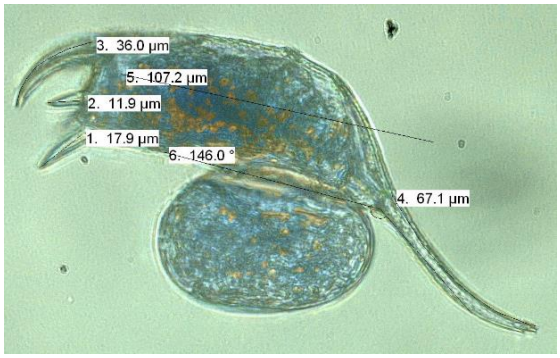

Fig. 10. Lorica measurements marked on a microscope picture of *K. cochlearis.*

2.3 DNA extraction

The DNA of every measured rotifer was extracted. For extraction, the Chelex rasin (InstaGeneMatrix, Bio-Rad, Hercules, CA, USA) was used (Walsh et al., 1991). The DNA extraction process was based on the protocol widely used for rotifer adults and rotifer diapausing eggs (Fontaneto et al., 2007; Obertegger et al., 2014). Choosing a proper extraction method out of many available is important because different extraction kits can result in different DNA extraction rate, and thus yield different estimates of biological diversity (Fontaneto et al., 2015). Each individual of *K. cochlearis* was carefully isolated with a glass pipette and cleaned few times with distilled water prior to extraction. Individuals with attached algae or fungi were not used. Despite careful cleaning and selection of individuals for DNA extraction, the extraction success rate varied for different populations of *K. cochlearis*, which is probably related to varying rotifer size and lorica thickness. More information on DNA extraction details can be found in article I.

2.4 COI sequencing

Choosing a barcoding marker gene is central for obtaining a reliable phylogenetic tree. The most important features of a good barcoding gene are: 1) significant species-level genetic divergence and variability, 2) universality i.e. possibility to be used for many different species, 3) conserved flanking sites for easiness of developing an universal primer, which can be applied to various taxonomic clades, 4) short sequence to facilitate DNA extraction and PCR (Kress & Erickson, 2008; Fontaneto et al., 2015). Woese (Woese & Fox, 1977; Woese, 1987) was one of the first to use ribosomal RNA to delimit domains of life. In the beginning, barcoding techniques of identification were mostly applied in studies on morphologically similar groups such as viruses, bacteria and protists (Hebert et al., 2003) but since the late 80s the technique gained popularity also for animals and plants. In recent years, one of the most commonly used barcoding gene is the mitochondrial cytochrome c oxidase subunit I (COX1 or COI) gene (Fontaneto et al., 2015). Mitochondrial genes usually lack introns, are generally haploid and exhibit limited recombination (Hebert et al., 2003). These features give them advantages as barcoding genes over nuclear genes. In most animal groups COI is very variable (Avise et al., 1987). Folmer et al. (2014) have developed a universal primer for the COI, which can be used for a wide variety of animals. Moreover, the short length of the COI of around 600 base pairs makes COI relatively easy to use for genetics. Furthermore, the relatively stable evolution rate of COI allowed estimating the rate of sequence divergence as approximately 2 % per million years (Hebert et al., 2003) and a barcoding gap of uncorrected genetic distance for most of the species (species threshold) of around 3 % (Hebert et al., 2003; Tang et al., 2012). Even though COI is very versatile, it still possesses some problems which are difficult to overcome. The gene has been shown to give confounding results when applied to plants (Cho et al., 2004) or some animals groups such as cnidarians where the whole mitochondrial genome appears to be very stable (Fontaneto et al., 2015). Moreover, because COI is a mitochondrial gene, it is only inherited through the maternal line. This can result in slower evolutionary rates compared to nuclear markers (Tang et al., 2012). Also mitochondrial genes can be subjected to mitochondrial introgression and be incorporated in other species as shown for *B. plicatilis* by Papakostas et al. (2016). Despite these problems, COI is still considered the most widespread gene for delimiting species of rotifers. Using COI

as a barcoding gene has the advantage of an easy access to sequence databases such as GenBank (NCBI, USA), which helps to compare studies with already existing ones. More information on sequencing details and methods using COI can be found in articles I and III.

2.5 ITS1 sequencing

Another very commonly used barcoding marker gene is the internal transcribed spacer 1 (ITS1). ITS1 is an intergenic region of DNA located in eukaryotes between the 18S, a part of the small ribosomal subunit and the 5.8S, a part of the large ribosomal subunit. ITS is an equivalent of ITS1 in bacteria and archaea. Intergenic regions (or intergenic spacers) are part of a noncoding DNA located between genes. It appears that both ITS regions (ITS1 and ITS2) play a role in rRNA processing; however, the details of this process are not yet fully understood (Coleman, 2015). There are many reasons why nuclear internal transcribed spacer regions are used as molecular markers; the most important one is because of their high degree of variation including intra-genomic multiple variants even between closely related species (Song et al., 2012). The ITS region is also relatively small in size, has a rapid pace of evolution, and highly conserved flanking sequences (Bena et al., 1998). All these characteristics result in a good resolution of species identification. Moreover, ITS1 is also relatively easy to amplify by PCR (Nilsson et al., 2012). Especially in funghi, a very high sequencing rate of ITS and difficulties with COI resulted in ITS becoming the main barcoding marker (Mahmoud & Zaher, 2015). However, ITS1 is usually more challenging to process than COI because there are two copies of this gene instead of one as in COI and because ITS1 has a secondary structure, which should be taken into account when aligning the sequence (Coleman, 2015; Wolf, 2015). Even though ITS often yields less phylogenetic groups than for example COI (Suatoni et al., 2006; Papakostas et al., 2016; Mills et al., 2017), and the reference base for the rotifer species is much smaller than for COI, it is still one of the commonly used markers in barcoding of rotifers (Fontaneto et al., 2015). ITS1 was chosen as the second marker of choice for the multilocus analyses of *K. cochlearis* cryptic species complex. More information on sequencing details and methods using ITS1 can be found in article III.

2.6 Life table experimental set-up

Life table experiments have been first applied to study human demography by Edmond Halley in the 17th century (Ciecka, 2008). In upcoming centuries this technique grew into a powerful statistical and demographical tool used to study life history traits and population dynamics of various organisms. Because life table experiments require usually many replicates and identical experimental conditions, this procedure is most often used for small organisms with a short lifespan. The main advantage of using life tables is the relative speed and ease in obtaining multiple replicates under chosen conditions. This approach allows studying population-level response to various environmental or biological factors. For small organisms such as rotifers or *Daphnia*, life table experiments are usually carried out in transparent plates with multiple wells allowing for easy microscopic observation (Fig. 11).

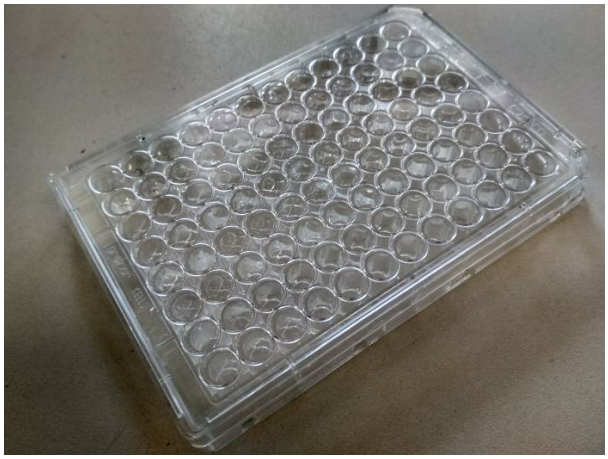

Fig. 11. 96-well plate used in our lifetable experiments. 96-wells CELLSTAR®, Greiner, Kremsmünster, Austria.

Life tables have been used in rotifer research to investigate population parameters (Stemberger & Gilbert, 1985), relationships between various plankton groups (Allan, 1976), and population dynamics (Walz, 1987). Life table experiments usually require recording of various life parameters in equal time intervals. Most commonly used parameters obtained with life tables are average lifespan, average number of offspring and sex of offspring. These parameters can be used to calculate so called population parameters, which describe the growth of the whole population. Most common population parameters are: instantaneous growth rate of the population, generation time, and net reproductive rate.

Due to replicability and ease to control experimental conditions, life table experiments are also used to investigate environmental preferences. This is especially useful when identifying ecological preferences of similar species or subspecies that are difficult to observe or distinguish in their natural environment, as it is the case for example with cryptic species. There is growing evidence that despite their close phylogenetic relationship, cryptic species often have different life history traits. For instance Ciros-Pérez et al. (2001) reported different intrinsic growth rates among three sympatric cryptic species of the *B. plicatilis* species complex that were cultured at the same temperatures. Cryptic species were also shown to have different environmental preferences; for example Gabaldón et al. (2015) described various preferences for salinity for two different cryptic species from the same cryptic species complex of *B. plicatilis*.

These different environmental preferences and demographical response of sibling species may be related to the diversity of ecological niches available in water bodies. Existence of various ecological niches and diverse ecological preferences of closely-related species may partially explain their co-existence in one environment (Angert et al., 2009; Montero-Pau et al., 2011). Therefore, life table analyses were chosen as a relatively easy tool for investigation of differences in population parameters of single cryptic species. More information on life table experimental set-up and performed calculations can be found in article II.

2.7 Zooplankton filming experimental set-up

Filming of life organisms is one the best methods to observe behavior of organisms under natural or experimental conditions. Observing animals in groups also creates a possibility to record interactions between individuals, which would be difficult to observe in nature. One of the pioneers of filming microscopic animals with a microscope was Jean Painlevé who produced a series of movies both for scientific purposes and for popular science (Thévenard & Tassel, 1948). Recording rotifers can definitely give more insight into the life of these animals. In the past decades few researchers (e.g. Gilbert, 1963; Viaud, 1940, 1943; Clément 1977a, b) recorded rotifers on cassettes to study behavior such as mating, response to light, speed, trajectory, and predation. Recording movies can also be used for statistical analyses of rotifer (and other organisms) movements. However, more complicated statistical analyses of movements require efficient computational systems of individuals tracking. Such systems started to appear only relatively recently with the advances in development of computers and computer image analyses. Apart of computers, another obstacle, which is often limiting a use of movement analyses to well equipped laboratories is the recording equipment. Until recently good digital cameras were expensive and often out of a reach for science facilities with smaller budget. However, with technological advances also in this field there is now a big choice of cheap equipment, which can be used for scientific purposes. Therefore, it is important that cheap ways of recording movies are developed, possibly using widely-available equipment. Constructing an easy and cheap set-up for recording rotifers will contribute to disseminate video recording as a scientific method and a way of promoting science. For that reason the common Canon DSL camera was used for recording movies. More information on camera and microscope set-up for recording movies can be found in article IV.

3. Results and summary of articles I-IV

The results of research carried out as part of this PhD are presented in four articles, which are summarized on the following pages: 29 -33.

Article I was published in *Hydrobiologia* and focuses on genetic and morphological diversity of *K. cochlearis*. COI gene was sequenced and lorica measured of 248 individuals of *K. cochlearis* sampled in various North Italian lakes. The results of COI-based phylogenetics indicate the existence of eight ESUs with some of them possible to be delimited purely based on morphometrics.

Article II was published in *Hydrobiologia* and focuses on life history and demographic differences between three haplotypes (belonging to two ESUs) of *K. cochlearis*. The results show significant differences for almost all the parameters for all three haplotypes, which indicates that genetically different haplotypes differ also in their life history and demography. Furthermore, the first case of an amphoteric female in *K. cochlearis* was documented.

Article III was published in *Zoologica Scripta* and focuses on mitonuclear discordance in three cryptic rotifer complexes: *K. cochlearis*, *Polyarthra dolichoptera* and *Synchaeta pectinata*. The results show different levels of mitonuclear discordance in all three cryptic species groups. Incomplete lineage sorting and unresolved phylogenetic reconstructions were recognized as most possible causes.

Article IV was published in *Journal of Limnology* and focuses on constructing a simple and cheap set-up and workflow for filming rotifers and *Daphnia* by using a widely-available digital camera and a stereomicroscope. Results highlight the easiness of constructing such a simple video system and its usefulness for both scientist and science educators. Moreover, first videos of *K. cochlearis* males were recorded and published online.

3.1 Article I

High diversity in *Keratella cochlearis* (Rotifera, Monogononta): morphological and genetic evidence.

Cieplinski, A., Weisse, T. and Obertegger, U. (2017). *Hydrobiologia*, 796(1), 145-159.

With advances in DNA phylogenetics there are increasingly more accounts on cryptic species among planktonic animals with rotifers being one of the most studied group. Number of cryptic species complexes are being described for various rotifers, which have previously been considered single species with the most pronounced example being *Brachionus plicatilis* complex with currently confirmed fifteen species (Mills et al., 2017). Here we tried to determine if *Keratella cochlearis* - one of the most widespread and under-studied freshwater rotifers - is in fact a cryptic species complex like many other rotifers from the class Monogononta. Another research question was if it is possible to delimit (and if yes, to what extend) cryptic species based only on morphology of the lorica. First, we sampled seventeen lakes (six lakes were sampled every month) of the Trentino-South Tyrol Region in Italy. Morphological measurements of 248 individuals were confronted with phylogenetics based on sequenced COI genes. Results obtained from three species delimiting methods; generalized mixed Yule coalescent approach, Poisson tree process model and automatic barcode gap discovery, showed consistence rendering all those three methods as valid and legit for rotifer cryptic species delimitation. All three methods resulted in delimitation of eight evolutionary significant units (ESUs) with average uncorrected genetic distance in COI between 12 and 30%, which is higher than the 3% threshold commonly used for delimiting species in most animals. These ESUs can be identified with cryptic species. Furthermore, multivariate analyses of morphological measurements indicated that it is possible to delimit only some of the evolutionary significant units based solely on lorica morphology. This finding corresponds with data of the *B. plicatilis* complex where it is possible to distinguish only few groups based on their morphology (L and S). Moreover, we found some of the detailed morphological features referred by some authors as discriminatory such as spinelets, bended median ridge, and posterior spine in various ESUs. Therefore, rendering them inadequate for cryptic species delimitation in *K. cochlearis.* However, we do not exclude the possibility that more detailed morphological data, possibly obtained with a scanning electron microscope could reveal more distinguishing features specific to only certain cryptic species. We took such pictures for two ESUs and identified few potential lorica features (bended ridge, lateral antennae, bumps in the middle of areolation). However, to confirm the usefulness of such pictures more detailed data is needed. Previous research stated that the correlation between lorica length and the posterior spine length is always positive in *K. cochlearis*. Our findings, however, indicate that there are in fact significant differences between various ESUs. These differences in correlation show that genetic differences in cryptic species complexes also corresponds with differences in morphology, although the exact scope of these differences needs further study. Finally, we found that many of the investigated ESUs co-occurred in the same lakes, but without clear pattern. This co-existence of ESUs corroborates similar data on co-occurrence of cryptic species from the *B. plicatilis* complex.

3.2 Article II

Life history traits and demographic parameters in the *Keratella cochlearis* (Rotifera, Monogononta) species complex.

Cieplinski, A., Obertegger, U., Weisse, T. (2018). *Hydrobiologia, 811*(1), 325-338.

In most cases cryptic species can only be delimited by using DNA-based taxonomic methods. However, some researchers described differences in environmental preferences even between sympatric species from the same cryptic species complex. Evidence is growing that different cryptic species have often different ecological preferences (for example salinity or temperature) and express different life history traits despite their phylogenetic relatedness. These diverse preferences and differences in demography may by important for co-existence in the same environment. To deepen our knowledge on differences between cryptic species we performed life table experiments on three haplotypes of *Keratella cochlearis*, a common freshwater rotifer, which was previously described by us as a cryptic species complex. The three selected haplotypes belonged to two evolutionary significant units (ESUs), were collected from three different lakes and were delimited by using the cytochrome c oxidase subunit 1 (COI) as a barcoding marker gene. Ninety-six rotifers from each haplotype were exposed to identical conditions and observed for the course of their lives. Instantaneous growth rate per day was positive in all three cases indicating growth in all three populations. However, all the population parameters such as generation time, net reproduction rate and the instantaneous growth rate were significantly different in all three haplotypes, which Indicates that populations of the three haplotypes develop at different pace and in a different way. Interestingly, differences were in general statically smaller for the two haplotypes that belonged to the same ESUs. Further, we also described and photographed for the first time deformed ill-swimming females, which most probably were not able to reproduce and were dying after just few days. These so called „abnormal" females were appearing in all the haplotypes but in one particular haplotype in much bigger numbers. We hypothesise that these females could have been an effect of mutations or degeneration of haplotypes. Moreover, we were able to observe - for the first time in *K. cochlearis* - an amphoteric female, which appeared in only one haplotype. Amphoteric females are females that are able to produce both males and females. Only few cases of such females were described for rotifers as they are quite rare and their existence can only be confirmed by conducting life table experiment. The results presented in this article were the first ones to link genetic differences and demographic data for *K. cochlearis* indicating that even small genetic differences can be linked to big differences in life histories. Such differences in life histories, demographic parameters and possibly also environmental preferences are possibly one of the reasons why *K. cochlearis* (and maybe some other cryptic complexes) are so widespread in various habitats. They can cope with a wide spectrum of conditions and adapt easily to new environments.

3.3 Article III

Mitonuclear discordance as a confounding factor in the DNA taxonomy of monogonont rotifers. Obertegger, U., Cieplinski A., Fontaneto D., Papakostas, S. (2018). *Zoologica Scripta,* 47(1), 122-132.

The difference in phylogenies obtained with nuclear and mitonuclear marker is called mitonuclear discordance and is an increasingly recognized phenomenon in many groups of animals. Mitonuclear discordance can lead to wrong species delimitation especially in animals in which morphological delimitation is difficult. Such is the case of cryptic species complexes of rotifers. Here, we investigated the occurrence of mitonuclear discordance in three rotifer species: *Keratella cochlearis, Brachionus plicatilis* and *Synchaeta pectinata.* We also tried to identify potential factors that can cause mitonuclear discordance in these three species complexes. At first we selected individuals from each of the three complexes and sequenced two barcoding genes: cytochrome c oxidase subunit I (COI) and the nuclear internal transcribed spacer 1 (ITS1). We used both maximum-likelihood (ML) and Bayesian inference (BI) approaches to construct phylogenetic trees for both genetic markers. We observed that COI delimitation produces more evolutionary significant units (ESUs) than delimitation based on ITS1. We found that mitonuclear discordance was present in all three species complexes. However, there were differences between cryptic complexes as in *K. cochlearis* incongruence were from the same nodes and for *B. plicatilis* and *S. pectinata* the discordance also appeared on deeper nodes. Our results indicate that in case of *K. cochlearis* ITS1 has a lower delimiting resolution than COI but the incongruence present in two other cryptic complexes are more difficult to explain. In order to investigate further if the mitonuclear discordances were caused by hybridization we performed tests but the results let us rule out this possibility. The inconsistence between topographies performed on two different genes indicate how difficult, but important it is to include multiple loci or even multiple data according to integrative taxonomy approach. We hypothesize that incomplete lineage sorting and unresolved phylogenetic reconstructions were recognized as the most likely drivers causing mitonuclear discordance.

3.4 Article IV

Filming of zooplankton: a case study of rotifer males and *Daphnia magna*.

Colangeli P, Cieplinski A, Obertegger U. (2016). *Journal of Limnology*, 75(1), 204-209.

Recording planktonic organisms on film can give a new inside to their lives since many of their characteristics cannot be fully depicted on pictures. Videos not only allow studying behavior of animals but also allow to see them from different angles giving perception of three dimensions. This is particularly important for small planktonic animals such as rotifers where most of the pictures are static and two dimensional. Movies can also allow to observe features, which are often difficult to notice on pictures, especially when specimen are delicate and do not preserve well through fixation procedure. Such is a case of some rotifer males on which knowledge is still limited. Males of rotifers from the class Monogononta usually appear only under certain conditions and thus are much less common than females. This results in relatively few published film recordings of life rotifer males and in case of some rotifers, such as *Keratella cochlearis* – lack of even photographic documentation of males. Here we built a cheap set-up using a commercial single-lens reflex camera and filmed the behaviors of males of *Keratella cochlearis*, *Brachionus plicatilis* and of *Daphnia magna.* One of the limits of using movies and high quality pictures in scientific work is the high cost of professional cameras. However, with the rapid development of technology, many widely available and cheap digital cameras are able to record very high quality films ready to use in scientific publications. We constructed the recording set-up in a way that can easily be reproduced also using different equipment. Our movies allowed us to observe small features, which are difficult to notice on pictures. For example in *B. plicatilis*, a retractable foot (on which retractility there was not agreement before), moving spermatozoa or excretion granules of *B.* males. We also tried to observed male-female interaction for both *K. cochlearis* and *B. plicatilis*. We observed one mating in *B. plicatilis* and no matings in case of *K. cochlearis*. The interesting fact about *K. cochlearis* is that there exists no records on mating between males and females in this species complex but still males occasionally appear. On one of our movies we were also able to observed a thread-like structure between mating pair of *B. plicatilis*. Very few records of this structure exist and our video is probably one of the first one. It is also the first published video of very poorly-studied *K. cochlearis* males. Further, we also constructed a simple set-up to record *D. magna's* migration away from UVA radiation. Our set-up does not require expensive equipment or technical skills and can easily be assembled and used outside research institutions, for example in schools and science centers or museums. Therefore, we placed all the videos on YouTube platform, where they can be easily accessible by everyone and possibly inspire wider audience to observe or perform research on plankton.

3.5 Conclusions

The purpose of this thesis was to assess and describe the genetic and morphological variety of the common freshwater rotifer *K. cochlearis* collected from various Alpine lakes. Further, I investigated if (and if yes, to what extend) these genetic differences are reflected in demography and reproductive strategies of this cryptic complex. This main objective of this thesis can be further described by five specific aims which were the basis for my research:

1) To assess the presence of a cryptic species complex in *K. cochlearis*

2) To investigate if genetic differences are reflected in morphological differences between cryptic entities.

3) To investigate if genetically different strains of *K. cochlearis* show differences in their life histories and demographic parameters.

4) To investigate mitonuclear discordance as a confounding factor in *K. cochlearis* delimitation.

5) To observe and record males of *K. cochlearis* and to record its mating behavior.

The result of the first article showed that using common barcoding gene - cytochrome c oxidase subunit I (COI) and three delimiting methods (generalized mixed Yule coalescent, the Poisson tree process model and the automatic barcode gap discovery) it is possible to delimit Evolutionary Significant Units (ESUs) (1^{st} aim of the PhD). These ESUs can be associated with cryptic species and that is why article I is the first one to describe cryptic species complex in *K. cochlearis*. The results of the three delimiting approaches were coherent, implying the existence of strong cladistic divisions between species from within the complex. Such matching data indicate the utility of each of the used delimiting approaches. Corresponding morphological data allowed to separate only two of eight ESUs rendering morphological data not adequate for delimiting *K. cochlearis* cryptic species (2^{nd} aim of the PhD). Even though cryptic species can be almost identical from a morphological point of view, the differences may still show up in life histories or environmental preferences. The results of article II indicate that indeed there are significant differences in almost all life and demographic parameters between not only ESUs but even haplotypes belonging to one ESU of *K. cochlearis* (3^{nd} aim of the PhD). The results of article II point to a very large demographical and behavioural variety within the cryptic complex of *K. cochlearis.* Since integrative taxonomy is gaining more popularity, multi-locus phylogenetic are favoured over single-locus delimitation approaches. However, using two different genes can lead to inaccuracies such as mitonuclear discordance. Results of article III indicate that for three common rotifer cryptic complexes, i.e. *K. cochlearis, Brachionus plicatilis* and *Synchaeta pectinata*, mitonuclear discordance is a common phenomenon (4^{th} aim of the PhD). Interestingly, in case of *K. cochlearis* ESUs delimited by COI nested within ESUs delimited by ITS1, whereas in *B. plicatilis* and *S. pectinata* there was a discordance also at deeper nodes. However, no traces of hybridization were found. The data also confirm earlier findings that in case of rotifers ITS1 has a lower taxonomic resolution than COI as it produces generally fewer ESUs. There are many possible factors that can cause mitonuclear discordance; however, incomplete lineage sorting was the most probable source for it in case of the three investigated cryptic complexes. However difficult it is to lump together phylogenies based on

few genes, integrative taxonomy yields more reliable results than the single-locus approach. In article IV we present for the first time movies of life males of *K. cochlearis,* which we recorded with a simple video set-up (5th aim of the PhD). However, we were unable to observe any male-female interaction in our strains, leaving the question whether sexual reproduction is common in *K. cochlearis* open for further research.

In conclusion, we described for the first time the cryptic species complex of *K. cochlearis* based on COI and ITS1 genes and detailed morphology. Further, we also showed how even slight genetic differences can translate into demographic differences, which can further explain a wide ecological tolerance of some cryptic species. Moreover, we were able to record first movies of *K. cochlearis* males but the existence of sexual reproduction remains in question and requires further studies.

3.6 Perspectives for future research

This study is the first comprehensive description, including genetic, morphological and lifetable data of the rotifer *Keratella cochlearis*. Based on these data, I developed perspectives for future research.

The first observation relates to integrative taxonomy. Based on genetic data (article I) combined with life history data (article II), large differences between cryptic species were identified. Therefore, I suggest that future research on cryptic species should incorporate (if possible) not only genetic data but also information from other fields following the approach of integrative taxonomy. I also recommend that more than one gene is used in phylogenetic analyses due to problems such as mitonuclear discordance described in article III.

Together with my collaborators, I was able to analyze life histories of only few haplotypes of *K. cochlearis*. As next step, life histories of other haplotypes of *K. cochlearis* should be analyzed and compared with phylogenetic data. Such experiments should enhance our knowledge on to what extent genetic variability translates to demographic variety and therefore, leading to an improved understanding of the evolution of cryptic species.

Sexual reproduction has been studied in many monogonont rotifers but not in *K. cochlearis*. In fact, most descriptions of sexual reproduction of *K. cochlearis* are based on comparisons with other species. During our observations - article IV - we were not able to observe any male-female interactions nor were any such interactions described in the literature. Therefore, the appearance of males in *K. cochlearis* is a puzzling phenomenon and requires further studies in order to establish if sexual reproduction appears in this species at all. As next step, a set of experiments aiming to record male-female interaction of *K. cochlearis* should be established to investigate if sexual reproduction occurs at all in this species. Preferably, various clones and ESUs of various age and relatedness should be used as all these characteristics may influence sexual reproduction. For recording movies of rotifers an easy and reliable system can be used, similar to the one described by us in article IV.

Next, I suggest a series of experiments in order to establish the trigger for male-appearance. Such triggers can differ, dependent on the species (King & Snell, 1980; Snell & Boyer, 1988; Gilbert & Schröder, 2004); however, the nature of such trigger in *K. cochlearis* remains elusive. Describing the male-appearance trigger could help us understand the mechanisms

of sexual reproduction (or the lack thereof) in *K. cochlearis*. Another approach to investigate if sexual reproduction appears in *K. cochlearis* would be to collect lake sediment from the lakes where *K. cochlearis* is abundant and trying to hatch individuals from resting eggs. Such eggs - products of sexual reproduction - are very common in monogonont rotifers and in other planktonic animals (e.g., *Daphnia, Cyclops*). However, resting eggs have never been described for *K. cochlearis*. Lakes to be chosen for such sampling should be preferably those that freeze over during winter or where conditions deteriorate rapidly in some periods during the year (for instance, lakes with strong seasonal change in water level). Such disturbing events usually impose a high pressure on zooplankton, and the survival in such environments is usually facilitated by resting eggs.

However, if resting eggs do not exist in *K. cochlearis* and there is no sexual reproduction, this could lead to a change in our understanding of reproduction in this species and in rotifers in general.

This thesis describes differences in various ESUs and haplotypes of *K. cochlearis* and, therefore, provides a basis for future research on this cryptic species complex. Moreover, the results of my articles indicate difficulties, which may appear in the delimitation process of cryptic species (such as mitonuclear discordance or co-existence).

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ARTICLE I

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ROTIFERA XIV

High diversity in *Keratella cochlearis* (Rotifera, Monogononta): morphological and genetic evidence

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Abstract Rotifers are ubiquitous freshwater animals for which many complexes of cryptic species (i.e. distinct species that are morphologically difficult to distinguish) are described. *Keratella cochlearis* occurs globally and shows a wide phenotypic diversity indicating the potential presence of a species complex. We sampled lakes of the Trentino-South Tyrol region (Italy) and investigated mitochondrial genetic diversity in *K. cochlearis* in relation to detailed lorica measurements. We sequenced the mitochondrial cytochrome *c* oxidase subunit I and used the generalised mixed Yule coalescent approach, Poisson tree process model and automatic barcode gap discovery to delimit mitochondrial groups, associated with putative evolutionary significant units (ESUs). Based on 248 sequences, eight putative ESUs were indicated that could only partially be delimited by lorica morphology. Specifically, several morphological characteristics (i.e. spinelets, bended median ridge, and posterior spine) were found in specimens of different putative ESUs, and thus, these characters seem to be of poor discriminatory value. Furthermore, different putative ESUs of *K. cochlearis* were found in the same lake. We conclude that the high mitochondrial genetic diversity may be linked to tolerance of *K. cochlearis* to varying environmental conditions.

Keywords Rotifera · GMYC · PTP · Lorica measurements · NMDS · Lauterborn

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Introduction

Biodiversity is currently under threat, and our perception of species loss is highly dependent on accurate estimates of species richness. However, estimates of species richness are often impaired by the occurrence of cryptic species (i.e. species that are impossible or difficult to distinguish based on their morphology) in diverse groups such as protists (Foissner, 2006), ants (Fournier et al., 2012), harvestmen (Arthofer et al., 2013), and rotifers (Gómez & Snell, 1996). Understanding how and why species occur is one of the fundamental aspects in ecology (Gaston, 2000).

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Evidence on cryptic species diversity in rotifers, subclass Monogononta, is growing and challenges our understanding of rotifer biodiversity. In monogonont rotifers, cryptic species complexes have been described for species such as *Brachionus plicatilis* (Gómez & Serra, 1995; Gómez & Snell, 1996; Gómez et al., 2002), *B. calyciflorus* (Schröder & Walsh, 2007; Xi et al., 2011), *Epiphanes senta* (Gilbert & Walsh, 2005), *Lecane* spp.(García-Morales & Elías-Gutiérrez, 2013), *Polyarthra dolichoptera* (Obertegger et al., 2014), *Synchaeta* spp. (Obertegger et al., 2012), and *Testudinella clypeata* (Leasi et al., 2013). The occurrence of cryptic species is often related to rotifer ubiquity and their wide tolerance to environmental parameters such as salinity (Ciros-Pérez et al., 2001a), temperature (Gómez & Snell, 1996; Ortells et al., 2003; Papakostas et al., 2012) or total phosphorus (Obertegger et al., 2012).

Keratella cochlearis Gosse, 1851 can be found in most freshwater lakes and ponds all over the world (Green, 1987). In fact, the whole genus *Keratella* is considered eurytopic and cosmopolitan (Segers & De Smet, 2008), and this makes the genus a good candidate for investigating the occurrence of cryptic species. Lauterborn (1900) described several morphotypes in *K. cochlearis*, and his detailed descriptions and drawings were the basis for following taxonomic work (e.g. Ahlstrom, 1943; Ruttner-Kolisko, 1974; Koste, 1978). The morphotypes described by Lauterborn (1900) encompass three series (*macracantha–typica–tecta*, *hispida*, and *irregularis*) and the group of *robusta*. These morphological varieties of *K. cochlearis* are different with respect to lorica length (LL), spine length, presence of spinelets on the lorica, and the course of the median ridge. Here, we give an overview of the Lauterborn (1900) series and a German to English translation of Lauterborn's (1900) descriptions. In the *macracantha–typica–tecta* series (Lauterborn's 1900, Figs. 1–10), the posterior spine is as long as the lorica or even longer, and the basis of the spine is so wide that it is difficult to decide where the spine begins and the lorica ends. The areolation is present on half of the spine, and only the distal part is smooth and pointed. In lateral view, the spine points to left or right, and this is according to Lauterborn (1900) not an important feature. Along the series, the reduction of the posterior spine is notable until it disappears completely. Lauterborn (1900) concluded that it is impossible to draw a line between the different morphotypes of the *macracantha–typica–tecta* series that only differ in size and posterior spine length (PSL). The morphotypes of the *hispida* (Lauterborn's 1900, Figs. 11–14) and *irregularis* series (Lauterborn's 1900, Figs. 15–20) show different morphological elements with respect to the *macracantha–typica–tecta* series, and size differences are not important. In the *hispida* series, small spines (called "Pusteln" after Lauterborn, 1900 and "spinelets" after Ahlstrom, 1943) are present and can be so dense that the areolation and the borders of the plates become invisible. The morphotypes of the *hispida* series can be considered the *forma punctata* of the *tecta* series. Only for Lauterborn's (1900), Fig. 11, closely related to *macracantha*, and for Lauterborn's (1900), Fig. 27, closely related to *tecta*, the name *forma punctata* is given. In the *irregularis* series, the ridge is bended to the left in dorsal view, and a displacement of the facets is visible that leads to pointed bumps (called "Höcker" by Lauterborn, 1900) on the facets and an additional facet (called facet *X* by Lauterborn, 1900). In addition, the basal margin is divided into small posterior carinal facets. Similar to the *hispida* series, the lorica has small pointed spinelets on the intersection of the areolation. The *robusta* group (Lauterborn's 1900, Figs. 21–23) is not a series because no direction of morphological variations can be distinguished. Characteristic for this group is the wide base of the posterior spine that is the elongation of the ventral part of the lorica, the hooked form of the anterior spines, and the slightly bended median ridge.

Considering the wide morphological variability of *Keratella* morphotypes, Lauterborn (1900) already hypothesised a subspecies status of some morphotypes. In fact, Ahlstrom (1943) and Eloranta (1982) erected the series *irregularis* and *hispida* to separate species. However, Hofmann (1983), who did not recognise transitional forms between the morphotypes *cochlearis*, *irregularis*, and *tecta* as described by Lauterborn (1900), questioned the validity of the Lauterborn cycles. Especially, the presence and length of the posterior spine seems to be a morphological character whose suitability for discriminating species is questionable. In eutrophic habitats, *K. cochlearis* tends to be smaller and has smaller posterior spines than in oligotrophic habitats (Green, 2007).

Furthermore, LL and PSL are longer with decreasing water temperature (e.g. Green, 1981, 2005; Bielanska-Grajner, 1995) and in the presence of predators (Conde-Porcuna et al., 1993; Green, 2005). Water conditioned with predators (i.e. *Asplanchna* spp., cyclopoid copepods) can induce spine formation in offspring of *tecta* (Stemberger & Gilbert, 1984). Derry et al. (2003) found a high mitochondrial genetic difference [4.4% cytochrome *c* oxidase subunit I (COI) sequence divergence] between spined and spineless individuals of *K. cochlearis* and hypothesised the presence of cryptic diversity within these morphotypes. Furthermore, the various morphotypes of *K. cochlearis* show different tolerances to temperature (Berziņš & Pejler, 1989a), oxygen content (Berziņš & Pejler, 1989b), trophic state, and conductivity (Berziņš & Pejler, 1989c). The wide tolerances to environmental conditions could also indicate that *K. cochlearis* is a cryptic species complex composed of species with narrower ecological preferences than when taken as a complex.

Here, we identified mitochondrial DNA (mtDNA) groups and compared their lorica morphology in a complementary approach as recommended by Schlick-Steiner et al. (2006), Fontaneto et al. (2015) and Mills et al. (2016). Combining genetic information with other species-bound aspects such as species morphology and ecology or biochemistry of species habitat can result in a more robust species delimitation than when using genetic information alone (Schlick-Steiner et al., 2006; Fontaneto et al., 2015; Mills et al., 2016). We hypothesised that *K. cochlearis* is a complex of putative evolutionary significant units (ESUs) and that it is possible to delimit ESUs based on lorica measurements. In fact, in *B. plicatilis* some clusters of cryptic species [*B. plicatilis* (sensu stricto) L., *B. rotundiformis* SS, *B. rotundiformis* SM] can be distinguished based on body length differences (Ciros-Pérez et al., 2001b). Closely related species might have similar niches according to the phylogenetic niche conservatism theory (e.g. Wiens & Graham, 2005; Wiens et al., 2010), and this may lead to competitive exclusion (Violle et al., 2011). Thus, ESUs with their close phylogenetic relationship might be especially prone to competitive exclusion; however, co-occurrence of rotifer cryptic species has been reported (Obertegger et al., 2014). Thus, we also investigated temporal co-existence of putative ESUs of *K. cochlearis* and hypothesised little co-occurrence.

Materials and methods

Sampling

From March to November 2014, six lakes in the Trentino-South Tyrol (Italy) region were sampled monthly. These lakes (called further the "core lakes") cover a wide range of environmental parameters (Table 1). In addition, we also sampled 11 additional lakes from Trentino-South Tyrol in the years 2010, 2013, and 2015 during summer and winter to cover a larger geographical area and altitudinal range (Table 1; Fig. 1). Environmental parameters were based on published data (IASMA, 1996–2000) and own analyses (Table 1). At the deepest site of each lake, plankton samples were collected with a 20 µm (Apstein) or 50 µm (Wisconsin) plankton net depending on lake depth. Both mesh sizes were small enough to effectively collect specimens of *K. cochlearis* (length >74 µm, width >60 µm; Lauterborn, 1900; Koste, 1978).

Measurements of specimens and morphological observations

For the core lakes and Lake Caldonazzo (July sample), single specimens of *K. cochlearis* were isolated under a stereomicroscope and photographed (Leica DC 300F camera, Leica IM1000 software) in dorsal and lateral view under a compound microscope. The following measurements were taken: PSL, LL excluding anterior and posterior spines, total LL (TLL) including all appendages, lorica width (LW) at its widest part, LW at the mouth opening region ("head width", HW), anterolateral dorsal spine length (ALS), anterointermediate dorsal spine length (AIS), anteromedian dorsal spine length (AMS), and posterior spine angle (PSA, Fig. 2). For the measured specimens, we also observed the main characteristics of the dorsal plate, important to discriminate morphotypes. Measured specimens were subject to DNA extraction and sequencing. However, we could not obtain sequences for all measured specimens.

DNA extraction and amplification

Specimens of *K. cochlearis* from the core and the additional lakes were sequenced to investigate presence of putative ESUs. Cryptic species complexes in

Table 1 Environmental data on sampled lakes

Lakes	Alti	Area	Depth	TP	NO_3	Si	SO_4	Cl	pH	Cond	Temp	Trophic states
Kaltern[c]	215	131	5	13	1,006	2	74	8	8.3	507	18	Meso
Terlago[c]	414	11.9	10	31	885	3.2	14.4	5.8	8.0	389	23	Eu
Levico	440	116.4	38	15	225	2.65	36	5	8	275	14	Meso
Caldonazzo	449	562.7	47	21	314	3.7	26.3	5.8	8.0	312	22	Meso
Großer Montiggler	492	17.8	12.5	50	13	0.55	9.6	8.5	7.9	293	6	Eu
Canzolino	540	7.1	15	56	510	3.6	27	4.5	7.4	257	23	Eu
Vahrn[c]	678	1.5	3.5	13	70	3.3	5.3	1.4	6.6	57	23	Meso
Raier Moos	835	0.7	5	39	0	2.7	19.6	7.9	8.3	368	19	Eu
Serraia	974	44.4	17	34	458	9.7	7.3	2.8	7.6	116	22	Eu
Völser Weiher	1,056	1.7	4	14	71	0.4	11.3	0.9	24	252	24	Meso
Lavarone	1,100	5.2	15	28	276	2.6	8	6.9	7.8	291	21	Eu
Wolfsgruben	1,176	3.9	5.4	33	55	2.0	9.3	3.1	8	114	8	Eu
Tovel[c]	1,178	38.2	39	4	318	1.3	1.7	0.3	7.9	192	15	Oligo
Antholz	1,642	43.3	38	7	226	2.6	12.6	0.5	7.5	90	17	Oligo
Glittner[c]	2,151	0.05	1	129	11	0.2	0.6	0.4	6.1	9	12	Meso
Radl[c]	2,258	0.8	6	13	21	0.5	15	0.4	7.7	92	13	Meso
Crespeina	2,374	0.6	7	11	30	0.2	1.5	0.2	8.8	157	12	Oligo

The superscript [c] indicates the core lakes ordered by altitude (alti, m above sea level): area (×10,000 m^2), depth (m), total phosphorus (TP, µg l^{-1}) at spring overturn, nitrate (NO_3, µg l^{-1}), reactive silica (Si, mg l^{-1}), sulphate (SO_4, mg l^{-1}), chloride (Cl, mg l^{-1}), conductivity (cond, µS cm^{-1}), mean summer surface temperature (temp), and trophic state (*eu* eutrophic, *meso* mesotrophic, *oligo* oligotrophic)

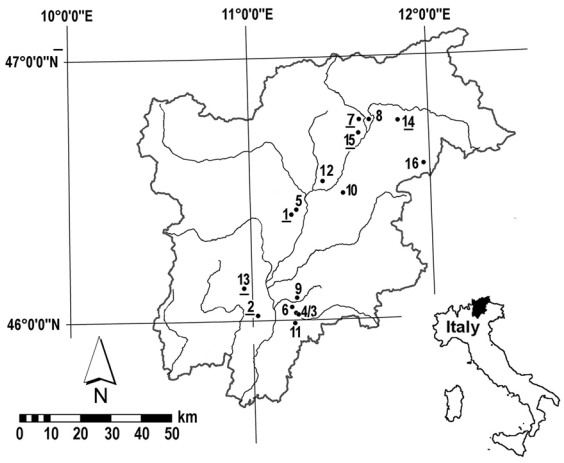

Fig. 1 Sampled lakes in the Trentino-South Tyrol region, (*1*) Kaltern[c], (*2*) Terlago[c], (*3*) Levico, (*4*) Caldonazzo, (*5*) Großer Montiggler, (*6*) Canzolino, (*7*) Vahrn[c], (*8*) Raier Moos, (*9*) Serraia, (*10*) Völser Weiher, (*11*) Lavarone, (*12*) Wolfsgruben, (*13*) Tovel[c], (*14*) Glittner[c], (*15*) Radl[c], and (*16*) Crespeina; core lakes (superscript [c]) are *underlined* on the *map*

rotifers are often inferred based on the mitochondrial COI (Suatoni et al., 2006; Obertegger et al., 2012, 2014; Leasi et al., 2013; Fontaneto, 2014; Malekzadeh-Viayeh et al., 2014). We extracted DNA from single live individuals with 35 µl of Chelex (InstaGeneMatrix, Bio-Rad, Hercules, CA, USA). The COI gene was amplified using LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTGG-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) primers (Folmer et al., 1994). PCR cycles consisted of initial denaturation at 95°C for 10 min, followed by 50 cycles at 95°C for 45 s, 46°C for 45 s and 72°C for 1.05 min, and a last step at 72°C for 7 min. For each sample, we used 2 µl of DNA extract and 23 µl of master mix solution. Master mix proportions for one sample were 12.7 µl distilled water, 2.5 µl of buffer, 3.5 µl $MgCl_2$ (25 mM), 1 µl primer HCOI2198, 1 µl primer LCOI1490, 2 µl dNTP (10 mM), and 0.3 µl AmpliTaq Gold® 360 DNA polymerase (Thermo Fisher Scientific, Italy). For post-PCR purification, we used ExoSAP-IT® PCR product cleanup (Affymetrix USB, USA).

Phylogenetic reconstruction

We constructed the phylogenetic tree using a maximum likelihood (ML) and Bayesian inference (BI)

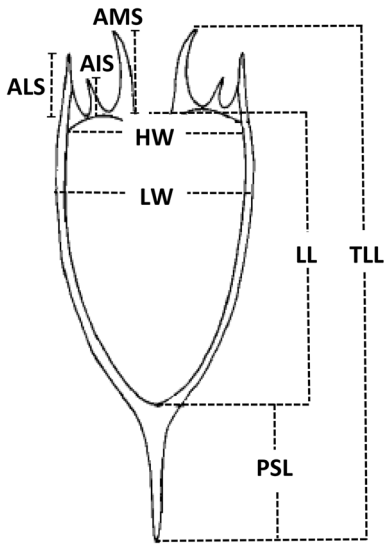

Fig. 2 Lorica drawing of *K. cochlearis* with measured parameters; lorica length excluding anterior and posterior spines (*LL*), posterior spine length (*PSL*), total lorica length including all appendages (*TLL*), lorica width at its widest part (*LW*), posterior spine angle (PSA), anterolateral dorsal spine length (*ALS*), anterointermediate dorsal spine length (*AIS*), anteromedian dorsal spine length (*AMS*), and lorica width beneath the anterior spines ("head width", *HW*)

approach. The model of evolution for the phylogenetic reconstruction was HKY + *I* + *G*, selected with ModelGenerator v0.85 (Keane et al., 2006). The selected model was implemented into PhyML 3.0 (Guindon & Gascuel, 2003) to perform ML reconstruction using the approximate likelihood ratio test to evaluate node support. For BI, we used BEAST v1.8.0 (Drummond et al., 2012) with the following settings: uncorrelated lognormal relaxed clock (mean molecular clock rate set as normal), HKY + *I* + *G* substitution model, and the birth–death model. The posterior probability distribution was estimated with Markov chain Monte Carlo (MCMC) sampling, which was run for 100 million generations, sampling every 10,000th generation. We used Tracer v1.5 (Rambaut et al., 2014) to investigate for convergence and the correctness of the MCMC model and to determine the burn-in. We used TreeAnnotator v1.7.5 to summarise trees and discard the first 2,000 trees as burn-in. As outgroup sequences, we used *B. urceolaris* (Genbank accession number EU499787), *B. rotundiformis* (JX239163), and *B. plicatilis* (JX293050), all belonging to the same family (i.e. Brachionidae) as *Keratella*.

Inference of mtDNA groups

We inferred mtDNA groups within *K. cochlearis* with the generalised mixed Yule coalescent (GMYC) approach (Fujisawa & Barraclough, 2013), the Poisson tree process model (PTP; Zhang et al., 2013), and the automatic barcode gap discovery (ABGD; Puillandre et al., 2012) and compared the results. For all methods, the outgroup was excluded prior to the analyses. We took the results of the GMYC approach as our baseline results because previously rotifer diversity was investigated by it for different species (Obertegger et al., 2012, 2014; Leasi et al., 2013; Malekzadeh-Viayeh et al., 2014). The GMYC approach is based on branching rates along an ultrametric tree (here from BEAST) to distinguish between species-level (Yule, slower) and population-level (coalescent, faster) branching rates. This model identifies GMYC ESUs. For the GMYC approach, we used R 3.0.2 (R Core Team, 2012), library splits (Ezard et al., 2009). The PTP model (http://species.h-its.org) uses a phylogenetic tree as input (here the ML tree produced in PhyML 3.0.) and applies coalescent theory to distinguish between population-level and species-level processes. Similarly to GMYC, PTP assumes that there are less intraspecific substitutions than interspecific substitutions because they have less time to accumulate. This method does not require an ultrametric tree and has been shown to match other methods of species delimitation in rotifers (Tang et al., 2014) and copepods (Blanco-Bercial et al., 2014). Two types of PTP were used: ML (PTP-ML) approach and Bayesian approach (PTP-BA). The ABGD (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html) deliminates species without any a priori assumptions. It detects the gaps in the distribution of genetic pairwise distances. This method has been successfully used to delimit species of the meiofauna (Tang et al., 2012; Leasi et al., 2013). Here, all aligned *K. cochlearis* sequences were used for ABGD.

We based our phylogenetic reconstructions and inference of mtDNA groups on a single mitochondrial gene (COI), and this may gave a biased estimate on genetic diversity. A higher evolutionary rate of COI with respect to other nuclear markers (Tang et al., 2012), mitochondrial introgression (reported for *B. calyciflorus* by Papakostas et al., 2016 but not for *E. senta* by Schröder & Walsh, 2010), and/or unresolved ancestral polymorphism (Funk & Omland, 2003)

could bias our inference on species diversity. Recently, it has also been shown that the methods we used give biased results in species poor datasets (Dellicour & Flot, 2015). Thus, considering this uncertainty, our statements are about putative ESUs based on the inference of mtDNA groups.

Statistical analysis of measurements in relation to putative ESUs

Green (1981, 1987) reports a positive correlation between LL and PSL in *K. cochlearis* from various lakes of the Auvergne region in France. To assess the general validity of this correlation, we considered only those specimens that were measured and for which we obtained COI sequences. We divided specimens into putative ESUs and investigated the sign and significance of the correlation (Pearson correlation coefficient; r_P) between LL and PSL.

We performed a univariate statistical analysis and a multivariate ordination method to investigate if putative ESUs could be distinguished based on morphology. As univariate statistical analysis, we used a one-way ANOVA and post hoc Tukey multiple comparisons. We performed generalised least squares modelling to allow for dependence of measurements of ESUs coming from the same lake and checked homogeneity of residuals graphically. As multivariate ordination method, we performed non-metric multidimensional scaling (NMDS). In NMDS, Bray–Curtis distance matrix was used on centred and standardised measurement data. In NMDS, the goodness of fit was investigated by the Shepard plot that shows the relationship between the inter-object distances in NMDS and Bray–Curtis dissimilarity. The residuals of this relationship were used to calculate Kruskal's stress (S); S values <0.2 are considered statistically meaningful (Quinn & Keuogh, 2002). We, furthermore, performed a linear discriminant analysis (LDA) to investigate the discriminatory power of lorica morphology to separate ESUs. We tested for homogeneity of within-ESU covariance matrices.

We also investigated the correlation between phylogenetic and morphological diversity. Phylogenetic diversity was calculated as distance matrix based on the ultrametric tree, and morphological diversity as a distance matrix based on mean morphological values of ESUs. The correlation between both distance matrices was investigated by a Mantel test.

For statistical analyses, we used the library nlme (Pinheiro et al., 2012), MASS (Venables & Ripley, 2002), vegan (Oksanen et al., 2015), and multcomp (Hothorn et al., 2008) in R 3.0.2 (R Core Team, 2012).

Results

Inference of putative ESUs

We obtained 248 sequences of the COI gene of *K. cochlearis* (Genbank accession number: supplementary material Table s1). These sequences comprised 57 haplotypes. The GMYC approach indicated eight ESUs (single threshold GMYC: likelihood of the null model = 261.4; likelihood of the GMYC approach = 269.5; $P < 0.001$; confidence interval = 8–14), that are hereafter called GMYC ESUs. Uncorrected genetic distances within GMYC ESUs were below 6.2% with ESU 5 showing the lowest and ESU 8 the highest within-ESU distance (Table 2). Distances between GMYC ESUs ranged from 9% (ESU 7 vs. 8) to 33% (ESU 8 vs. 3) with an overall average value of 21% (Table 3).

The ABGD and the PTP-ML grouped the same haplotypes in the same ESUs as GMYC (Fig. 3). However, PTP-BA, split GMYC ESU 3 into three and ESU 6 into five units (Fig. 3).

GMYC ESUs occurrence in lakes

GMYC ESUs 3 and 7 were found in seven lakes, ESU 8 in six, ESU 5 in five, and ESU 4 and 1 were found only in two and ESU 2 only in one lake (Fig. 3; Tables s2, s3 supplementary material). Considering temporal co-existence of GMYC ESUs in the core lakes, no clear pattern emerged (Table s3 supplementary material). Generally, GMYC ESUs co-occurred, except for ESU 2 that was found only once in Lake Radl, despite monthly sampling during summer 2013. ESUs 3 and 7 co-occurred most often in different lakes. ESU 3 was almost always present throughout the sampling period in Lakes Kaltern and Terlago (Table s3 supplementary material); similarly, ESU 5 in Lake Glittner and ESU 6 in Lake Tovel were present

Table 2 Report of the uncorrected genetic distances within GMYC ESUs of *K. cochlearis*, number of haplotypes, number of individuals, and mean, median, minimum (min), and maximum (max) of distances

GMYC ESUs	Individuals	Number of haplotypes	Mean	Median	Min	Max
ESU 1	2	1				
ESU 2	5	1				
ESU 3	60	13	0.02	0.04	0.000	0.05
ESU 4	8	4	0.01	0.02	0.002	0.02
ESU 5	65	3	0.01	0.01	0.010	0.01
ESU 6	67	8	0.02	0.01	0.002	0.04
ESU 7	13	12	0.02	0.01	0.002	0.04
ESU 8	28	15	0.02	0.02	0.002	0.06

Table 3 Report of the uncorrected genetic distances between GMYC ESUs of *K. cochlearis*, mean, and median values equal to the second decimal point (mean ≈ median), minimum, and maximum (min|max) values of distances

	ESU 1	ESU 2	ESU 3	ESU 4	ESU 5	ESU 6	ESU 7
Mean ≈ median							
ESU 2	0.29						
ESU 3	0.21	0.28					
ESU 4	0.22	0.27	0.18				
ESU 5	0.22	0.28	0.18	0.19			
ESU 6	0.23	0.28	0.20	0.20	0.12		
ESU 7	0.21	0.30	0.18	0.19	0.15	0.15	
ESU 8	0.22	0.27	0.18	0.19	0.15	0.14	0.13
Min|Max							
ESU 2	0.29						
ESU 3	0.20|0.23	0.27|0.28					
ESU 4	0.22|0.23	0.27|0.28	0.16|0.19				
ESU 5	0.22	0.28|0.28	0.18|0.19	0.19|0.20			
ESU 6	0.22|0.24	0.28|0.29	0.19|0.20	0.18|0.21	0.11|0.13		
ESU 7	0.18|0.23	0.29|0.34	0.15|0.21	0.16|0.21	0.12|0.16	0.11|0.17	
ESU 8	0.20|0.24	0.26|0.33	0.16|0.20	0.18|0.23	0.14|0.17	0.09|0.17	0.11|0.15

throughout the sampling period (Table s3 supplementary material).

Morphology

We obtained lorica measurements from 138 individuals of *K. cochlearis* that could also be attributed to GMYC ESUs based on their COI sequence (Table 4; Table s3 supplementary material). For ESUs 1 and 2, no measurements were obtained, and for ESU 7, only one specimen was measured (Table 4). All specimens of ESU 4 and three specimens of ESU 6 did not have a spine, while the other measured specimens had a spine of varying length (Table 4).

The correlation between LL and PSL was different when based on all specimens ($r_P = 0.68$; $P < 0.001$) compared to splitting it into GMYC ESUs: for ESUs 3 and 6, the correlation was higher ($r_P = 0.76$ and 0.77, respectively; $P < 0.001$) than the overall one, and no correlation was found for ESU 4 (spineless specimens), ESU 5 ($r_P = 0.13$; $P = 0.41$), and ESU 8 ($r_P = 0.76$; $P = 0.13$; Fig. 4).

We tested for significant differences in LL, PSL, and PSA between GMYC ESUs by ANOVA and following post hoc multiple comparisons tests by mixed modelling. LL and PSA were different between four ESUs, and PSL differed between three ESUs (Table 5). Based on all three measurements, ESU 8 was different from ESUs 3 and 5 (Table 5).

In NMDS with all measurements ($S = 0.13$), a gradient from specimens of ESU 5 to specimens of ESU 4 and spineless specimens of ESU 6 was evident. To get a clearer picture on the relationships between ESUs with spines, we excluded ESU 4 and the three

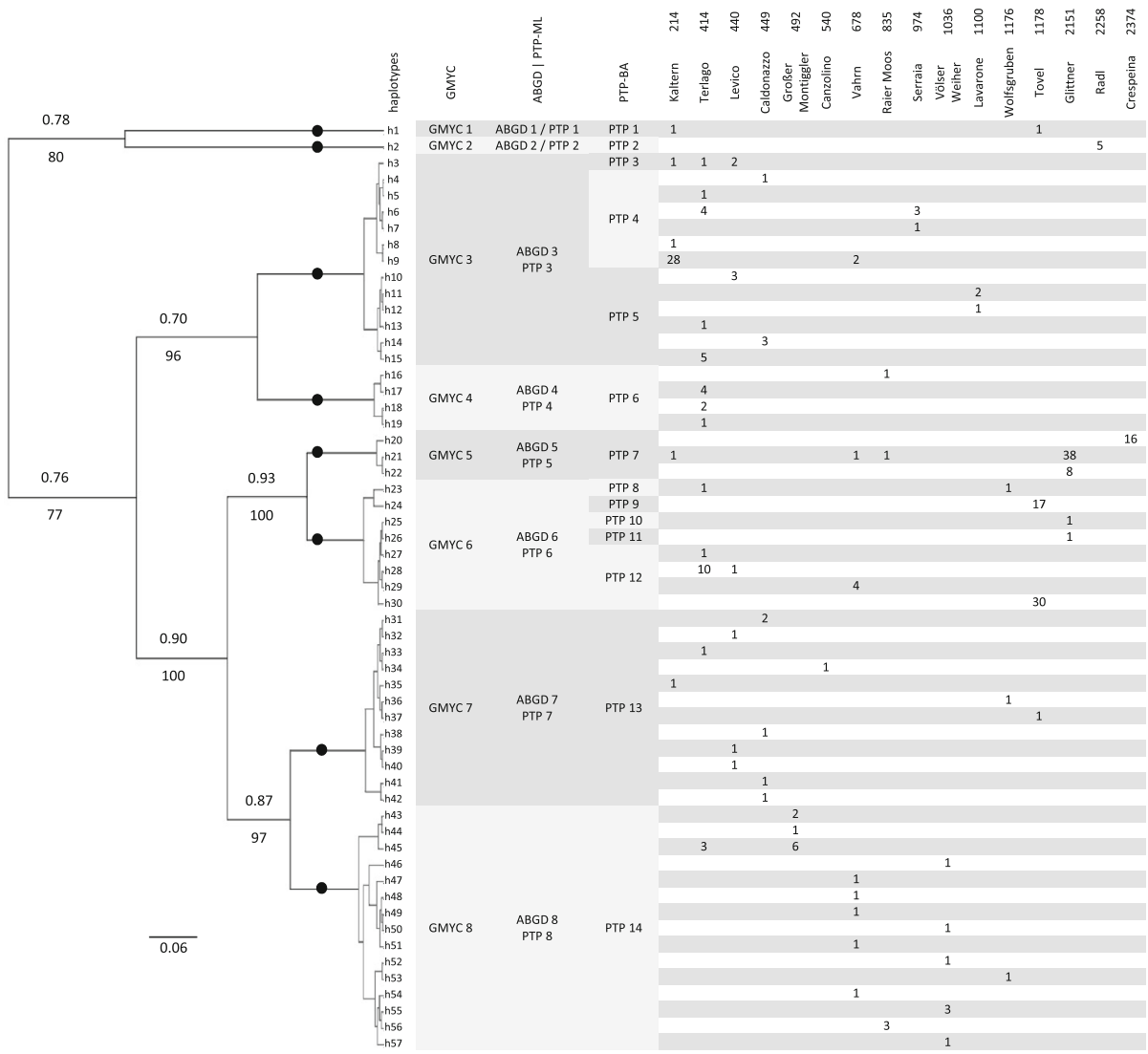

Fig. 3 Phylogenetic relationships of the 57 COI haplotypes of *K. cochlearis*. The phylogenetic *tree* was created with Bayesian interference analysis showing all compatible groupings and with average branch lengths proportional to numbers of substitutions per site under a HKY + I + G substitution model. Posterior probabilities from the Bayesian reconstruction and approximate likelihood ratio test support values from the maximum likelihood are shown below and above each *branch*, respectively. The inference of putative ESUs by *GMYC*, *ABGD*, and *PTP* based on maximum likelihood (PTP-ML) and Bayesian inference (PTP-BI) is shown. Lakes were sorted according to increasing altitude (elevation in the *upper line*, metres above sea level). The *number* of sequences for each haplotype per lake is given in each *line*

spineless specimens from ESU 6 from the NMDS analysis. In this NMDS with measurements of spined individuals ($S = 0.17$), specimens of ESUs 5 and 8 formed distinct clusters while specimens from ESUs 3 and 6 were mixed (Fig. 5). In the LDA based on PSL, LL, and PSA, the percent correct assignment of ESUs varied (ESU 3: 69%, ESU 5: 83%, ESU 6: 53%, ESU 8: 50%).

We noted the presence of spinelets (Fig. 6), additional facets, and bending of the ridge (Fig. 4) in some specimens and linked these characteristics to their association to GMYC ESUs. We observed across ESUs the presence of spinelets, additional facets, and bending of the ridge (Table 6). In addition, we observed small humps in the middle of the areolation section and the symmetrically situated lateral antenna (Fig. 6).

Table 4 Length measurements of main lorica characteristics based on 138 specimens of *K. cochlearis*, lorica length (excluding anterior and posterior spines, LL), posterior spine length (PSL), total lorica length including all appendages (TLL), lorica width at its widest part (LW), posterior spine angle (PSA), anterolateral dorsal spine length (ALS), anterointermediate dorsal spine length (AIS), anteromedian dorsal spine length (AMS), and lorica width beneath the anterior spines ("head width", HW)

	LL	PSL	TLL	LW	PSA	ALS	AIS	AMS	HW
ESU 3									
Mean	99.5	52.8	182.5	67.4	159.3	20.5	14.5	30.5	58.2
Median	100.0	52.6	182.9	68.2	159.0	20.8	14.3	30.0	58.8
Min	87.4	24.9	131.3	51.5	145.9	15.1	10.5	25.6	49.7
Max	115.0	90.3	231.5	77.1	174.2	24.3	18.2	36.1	66.8
Lakes: Caldonazzo (2), Kaltern (21), Terlago (13), Vahrn (2)									
ESU 4									
Mean	92.8	0.0	114.8	63.5	0.0	14.9	11.9	22.0	51.8
Median	88.6	0.0	113.3	61.0	0.0	14.1	11.7	21.0	51.8
Min	82.8	0.0	106.0	59.4	0.0	12.3	11.0	17.0	49.3
Max	109.2	0.0	126.2	72.1	0.0	19.2	13.4	29.5	54.5
Lake: Terlago (7)									
ESU 5									
Mean	111.1	71.9	215.3	71.7	143.1	15.9	11.6	32.3	65.4
Median	109.9	75.4	220.3	75.0	144.0	16.9	11.4	32.7	67.8
Min	93.5	0.0	140.3	40.9	0.0	8.8	4.9	11.8	50.0
Max	129.2	113.1	266.8	83.2	165.5	20.9	16.0	43.5	75.3
Lakes: Glittner (43), Kaltern (1), Vahrn (1)									
ESU 6									
Mean	106.8	54.4	194.9	70.8	144.3	20.9	15.3	33.8	62.5
Median	108.7	57.4	199.3	72.3	155.1	20.7	15.2	33.8	63.2
Min	88.2	0.0	116.2	52.0	0.0	15.1	9.0	24.9	54.5
Max	125.7	81.0	229.7	77.9	168.3	26.6	19.5	41.4	68.4
Lakes: Terlago (8), Tovel (30), Vahrn (4)									
ESU 7									
	84.9	35.4	151.9	50.0	161.6	18.4	14.6	31.6	48.9
Lake: Caldonazzo (1)									
ESU 8									
Mean	81.4	25.9	135.6	50.8	165.8	16.8	12.5	28.2	51.0
Median	83.6	26.0	138.0	51.7	166.1	16.9	12.9	28.0	51.9
Min	74.3	24.0	125.6	45.7	163.7	15.2	11.3	25.4	46.1
Max	87.1	28.8	145.3	52.7	167.2	18.0	13.1	31.6	53.4
Lake: Vahrn (5)									

The number of individuals measured is given between brackets next to the lake name

No correlation was found between phylogenetic and morphological diversity (Mantel $r = 0.07$; $P = 0.41$).

Discussion

Our study indicated that eight putative ESUs of *K. cochlearis* occurred in lakes of the Trentino-South Tyrol region. This diversity may be responsible for the apparent tolerance of *K. cochlearis* to varying environmental conditions. The putative ESUs of *K. cochlearis* had an average uncorrected genetic distance in COI between 12 and 30%, which is higher than the 3% threshold commonly used to separate species for most animals (Hebert et al., 2003; Tang et al., 2012). The general good agreement of the various methods that we used to infer putative ESUs corroborated our results. We did not consider the splitting of GMYC ESUs 3 and 6 by PTP-BA because it was not supported by the branching pattern of the tree and the other methods of species delimitation.

The wide morphological variability in *K. cochlearis* that led to the description of morphotypes

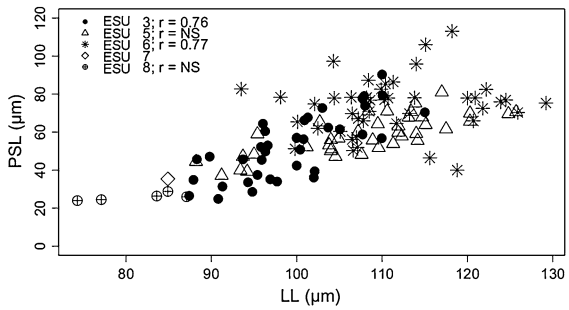

Fig. 4 Relation between posterior spine length (PSL) and lorica length (LL) for different GMYC ESUs. *Numbers* on *axis* represent length in µm. Values of significantly important ($P < 0.05$) correlation coefficients are reported next to ESUs *symbols*

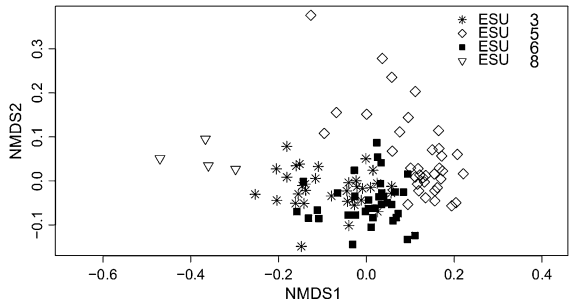

Fig. 5 Non-metric multidimensional scaling (NMDS) plot of all morphological variables. GMYC ESUs 1, 2, and 7 are excluded due to absence of morphometric data. GMYC ESU 4 and spineless specimens of ESU 6 are excluded due to lack of the posterior spine

Table 5 Morphological parameters showing statistical significant differences in ANOVA between different GMYC ESUs (only significant comparisons are shown), lorica length (excluding anterior and posterior spines, LL), posterior spine length (PSL), and posterior spine angle (PSA), degrees of freedom (df), 138 specimens were measured, but ESU 7 was excluded from analyses because only specimen was measured, for ANOVA on PSL, specimens without spine were excluded (7 of ESU 4 and 3 of ESU 6), in mixed modelling of ANOVA for PSL and LL, measurements from the same lakes were modelled as correlated and in mixed modelling of ANOVA for PSA, residuals were allowed to have a different spread per lake

Comparison	df	*t*-ratio	*P*
PSA			
ESUs 3–5	118	6.23	<0.001
ESUs 3–8	118	−2.70	0.038
ESUs 5–6	118	−4.72	<0.001
ESUs 5–8	118	−7.01	<0.001
ESUs 6–8	118	−4.29	<0.001
PSL			
ESUs 3–5	121	−4.38	<0.001
ESUs 3–8	121	2.98	0.028
ESUs 5–6	121	3.23	0.013
ESUs 5–8	121	5.53	<0.001
ESUs 6–8	121	3.64	0.004
LL			
ESUs 3–8	132	3.14	0.017
ESUs 4–5	132	−2.90	0.035
ESUs 5–8	132	4.20	<0.001
ESUs 6–8	132	3.47	0.006

by Lauterborn (1900) has been investigated by many researchers who tried to understand factors influencing morphology such as temperature (Green, 2005), predation (Conde-Porcuna et al., 1993), maternal effect (Stemberger & Gilbert, 1984), or presence of distinct species (Ahlstrom, 1943; Eloranta, 1982). Our study indicated that neglecting presence of ESUs of *K. cochlearis* might have led to biased conclusions on their morphological variability and global distribution. For example, the correlation between LL and PSL is not always positive as stated by Green (2005) but seems to differ between ESUs showing no correlation or varying positive correlation. Furthermore, Green (2005) underlined that specimens with a LL of around 80 µm show a wide variability in PSL. We observed an overlap of specimens of different ESUs in the range of 80–90 µm. Thus, neglecting ESUs of *K. cochlearis* may lead to underestimating their phenotypic diversity.

An important characteristic for the delimitation of *K. cochlearis* morphotypes is the presence and length of the posterior spine. Our study indicated that spined and unspined (=*tecta*) specimens occurred in the same and different ESUs (i.e. ESUs 3 and 6, respectively). Hofmann (1983) and Green (2005, 2007) noted that *tecta* specimens could not be explained by allometric growth because specimens with spines were smaller than those without spines. Green (2005) presented three hypotheses of the origin of spineless *K. cochlearis*: 1, true *tecta* (appearing only in colder periods of the year as the "end" of the posterior spine reduction); 2, *aspina* (truly spineless, absent in the winter, LL longer than in spined form); 3, *ecaudata* (the same dorsal structure, occurring in summer, LL longer than in spined form). Coherent with Green's (2005) hypothesis 1 of *true tecta*, our study indicated based on ESU 6 that spineless forms have the same and

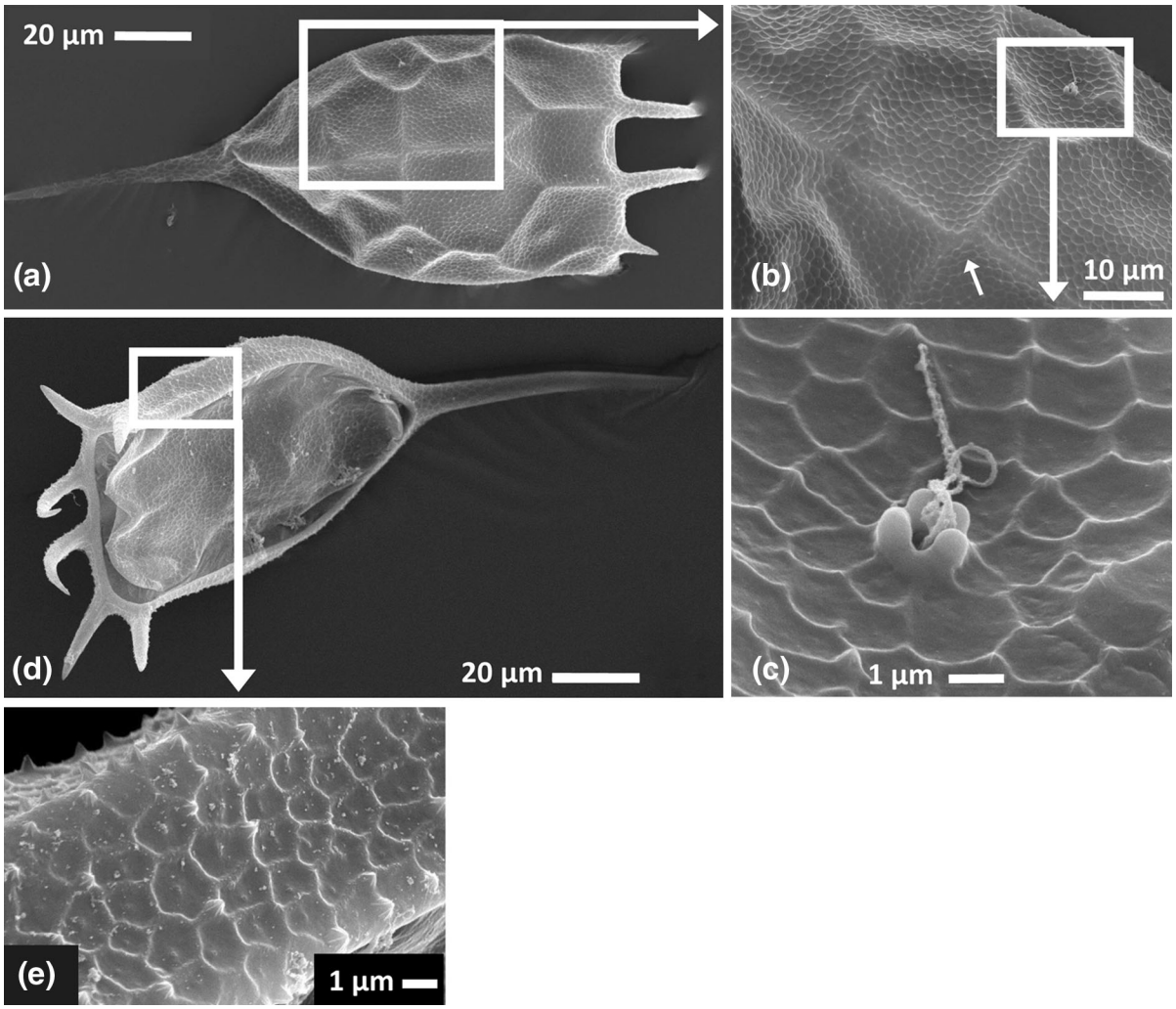

Fig. 6 SEM pictures of *K. cochlearis*, **a** dorsal view, **b** detail of bended ridge (indicated by *arrow*), lateral antenna, **c** detail of lateral antenna, **d** ventral view, and **e** detail of spinelets on the intersection of the areolation and of bumps in the middle of areolation, GMYC ESU 5: (**a**–**c**), and GMYC ESU 8: (**d**, **e**)

smaller LL than specimens from the same ESU across different habitats. Coherent with Green's (2005) hypotheses 2 and 3, spineless specimens of ESU 4 were smaller and larger than spined morphotypes across habitats and those from the same lake. Thus, neglecting the co-occurrence of different ESUs in *K. cochlearis* leads to the odd situation that spineless specimens seem larger than spined ones. We suggest that *tecta* morphotypes can actually have at least two possible origins (Green's hypotheses 1 and 2/3) but delimiting true *tecta* from spineless *aspina* or *ecaudata* based on morphology seems quite tricky. We, furthermore, hypothesise that detailed SEM pictures of lorica facets might reveal features (such as the *X*-facet or carinal facets described by Lauterborn, 1900) helpful for delimiting putative ESUs.

Spinelets and the bended ridge are other morphological features that are used in morphotype delimitation (Lauterborn, 1900) but the usefulness of spinelets was already questioned by Hofmann (1980). According to Lauterborn (1900) spinelets are characteristic for the *hispida* and *irregularis* series. However, specimens from GMYC ESUs 3 and 6 did and did not have spinelets. According to Hofmann (1980), the size of spinelets increases from spring to summer and are almost invisible during winter. In fact in our samples, specimens with spinelets occurred during summer and spring (only one was collected

Table 6 Observed combinations of morphological characteristics present in individuals of the respective GMYC ESUs

	Bended ridge	Spinelets	Posterior spine	Additional facets
ESU 3	Yes	No	Yes	No
ESU 3	Yes	Yes	Yes	No
ESU 3	No	No	Yes	No
ESU 4	Yes	Nv	No	Yes
ESU 5	Yes*	No**	Yes	No
ESU 6	Yes	Yes	No	Yes
ESU 6	Nv	No	Yes	No

Different lines were used if more than one combination was observed in a given ESU

nv Not visible

* Shown in Fig. 6b, ** shown in Fig. 6e

from Lake Terlago during November), but we cannot exclude that we missed the presence of spinelets in some specimens as they were very difficult to observe. However, it seems that spinelets are only appearing (and changing in length) in some ESUs because no spinelets were ever observed in ESU 5 regardless of sampling time. Thus, we suggest that the presence of spinelets is no valid criterion for delimitating morphotypes or putative ESUs. Ahlstrom (1943) and Eloranta (1982) already pointed out that the presence of spinelets shows high variability in most *K. cochlearis* species, and here we corroborated their statement with genetic data. More detailed SEM pictures of various putative ESUs taken from different seasons are, in any case, needed in order to investigate the temporal appearance of spinelets. According to the bended ridge, specimens of ESUs 4 and 5 always showed it while it was present or absent in specimens of ESU 3. We conclude that the bended ridge is also not a valid character to delimitate ESUs. In addition to spinelets and the bended ridge, we observed small humps in the middle of the areolation section. To the best of our knowledge, we do not know about any reference to these structures. We refrain from hypothesising on their function, and if they grow, they seem to be an overlooked feature of lorica morphology. Furthermore, we provided detailed SEM images on the lateral antenna that was previously only shown by Garza-Mouriño et al. (2005, their plate 1c).

Taking into account all information on lorica morphology, different ESUs showed different morphological variabilities. Both univariate and multivariate analyses indicated that ESUs 3 and 6 were not unambiguously distinguishable based on lorica measurements showing a wide phenotypic plasticity. Contrarily, ESU 8 could be distinguished from ESU 5 based on morphology based on single measurements and NMDS. In LDA, only specimens of ESU 5 were correctly assigned in most cases, while specimens of ESU 8 did not perform that well. Specimens of ESU 8 were smaller with respect to measured characters than specimens of ESU 5. Therefore, it is possible to delimit only some putative ESUs having a more restricted phenotypic plasticity with respect to other ESUs based on detailed lorica measurements. We suggest that an analysis of specimens sampled separately during cold and warm seasons in specific water layers could provide insights into the effect of water temperature on spine development of ESUs that we may have missed by our sampling strategy.

In many of our study lakes, different ESUs of *K. cochlearis* co-occurred. Generally, it is assumed that species with similar morphology and close phylogenetic relationship might have similar niches (e.g. Wiens & Graham, 2005; Wiens et al., 2010) and this would lead to competitive exclusion (Violle et al., 2011; Gabaldón et al., 2013). Cryptic species are not only morphologically similar but also phylogenetically closely related, and thus, the co-occurrence of cryptic species should be rarely encountered. However, cryptic species of *B. plicatilis* occur in temporal co-existence or in overlap, and their co-existence is mediated by disturbance and food partitioning (Ciros-Pérez et al., 2001a). Not only in the genus *Brachionus* but also in *P. dolichoptera* (Obertegger et al., 2014) co-existence of cryptic species has been observed. We found that several morphologically similar putative ESUs of *K. cochlearis* co-occurred but, at the moment, cannot infer their niche

partitioning because of missing information regarding their depth distribution. Furthermore, our study indicated no link between phylogenetic and morphological diversity of putative ESUs. Similarly, Gabaldón et al. (2013) found no difference between cryptic species of *B. plicatilis* for key parameters (i.e. clearance rates, starvation tolerance and predation susceptibility) related to body size. Recently, co-existence of cryptic species was linked to a negative feedback based on sex-based mechanisms that lead to stable co-existence (Montero-Pau et al., 2011).

In conclusion, our study indicates that *K. cochlearis* is composed of eight putative ESUs based on mtDNA, as indicated by three different methods. The generally good agreement between these methods enhances our inference on species diversity. Several morphological characteristics such as presence/absence of the posterior spine, spinelets, and bended ridge seem to be of poor value to discriminate ESUs. However, when all lorica measurements are taken together in a multivariate statistical approach, ESU 5 could be distinguished from ESU 8. More detailed morphological research is needed for a longer period to understand the morphological variations of *K. cochlearis* ESUs.

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ARTICLE II

Hydrobiologia
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PRIMARY RESEARCH PAPER

Life history traits and demographic parameters in the *Keratella cochlearis* (Rotifera, Monogononta) species complex

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Abstract A recent study based on DNA taxonomy indicated that the widespread rotifer *Keratella cochlearis* comprises several evolutionarily significant units (ESUs). Identification of ESUs based on DNA taxonomy alone is problematic and usually requires morphological, demographic, and/or ecological evidence. We isolated three haplotypes belonging to two ESUs of *K. cochlearis* and conducted life table experiments to investigate if this genetic diversity is reflected in demography. We found significant differences between haplotypes in life history traits (average lifespan, number of offspring, and percent of rejected eggs) and in demographic parameters (instantaneous growth rate, generation time, and net reproductive rate of the populations). During the experiments, all the haplotypes produced abnormal females with a deformed lorica, which was never reported before in *K. cochlearis*. We also report the first case of an amphoteric female (producing both females and males) in *K. cochlearis*. We hypothesize that *K. cochlearis* haplotypes and thus ESUs may exhibit niche differentiation through their different life histories. The link between demographic parameters of *K. cochlearis* and niche utilization requires further research.

Keywords Life table · Cryptic species · Abnormal females · Rotifers · Lake Tovel

Introduction

Rotifers are among the most abundant planktonic metazoans and constitute a crucial link between lower and higher trophic levels in most freshwater ecosystems around the world (Wallace et al., 2006). Rotifer biodiversity has been studied for over two hundred years, and so far about 2000 species have been described (Koste & Hollowday, 1993; Segers & De Smet, 2008). With the advent of molecular techniques and the introduction of DNA taxonomy, many rotifer species, traditionally considered as one species, proved to be complexes of cryptic species. Cryptic

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species, defined as genetically distinct but morphologically difficult-to-distinguish species (Gomez et al., 2002; Fontaneto et al., 2009; Birky et al., 2011; Obertegger et al., 2012, 2014; Cieplinski et al., 2017), appear to be widespread among both microorganisms and macroorganisms and have been reported in many groups such as protists (Foissner, 2006), ants (Fournier et al., 2012), harvestmen (Arthofer et al., 2013), and rotifers (Gomez & Snell, 1996; Gomez et al., 2002; Fontaneto et al., 2009; Birky et al., 2011; Obertegger et al., 2012, 2014; Cieplinski et al., 2017). According to the niche conservatism theory, the closer the related species are, the more profound is their niche conservatism (i.e., a higher tendency to retain their ancestral traits) and the stronger is their competition (e.g., Darwin, 1859; Violle et al., 2011). Therefore, cryptic species should show strong interspecific competition and little co-existence (Wiens & Graham, 2005; Losos, 2008; Violle et al., 2011). However, co-existence of closely related species is a difficult-to-explain phenomenon (Leibold & McPeek, 2006), but has been observed in nearly 60% of rotifer complexes (Gabaldón et al., 2017). Yet, especially with small aquatic organisms we can never fully account for the n-dimensionality of the species niche, and, therefore, inferences about real co-existence are difficult.

Evidence is growing that cryptic species in rotifers often have different life history traits despite their close phylogenetic relationship and that these differences may play a role in their co-existence in the same environment (Gabaldón et al., 2015). Consequently, our knowledge on biodiversity, biogeography, and ecology of certain species might be biased because several cryptic species with different ecological requirements and characteristics are lumped into one species. Differences in life histories of closely related species that are linked to niche differentiation may thus add to the co-existence and evolution of cryptic species (Angert et al., 2009; Montero-Pau et al., 2011). Therefore, analyses of life histories in cryptic species complexes may help understand competitive abilities between those species.

Life table experiments represent one of the most widespread methods to study life history traits and population dynamics (King, 1970; Allan, 1976; Walz, 1983, 1987; Gribble & Welch, 2013; Xi et al., 2013; Xiang et al., 2016a, b). Life history traits are those parameters that are directly derived from the life table of an organism (Stearns, 1992). Demographic parameters (also known as "population parameters", "population traits", etc.) are the key parameters of population dynamics (e.g., instantaneous growth rate, net reproductive rate, and generation time) (Begon et al., 1996). Various studies reported interspecific differences in life history traits for several rotifer species and cryptic species in response to abiotic factors such as salinity or temperature. Temperature is one of the most important abiotic factors influencing life histories of rotifers (Bottrell et al., 1976). Gabaldón et al. (2015) showed that the brackish water cryptic species *Brachionus manjavacas* exhibits—irrespective of salinity—higher growth rates than its sibling cryptic species *B. plicatilis.* Gabaldón and Carmona (2015) demonstrated that asexual females of *B. manjavacas* have higher survival rates in both middle and old age classes and, consequently, a longer mean lifespan than asexual females of *B. plicatilis* from the same lake. In spite of this demographic advantage of *B. manjavacas* with respect to *B. plicatilis*, the two cryptic species can co-exist stably (Gomez et al., 2002, 2007). Ciros-Pérez et al. (2001) reported different intrinsic growth rates in three sympatric cryptic species of the *B. plicatilis* species complex that were cultured at the same temperatures. Similarly, demographic parameters were different for eight closely related Chinese populations of *B. calyciflorus* exposed to different temperatures, and these differences were linked to adaptations of populations to different environmental conditions (Ma et al., 2010).

Life history traits and demographic parameters are also influenced by biotic factors such as food quality (Korstad et al., 1989) and quantity (Robertson & Salt, 1981; Xi & Huang, 1999; Sarma et al., 2001). Hu and Xi (2008) showed that the intrinsic growth rate, generation time, and average lifespan of two strains of *B. plicatilis* and one strain of *B. calyciflorus* were all significantly different under different food regimes. Differences in life history traits and demographic parameters were observed not only between cryptic species inhabiting the same environment (e.g., Gabaldón et al., 2015) but also between geographically isolated populations of the same species of *B. calyciflorus* (Wang et al., 2014).

Compared to the well-studied *Brachionus* spp., little is known about the life history of the freshwater rotifer *Keratella cochlearis* (Gosse, 1851). This is astounding considering that *K. cochlearis* is globally

distributed in lakes and ponds and is one of the most common pelagic species worldwide (Pourriot, 1965). One of the reasons why *K. cochlearis* is understudied in contrast to *Brachionus* spp. is because it is much more difficult to culture (Lindström & Pejler, 1975; Pourriot, 1980; Stemberger, 1981). Among the few existing studies on *K. cochlearis* (Edmondson, 1965; Zimmermann, 1974; Walz, 1983; Gilbert & Stemberger, 1985; Walz, 1983, 1986, 1987), those performed by Walz (1983, 1987) are the most extensive ones. This author reported changes in the life history of *K. cochlearis* dependent on temperature and food regimes. Recently, the existence of a cryptic *K. cochlearis* species complex has been hypothesized by Cieplinski et al. (2017) based on DNA taxonomy; these authors also demonstrated that it is possible to delimit several distinct evolutionarily significant units (ESUs) based solely on morphological differences between ESUs.

Here, we investigated differences in life histories between three haplotypes of *K. cochlearis*. These haplotypes belong to two putative evolutionarily significant units (ESUs)—ESU 3 and ESU 6 of *K. cochlearis*, described in Cieplinski et al. (2017) and in Obertegger et al. (2017). We hypothesized different life history traits and demographic parameters in at least two haplotypes of *K. cochlearis* belonging to different ESUs, assuming that the existence of cryptic species was correctly inferred by DNA taxonomy.

Materials and methods

Rotifer isolation, haplotypes, and ESU

We focused on three haplotypes belonging to two ESUs that were discriminated based on their mitochondrial cytochrome oxidase subunit 1 gene (COI) by Cieplinski et al. (2017). Discrimination for these ESUs and haplotypes was later confirmed by phylogenetic analyses with a nuclear marker (internal transcribed spacer 1, ITS1) by Obertegger et al. (2017). These haplotypes were isolated from lakes Tovel, Kaltern, and Terlago (N. Italy) during a detailed sampling conducted between 2013 and 2015 (for details, see Cieplinski et al., 2017).

For simplicity, we refer to two ESUs as "ESU 3" and "ESU 6" as previously described by Cieplinski et al. (2017) and to the haplotypes as "Hap A" (belonging to ESU 6), "Hap B," and "Hap C" (both belonging to "ESU 3," Table 1). The sampled lakes, although geographically relatively close, represent different environmental conditions (Table 2). Lake Tovel, despite its mid-altitude location, has the characteristics of an alpine lake (Obertegger & Flaim, 2015), while Lakes Kaltern and Terlago are lowland lakes embedded in an agricultural landscape.

The three haplotypes were regularly observed during the monthly sampling period in 2014 (supplementary material Table s1). Hap B was isolated from Lake Kaltern but was also found once in Lake Vahrn (supplementary material Table s1). Moreover, Hap A was present in Lake Tovel, Hap B in Kaltern, and Hap C in Lake Terlago in all samples indicating that these particular haplotypes are not only temporarily occurring but are in fact parts of permanent *K. cochlearis* communities in the studied lakes (Cieplinski et al., 2017).

Samples were taken at the deepest site of each lake with a 50-µm Wisconsin-type plankton net. Rotifers were collected from Lake Terlago on September 23, 2014 and from both Tovel and Kaltern on March 2, 2015. One clonal culture per lake was established from a single female collected from that lake and continuously maintained in the laboratory. Clonal rotifer cultures were kept inside an incubator in 6-well plates (Biomedica, Vienna) in modified WC medium (Guillard & Lorenzan, 1972) at an average temperature of 14.5 °C and a 16:8 h light–dark photoperiod. The same medium was also used to cultivate *Cryptomonas* sp. strain no. 26.80 obtained from the culture collection of algae in Göttingen, Germany. This *Cryptomonas* sp. served as the only food source for all rotifer clones before and during the life table experiments. Algal concentration was measured with an electronic particle counter (CASY 1-Model TTC, Schärfe System) according to Weisse and Kirchoff

Table 1 Haplotypes used for life table experiments: Haplotype, COI-ESU (terminology for haplotypes and COI-ESUs according to Cieplinski et al., 2017), ITS1-ESU (terminology for ESUs according to Obertegger et al., 2017), and Hap (terminology for haplotypes in this study)

Lake	Haplotype	COI-ESU	ITS1-ESU	Hap
Tovel	h30	ESU 6	ESU α	A
Kaltern	h5	ESU 3	ESU β	B
Terlago	h14	ESU 3	ESU β	C

Table 2 Environmental data of sampled lakes: altitude (alti; m above sea level), area (ha), depth (m), mean summer surface temperature (temp; °C), conductivity (cond; μS cm^{-1}), and trophic state (eutrophic—eu; mesotrophic—meso; oligotrophic—oligo)

Lakes	Geographical coordinates	Alti	Area	Depth	Temp	Cond	pH	Trophic state
Tovel	46° 15′ 43.2432″ N 10° 56′ 41.6472″ E	1178	38.2	39	15	192	7.9	oligo
Kaltern	46° 22′ 43.1724″ N 11° 15′ 50.2092″ E	215	147	5	18	507	8.3	meso
Terlago	46° 5′ 56.3568″ N 11° 3′ 21.258″ E	414	11.9	10	23	289	8	eu

(1997). Most complete life table experiments with *K. cochlearis* were performed by Walz (1983, 1987) on specimen coming from the small pond Fasaneriesee in southern Germany. Walz (1983, 1987) reported 15 °C as the optimum temperature for his cultures. Therefore, we conducted all experiments at 15 °C.

Initially, many more clones and haplotypes were selected for culturing for each of the ESUs, also including various haplotypes from the same lakes. However, owing to general difficulties in culturing *K. cochlearis*, we were not able to maintain them in cultures and most of the clones died regardless of culturing efforts.

Life table experiments

Life table experiments for all three haplotypes of *K. cochlearis* were performed using exactly the same experimental setup, including food concentration, temperature, and light conditions. Depending on the size of the wells, we placed two to four flakes of cetyl alcohol on the surface of each well to reduce surface tension (see Desmarais, 1997; Stelzer, 1998), and thus to lower the probability that rotifers were caught in the surface film.

Each experiment comprised three phases:

(1) standardization—performed in 30 Petri dishes to standardize conditions and to minimalize maternal effects;
(2) synchronization—performed in 24-well plates for female synchronization;
(3) life table experiment—performed in 96-well plates, similarly to the experiment conducted by Walz (1983).

In monogonont rotifers, a switch from asexual to sexual reproduction is generally attributed to the accumulation of mixis-inducing proteins released into the environment by the rotifers themselves (Stelzer & Snell, 2003; Snell et al., 2006). Sun and Niu (2012) observed for *B. calyciflorus* that maternal crowding of amictic (asexually reproducing) females can enhance the propensity of offspring to produce mictic (sexually reproducing) females. Therefore, the main purposes of the acclimation period (phase 1) were to minimize the probability of mictic female appearance in phases (2) and (3) and to standardize the starting conditions for all three haplotypes. Because the exact sex-inducing female density has not yet been described for *K. cochlearis*, the number of females used for phase (1) was based on earlier observations in our laboratory. The duration of phase (1) was long enough to rear several generations of rotifers. To initiate phase (1), five individuals were placed into 30 Petri dishes containing 30 mL of medium with abundant food (*Cryptomonas* sp. > 30,000 cells mL^{-1}) and cultured for approximately 14 days. Rotifer abundance was monitored until the total number of rotifers in all dishes reached more than ~ 300 individuals, which was a prerequisite to start phase (2).

For phase (2), single young females from phase (1) were pipetted into eight 24-well plates containing 2 mL of medium with *Cryptomonas* sp. (> 30,000 cells mL^{-1}). The criteria for selecting young females were transparency, smaller body size than adult females, and lack of eggs. These features allowed us to discriminate young females from adult ones that recently gave birth and carried no eggs. Each rotifer during phase (2) was observed two times per day, in the morning and late afternoon, to record the most proximate time of offspring production. The general purpose of phase (2) was to produce many females of similar age whose offspring born at approximately the same time were then used for phase (3); accordingly, we used the term "female synchronization" for phase (2). Newly hatched offspring from the 1st clutch (i.e., a cohort) of phase (2) females were immediately removed and used for phase (3). Phase (2) lasted for a period of approximately 14 days, which was sufficiently long enough to ensure that females produced

several generations of offspring. Only offspring from the same 1st clutch were selected (possible due to the synchronization of their mothers) for the life table experiment (phase 3) to further standardize initial conditions. For phase (3), single females born at approximately the same time were placed into wells of a 96-well plate containing 230 µL of medium and food solution with *Cryptomonas* sp. > 30,000 cells mL^{-1}. The initial number of females was always 96. Rotifers were observed twice per day to record the number of eggs, dead individuals, and the number and sex of offspring. All specimens were transported to fresh medium with food every fourth day, which represented a compromise between culture maintenance and preventing specimen loss due to death by mechanical interference. Newly hatched juveniles were removed immediately and discarded. Apart from females and males, abnormally swimming and non-loricated individuals were also counted; these specimens were called abnormal females. Amictic (parthenogenetic) eggs that did not hatch and instead decomposed in the course of several days at the bottom of the Petri dish were also counted and called rejected eggs.

Analyses of life table data

The life history traits and demographic parameters were calculated based on a sample size of 96 individuals for each haplotype. All females, irrespective of offspring production and all offspring, females, males, and abnormal females were included in calculations. Demographic parameters were calculated according to Birch (1948) and Walz (1983): average lifespan (L) was reported in days, survivorship (l_x) was the percentage of surviving females on day x, age-specific birth rate (m_x) was the fraction of all the surviving offspring on day x, and age-specific fecundity rate ($l_x m_x$) was the product of l_x and m_x.

The net reproduction rate (R_0) was the sum of $l_x m_x$ over the entire experiment:

$$R_0 = \Sigma l_x m_x$$

Generation time (T) is the time from hatching from an egg to producing an offspring and is calculated according to

$$T = \ln(R_0)/r$$

The instantaneous growth rate per day (r) was estimated by solving Lotka's equation (Lotka, 1907) iteratively, assuming exponential growth (see Birch, 1948):

$$\sum_{x \geq 1} e^{r(x+0.5)} l_x m_x$$

where e is the Euler constant (2.71828), x the age in days, l_x the age-specific survival rate, (i.e., the proportion of surviving females at day x), relative to the initial number of females, and m_x the age-specific fecundity rate, i.e., the mean number of offspring produced on day x by a female of age x.

In the case of R_0, T, and r, bootstrapping was used to obtain estimates of means and standard deviation. Bootstrapping was done by randomly resampling the same sampling size ($n = 96$) with replacement from the original sample (Quinn & Keough, 2002). Bootstrapping with replacement generates robust representative statistics (Dixon, 2002) as shown for growth rates of cladocerans (Meyer et al., 1986). Here, we used 1000 bootstrapped samples.

Mean values of all life table parameters were tested for significant differences between haplotypes by non-parametric Kruskal–Wallis one-way analysis of variance. For pairwise comparisons of values which did not show normal distribution, the Dunn's post hoc test (95% family-wise confidence levels) was used. Normal distribution was found only for T, and therefore in this case ANOVA was used for analysis of variance and Tukey's post hoc test (95% family-wise confidence levels) was used for pairwise comparisons. All statistical analyses were performed using R 3.4.1 (R Core Team, 2017).

Results

Life history traits and demographic parameters of different haplotypes and ESUs

All females (i.e., amictic, mictic, females producing abnormal females) and all the offspring (females, males, and abnormal females) were included in the analyses of life table data. Excluding mictic females or females producing abnormal females did not change the results in a meaningful way (supplementary

material Table s2). All three haplotypes showed statistically significant differences in all demographic parameters except for L (Table 3). Specifically, Hap A produced more offspring (average number of offspring: 8.1 for Hap A, 2.0 for Hap B, 3.3 for Hap C) and showed a lower percentage of rejected eggs (2.15% with respect to 12.61% for hap B and 9.67% for Hap C) and a higher L compared to Hap B and Hap C (17.0 days with respect to 11.3 and 13.4 days; Table 3). Males were only observed in Hap A (Table 3). All haplotypes showed positive r with Hap A showing the highest r and Hap B the lowest r (0.23 and 0.08 days, respectively) (Table 3). T was the shortest in Hap C (7.99 days) and the longest in Hap A (9.22 days); there were significant differences between all haplotypes. Similarly, R_0 was significantly different between all haplotypes.

Age-specific survival rate (l_x) of Hap C decreased less, relative to Hap B or Hap A during the first nine days (Fig. 1). After this initial phase, Hap C showed a distinctly faster decline than Hap A and Hap B. Furthermore, after day 23 no specimens of Hap C were alive, in contrast to Hap A and Hap B. The shape of the l_x curve for Hap A and Hap B was similar, but Hap A specimens lived longer than Hap B specimens.

Hap A showed higher $l_x m_x$ and m_x values and more frequent and regular cyclical patterns (Fig. 2A1, A2) than Hap B and Hap C (Fig. 2B1, B2; Fig. 2C1, C2). In all three haplotypes, a sharp initial peak appeared after 3 to 4 days, corresponding to the time needed for specimens to reach maturity. Due to increasing mortality of the mothers, the peak height declined in all experiments until the whole population had died.

Occurrence of abnormal and amphoteric females in experiments and in routine cultures

Amictic females showing an undeveloped, non-rigid lorica, impaired swimming abilities, and short (maximum 1 day) lifespan were classified as abnormal females (Fig. 3). We exclude the possibility that these females were males because they were larger than males and had visible and moving trophi (Fig. 3c). Furthermore, both penis and setae were absent in the photographed females. The lorica of these deformed females lacked structures such as plates, ridges, and ornamentation. However, a small, deformed posterior spine was present (Fig. 3e). In routine cultures, such deformed females were very rare. During the experiments, the highest number of abnormal females was

Table 3 Demographic parameters reported for COI haplotypes: lifespan (L; days), instantaneous growth rate of the population (r; d^{-1}), generation time (T; days), and net reproductive rate (R_0)

	Hap A	Hap B	Hap C
COI-ESU	**6**	**3**	**3**
L (days)	$17.0^{aa} \pm 8.49$	$11.3^{bb} \pm 6.94$	$13.4^{bb} \pm 3.35$
	17.5	11.75	13.5
Average number of offspring	$8.1^{aa} \pm 4.52$	$2.0^{bb} \pm 2.02$	$3.3^{cc} \pm 1.38$
	9.5	2	3
r (d^{-1})	$0.23^{a} \pm 0.001$	$0.08^{b} \pm 0.001$	$0.15^{c} \pm 0.001$
	0.228	0.077	0.149
T (days)	$9.22^{aa} \pm 0.02$	$9.24^{bb} \pm 0.05$	$7.99^{cc} \pm 0.03$
	9.22	9.24	7.99
R_0	$8.2^{a} \pm 0.05$	$2.0^{b} \pm 0.03$	$3.3^{c} \pm 0.02$
	8.16	2.03	3.29
Number of female offspring (total)	769	194	302
Number of male offspring (total)	7	0	0
Number of abnormal females (total)	1	1	14
% of abnormal females in all offspring	0.64^{ac}	0.51^{a}	4.43^{c}
% of rejected eggs	2.15^{aa}	12.61^{bb}	9.67^{b}

Reported are mean ± standard deviation (upper row values) and median (lower row value) for each parameter; all values followed by different superscripts are statistically different; values followed by a single superscript are statistically different at $P < 0.05$, and values followed by double superscript are statistically different at $P < 0.001$. COI-ESU coding is according to Cieplinski et al. (2017)

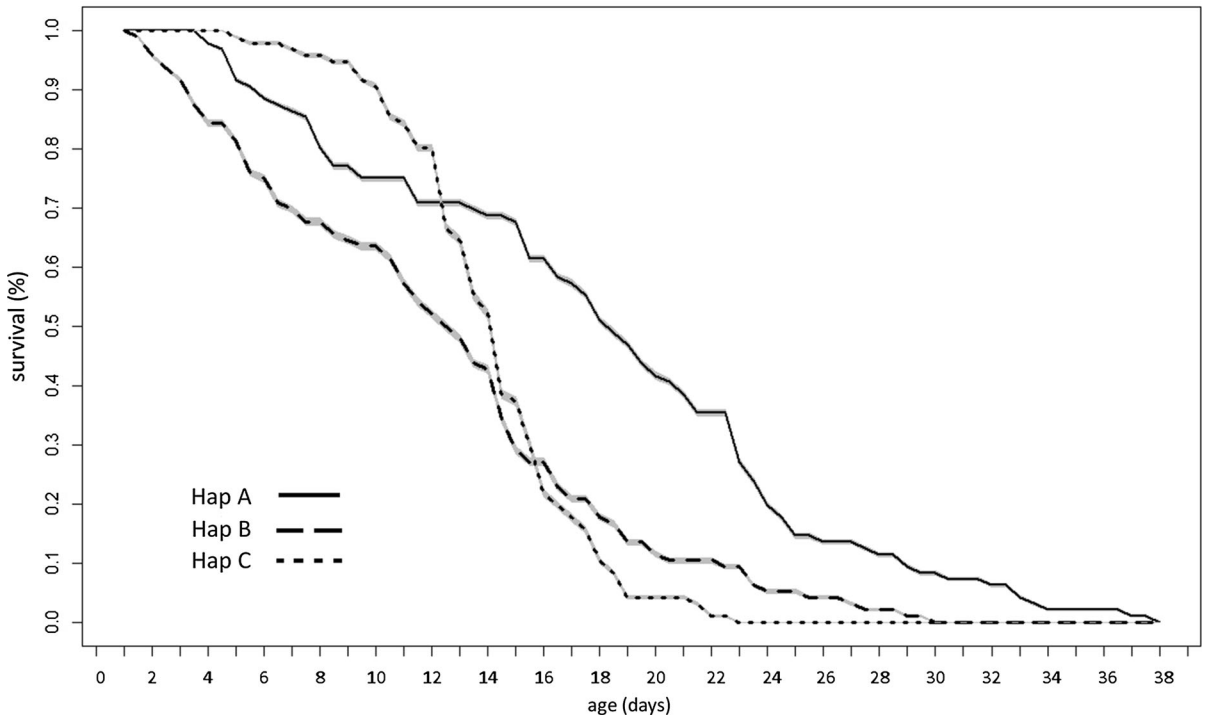

Fig. 1 Age-specific survivorship (l_x) for Hap A, Hap B, and Hap C. Hap A belongs to ESU 6 and Hap B and C to ESU, and 3. 95% confidence intervals are shown as shaded area, but are quite small

recorded for Hap C with a total of 14 specimens (Table 3). For Hap A, one amphoteric female was observed. This female produced two female offspring on days 5 and 10, and 6 male offspring on several days (i.e., 6, 8, 11, 12, 14, 19). No other amphoteric females have been observed in our cultures. The percent of amphoteric females was therefore 0.35% for all the rotifers (288 neonates for three haplotypes together). We did not observe any morphological differences between the single amphoteric female and amictic females.

Discussion

Differences in life history traits and demographic parameters in cryptic species of K. cochlearis

The total diversity of haplotypes of *K. cochlearis* within one ESU and the diversity of ESUs is unknown and requires further research. Even though our study did not evaluate intra-haplotype variability of demography, it is the first study that investigates demographic differences in haplotypes in rotifer species other than *Brachionus*. We demonstrated that genetically different (see Cieplinski et al., 2017) haplotypes of *K. cochlearis* differ also demographically. Combining molecular and demographic data for cryptic species is essential to correctly delimit species using an integrative taxonomy approach recommended by Schlick-Steiner et al. (2010), Fontaneto et al. (2015), and Papakostas et al. (2016). Very few experimental studies on *K. cochlearis* exist; moreover, they did not consider cryptic diversity. These earlier studies focused on the instantaneous rate of population growth (Table 4). Only Walz (1983, 1987) provided complete life table data for *K. cochlearis*, and thus most of our comparisons relate to his studies. The instantaneous rate of population growth (r) is a comprehensive parameter and is often considered a proxy for fitness, representing the ability of a rotifer population to grow and prosper in an environment (Campillo et al., 2011). In our study, r values of the three haplotypes mostly fell within the range known from previous studies of *K. cochlearis* (Table 4) and were all positive, indicating population growth;

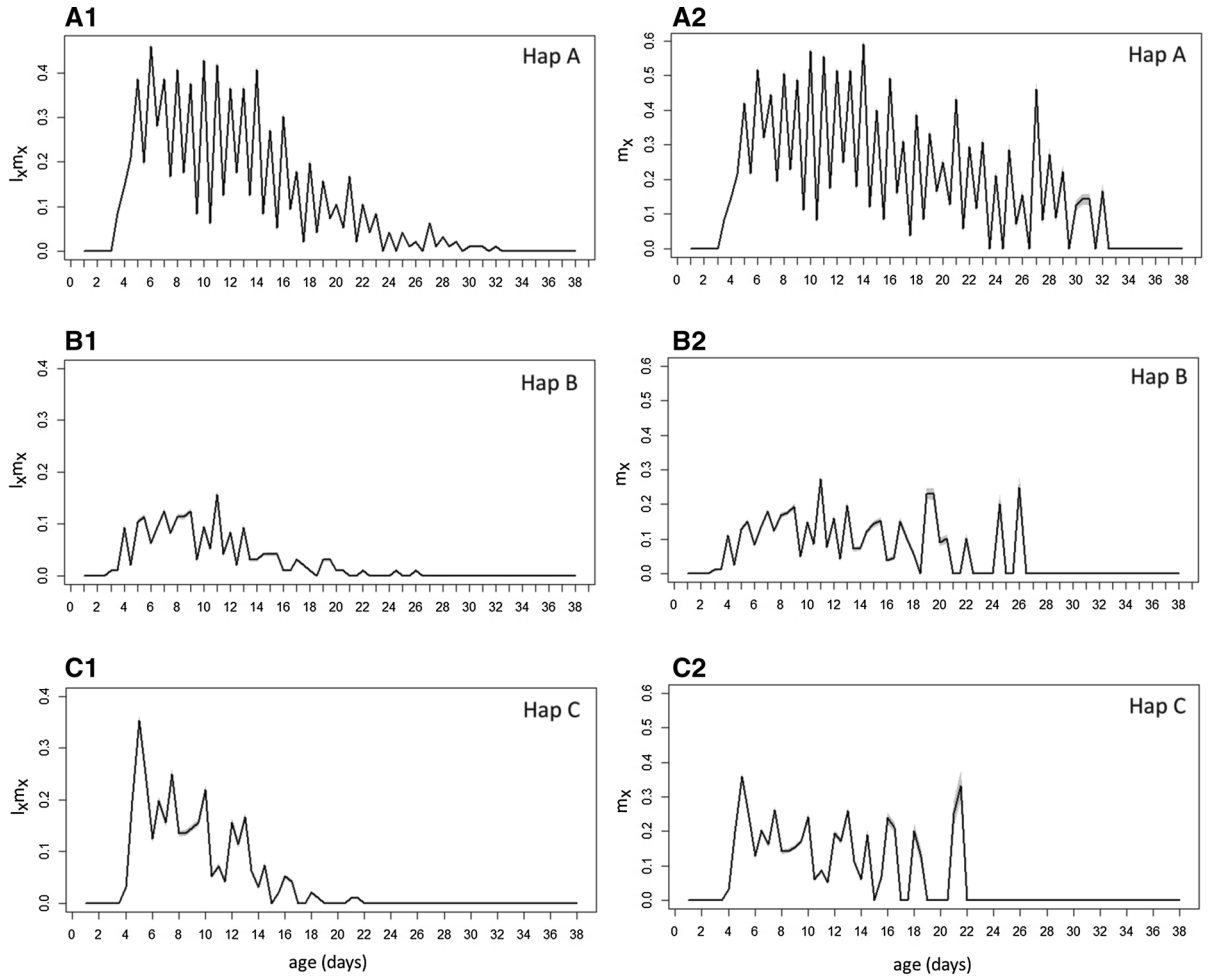

Fig. 2 Age-specific fecundity (l_xm_x) and age-specific birth rate (m_x) for Hap A (**A**1 and **A**2), Hap B (**B**1 and **B**2), and Hap C (**C**1 and **C**2). Hap A belongs to ESU 6 and Hap B and C to ESU, and 3. 95% confidence intervals are shown as shaded area, but are quite small

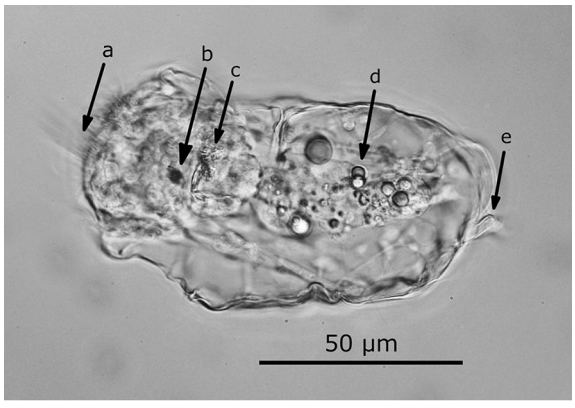

Fig. 3 Light microscopic picture in dorsal view of a deformed *K. cochlearis* female: **a** ciliae, **b** eyespot, **c** trophy, **d** lipid globules, and **e** deformed posterior spine. Dorsal view. Scale bar: 50 µm

however, we also observed the lowest *r* value (for Hap B) ever reported for *K. cochlearis.* The *r* of Hap A (0.228) was also comparable with *r* reported by Weisse and Frahm (2001) for *K. quadrata* (0.223 ± 0.21 fed with *Cryptomonas* sp. $> 30{,}000$ cells mL^{-1}). Walz (1995) reported slightly higher *r* values for other *Keratella* species, i.e., 0.32 for *K. quadrata*, 0.3 for *K. earlinae*, and 0.28 for *K. crassa*.

The time span to reach reproductive maturity is indicated by the T value. Walz (1983) reported T for *K. cochlearis* to be 8.1 days at 15°C; this value is slightly lower than those for Hap A and Hap B but comparable with that for Hap C. Differences in T and in *r* between the haplotypes were also reflected by their offspring number. Correspondence between T and offspring number was found by Ma et al. (2010) for

Table 4 Population parameters for *K. cochlearis* reported in various studies where food and temperature varied depending on the experimental setting: instantaneous growth rate of populations (r; days), net reproductive rate (R_0), generation time (T; days), lifespan (L; days), lake (refers to the lake of population's origin), lake altitude (Alti; m above sea level), lake depth (Depth; m), and trophic state (eutrophic—eu; mesotrophic—meso; oligotrophic—oligo)

r	R_0	T	L	Lake	Alti	Depth	Trophic state	Authors
~ 0.17[a]				Windermere N. Basin; Windermere S. Basin; Esthwaite; Blelham	39; 39; 65.2; 42	25.1; 16.8; 6.4; 6.8	all lakes eu	Edmondson (1965)
0.35				Sempach	504	87	eu	Zimmermann (1974)
0.095	2.5	8.1	15.4	Fasaneriesee	494	5.7	eu	Walz (1983)
0.28 (food: *Rhodomonas*)				Post Pond	134	38	meso	Stemberger & Gilbert (1985)
0.35 (food: *Cryptomonas*)				Post Pond	134	38	meso	Stemberger & Gilbert (1985)
0.3				Fasaneriesee	494	5.7	eu	Walz (1986)
0.095				Fasaneriesee	494	5.7	eu	Walz (1987)
0.214				Schöhsee	22	29	meso	Weisse & Frahm (2001)
0.23;	8.2;	9.22;	17.0;	Tovel;	1178;	39;	oligo;	This study
0.08;	2.0;	9.24;	11.3;	Kaltern;	215;	5;	meso;	
0.15	3.3	7.99	13.4	Terlago	414	10	eu	

In the present study, means were reported for demographic parameters. Please note that values are as reported in original papers

[a]Edmondson (1965) reports "population reproductive rate" which has a similar meaning as r; its value was derived from the graph

different cryptic species of *B. calyciflorus*. Walz (1995) reported T for *K. quadrata* to be 4.8 days at 15 °C, which is comparable with T in our study.

Walz (1983) calculated $R_0 = 2.15$. In our study, R_0 for Hap B was 2.0 and that for Hap C was 3.29, while R_0 for Hap A was much higher. Such differences in amictic offspring production under identical culture conditions were also reported for the *B. plicatilis* cryptic complex (Kostopoulou & Vadstein, 2007). Lifespan (L) reported by Walz (1983) was 15.4 ± 1 days at 15°C, which is comparable to our fastest developing Hap A. Regarding reproductive curves, both $l_x m_x$ and m_x did not overlap for Hap A and for the two other haplotypes but were almost identical between Hap B and Hap C. However, l_x did not overlap between all haplotypes indicating differences in survivorship of all three haplotypes. This suggests that for *K. cochlearis* some demographical parameters may differ more between ESUs than between closely related haplotypes from the same ESU.

None of the haplotypes tested showed all life table parameters similar to the ones reported by Walz (1983, 1987). Large demographic differences in the cryptic species complex of *K. cochlearis* may indicate that the *K. cochlearis* populations previously described were composed of various ESUs and/or haplotypes that differed from those used in the present study.

In rotifers, the appearance of males has been associated with mixis-inducing proteins that are released by females when the population density reaches a species-specific threshold (Carmona et al., 1993; Stelzer & Snell, 2003, 2006; Snell et al., 2006). We observed males only in Hap A, regardless of identical culture conditions for all haplotypes. We hypothesize that this could be related to different density thresholds that trigger sexual reproduction in haplotypes (or in ESUs); this issue requires further study.

We observed some rejected, detached eggs for all the haplotypes. Keen and Miller (1977) indicated that in *K. cochlearis* amictic eggs that are always attached to their mother hatch at different intervals. This indicates that detached amictic eggs in our study were no more viable. We also excluded the possibility of those eggs being pseudo-sexual eggs (resting eggs produced in parthenogenesis) similar to those observed for *K. hiemalis* (Ruttner-Kolisko, 1946) and for *Synchaeta pectinata* (Gilbert, 1995) because these rejected, detached eggs were morphologically identical to amictic eggs and clearly decomposed after some time on the bottom of the container. Moreover, pseudo-sexual eggs have never been reported in *K. cochlearis* and neither did we observe them in our laboratory. This result is unexpected as Keen and Miller (1977) reported a hatching rate for amictic eggs of *K. cochlearis* of 100%. The percent of rejected amictic (parthenogenetic) eggs did vary significantly between Hap A and Hap B and between Hap A and Hap B but not between Hap B and Hap C. Such differences in hatching rates for different cryptic species have been found for diapausing eggs. Gabaldón et al. (2015) reported that the hatching rate of diapausing eggs differs depending on salinity between different cryptic species of *B. plicatilis* and *B. manjavacas*. In our case, culture conditions were constant; therefore, we associated varying hatching rates with population differences between the haplotypes.

The pre-experiment phases (1) and (2) lowered the maternal effect related to crowding (see Lynch & Ennis, 1983) and standardized initial conditions allowing us to observe what we interpret as phenotypic differences unrelated to culture conditions. The observed vast phenotypic and genetic diversity may result from genetically fixed, adaptive evolution (Olson-Manning et al., 2012) related to life in a fast-changing and harsh environment of alpine lakes (represented in our study by lake Tovel). Alpine lakes often experience large environmental changes within short time scales (Sommaruga, 2001), which may trigger intraspecific variation and promote changes in species composition over relatively short evolutionary time scales (Weckström et al., 2016).

Relevance of abnormal females

Abnormal and deformed females, usually appearing as a response to toxins, were reported for *K. cochlearis* by Żurek (2006), for *Plationus patulus* by Rios-Arana et al. (2007), and for *B. calyciflorus* by Alvarado-Flores et al. (2015). While the latter two studies were performed in the laboratory experimentally exposing rotifers to toxins, the former study found deformed spines in *K. cochlearis* due to exposure to sulfides or its derivates present in water of a mine impoundment. To the best of our knowledge, deformed females without induction of any toxins are not known for *K. cochlearis*. These deformed females did not show any similarity with males. Wesenberg-Lund (1923) described that any trace of an alimentary canal and trophi have never been observed in males of *K. cochlearis* (previously described as *Anurae cochlearis*). Moreover, as described by Wesenberg-Lund (1923), males of *K. cochlearis* have a long flexible penis with two setae at the end, and the penis cannot be withdrawn. In the present study, the larger size, the presence of trophi, and the absence of a penis and setae let us conclude that these specimens were indeed females, not males. In our study, all three haplotypes of *K. cochlearis* produced abnormal females under standard experimental conditions. Moreover, the percent of abnormal females in Hap C was much higher than that for the other two haplotypes. We cannot exclude the possibility that the genotype of Hap C had some mutations leading to a higher number of abnormal females than in the case of Hap A and Hap B. Most probably, abnormal females did not reproduce because they were never observed carrying eggs and their lifespans were shorter than that of normal females. Therefore, in the long run, the occurrence of abnormal females would result in fitness reduction of the population, relative to a population that produces only fertile females per generation.

We used identical conditions for all cultures and could not identify any proximate factor triggering the occurrence of abnormal females. Therefore, we conclude that the production of deformed females was due to intrinsic factors. One possible intrinsic factor is the accumulation of deleterious mutations (Lynch et al., 1999). Henry et al. (2011) and Barraclough et al. (2007) showed that deleterious mutations are more prevalent in asexually reproducing populations.

Furthermore, deleterious mutations can accumulate due to low genetic variance also in small, at times sexually reproducing populations (Ridley, 2008) such as in *Daphnia* (Berg, 2005). Therefore, differences in the occurrence of abnormal females between the haplotypes of *K. cochlearis* may reflect differential genetic variability and accumulation of mutations because our cultures have been kept for approximately 2 years in the laboratory. Furthermore, we cannot exclude the possibility that we accidently selected haplotypes prone to genetic mutations.

Occurrence of amphoteric females

Amphoteric females can produce eggs both by mitosis and meiosis, and are thus able to produce both female and male offspring (King & Snell, 1977). Amphoteric females have only been described for six rotifer species (Rico-Martínez & Walsh, 2013): *Asplanchna herricki* (Mrázek, 1897), *A. priodonta* (Sudzuki, 1955), *Sinantherina socialis* (Bogoslavsky, 1958), *Conochiloides coenobasis* (Bogoslavsky, 1960), *A. girodi* (King & Snell, 1977), and *Trochospaera solstitialis* (McCullough & Lee, 1980). Therefore, to the best of our knowledge, this is the first record on the appearance of amphoteric females in the genus *Keratella*. Only by careful observation of single females for longer time periods, the existence of amphoteric females can be confirmed; therefore, it is possible that also in other genera and species amphoteric females occur. In our study, we observed only one amphoteric female corresponding to 0.35% females in our population; this is similar ratio to the ratio reported by King and Snell (1977), who observed seven amphoteric females of *A. girodi* among 1386 neonates. However, Rico-Martínez and Walsh (2013) observed three amphoteric females of *S. socialis* among only 12 neonates; the exact mechanisms behind the production of amphoteric females remain unknown (Rico-Martinez & Welsh, 2013). Therefore, more observations with different *Keratella* populations are required to investigate this phenomenon in more detail.

In conclusion, this is the first study on *K. cochlearis* that combines demography with genetics-based taxonomy and investigates demographic differences between *K. cochlearis* haplotypes and ESUs. The three investigated haplotypes showed large differences in almost all life history traits and demographic parameters. Furthermore, smaller (and possibly biologically less relevant) differences were recorded between the two haplotypes from ESU 3, which may point to their closer relatedness. Thus, our hypothesis of significant differences in life history parameters between different haplotypes of *K. cochlearis* was confirmed. Although widespread around the world, *K. cochlearis* is an understudied species of monogonont rotifers, probably because of difficulties in culturing. Our study includes a detailed description of *K. cochlearis* culturing methods, which may be useful for future research on this species. The occurrence of abnormal and amphoteric females in *K. cochlearis* deserves further investigation. Because haplotypes used in this study were collected from various lakes, it is difficult to derive any conclusions regarding possible co-existence of these haplotypes in their natural environment. Therefore, more research is needed with more *K. cochlearis* haplotypes per ESU and ESUs derived from the same lake and season.

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ARTICLE III

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ORIGINAL ARTICLE

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Mitonuclear discordance as a confounding factor in the DNA taxonomy of monogonont rotifers

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Discordance between mitochondrial and nuclear phylogenies is being increasingly recognized in animals and may confound DNA-based taxonomy. This is especially relevant for taxa whose microscopic size often challenges any effort to distinguish between cryptic species without the assistance of molecular data. Regarding mitonuclear discordance, two strikingly contrasting scenarios have been recently demonstrated in the monogonont rotifers of the genus *Brachionus*. While strict mitonuclear concordance was observed in the marine *B. plicatilis* species complex, widespread hybridization-driven mitonuclear discordance was revealed in the freshwater *B. calyciflorus* species complex. Here, we investigated the frequency of occurrence and the potential drivers of mitonuclear discordance in three additional freshwater monogonont rotifer taxa, and assessed its potential impact on the reliability of DNA taxonomy results based on commonly used single markers. We studied the cryptic species complexes of *Keratella cochlearis*, *Polyarthra dolichoptera* and *Synchaeta pectinata*. Phylogenetic reconstructions were based on the mitochondrial barcoding marker cytochrome *c* oxidase subunit I gene and the nuclear internal transcribed spacer 1 locus, which currently represent the two most typical genetic markers used in rotifer DNA taxonomy. Species were delimited according to each marker separately using a combination of tree-based coalescent, distance-based and allele-sharing-based approaches. Mitonuclear discordance was observed in all species complexes with incomplete lineage sorting and unresolved phylogenetic reconstructions recognized as the likely drivers. Evidence from additional sources, such as morphology and ecology, is thus advisable for deciding between often contrasting mitochondrial and nuclear species scenarios in these organisms.

KEYWORDS
biodiversity, coalescent theory, cyclical parthenogenesis, introgression, molecular systematics, taxonomic conflict

1 | INTRODUCTION

The phylum Rotifera is comprised by at least 2,000 species defined by morphological characters (Segers, 2008). These morpho-species often harbour a great amount of cryptic diversity that is commonly recognized based on DNA taxonomy (e.g., Gabaldón, Fontaneto, Carmona, Montero-Pau, & Serra, 2017; Kordbacheh, Garbalena, & Walsh, 2017; Suatoni, Vicario, Rice, Snell, & Caccone, 2006). However, detailed morphological and ecological information is rare at the level of cryptic rotifer species as it is more challenging to obtain than DNA sequences (Leasi, Tang, De Smet, & Fontaneto, 2013; Mills et al., 2017; Papakostas, Michaloudi, Triantafyllidis, Kappa, & Abatzopoulos, 2013). Cryptic

rotifer species identification thus largely depends on the assumption that phylogenetic reconstructions using selected single genetic markers correctly reflect the genealogy of the studied taxa.

Generally, the combined use of nuclear and mitochondrial markers is advocated to infer robust phylogenetic relationships. However, phylogenetic discordance between nuclear and mitochondrial markers, coined mitonuclear discordance (Avise, 2004; Petit & Excoffier, 2009; Toews & Brelsford, 2012), is notorious across different animal phyla and has been suggested to confound species delimitations (Degnan & Rosenberg, 2009; Mallet, Besansky, & Hahn, 2016; Wielstra & Arntzen, 2014). Mitonuclear discordance generally describes different types of incongruences in the phylogenies between mitochondrial and nuclear markers (Toews & Brelsford, 2012). Furthermore, other types of discrepancies such as phylogeographic disparities can also be broadly categorized as evidence for mitonuclear discordance (Toews & Brelsford, 2012). Because DNA taxonomy often relies on phylogenies to decide on species boundaries, we may also consider differences in the number of species estimates between mitochondrial and nuclear markers as mitonuclear discordance. Whichever the case may be, mitonuclear discordance can have profound implications in assessments of the morphology or the ecology of the delimited species (Papakostas et al., 2016; Wielstra & Arntzen, 2014).

Phylogenetic mitonuclear discordance may be attributed to several causes including introgressive hybridization, horizontal gene transfer (HGT), androgenesis, incomplete lineage sorting (ILS), and unresolved phylogenetic polytomies. Mitochondrial introgression refers to the interspecific movement of mitochondria by hybrid backcrossing and has been recognized in several animal taxa as a driver of mitonuclear discordance (Petit & Excoffier, 2009; Toews & Brelsford, 2012). HGT typically involves relatively few genes at a time and refers to the movement of genetic material between diverged taxa, and as such, it may produce strong phylogenetic incongruences (Keeling & Palmer, 2008; Mallet et al., 2016). However, HGT is generally considered to be a rare event in animals compared to prokaryotes. Androgenesis involves the asexual reproduction of the nuclear genome and thus may also result in mitonuclear discordance, but, like HGT, its role has been suggested to be limited amongst animal taxa (Hedtke & Hillis, 2011). ILS is the process by which ancestral polymorphism is retained through speciation. ILS may obscure phylogenetic signal, cause phylogenetic conflict, and produce dissimilar species delimitations between markers (Chaudhary, Boussau, Burleigh, & Fernandez-Baca, 2015; Degnan & Rosenberg, 2009; Pamilo & Nei, 1988). ILS is considered a common source of phylogenetic discrepancy for a wide range of animal taxa, including rotifers (Meyer, Matschiner, & Salzburger, 2017; Papakostas et al., 2016; Rogers & Gibbs, 2014; Suh, Smeds, & Ellegren, 2015). Altogether, the challenges posed by mitonuclear discordance may be exceptionally crucial for species delimitation and identification. This is especially true when it comes to microscopic animals for which DNA sequence data are relatively easy to obtain, but morphological and ecological information is scarce.

In rotifers, DNA taxonomy is extensively applied using single markers, usually the barcoding mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene or the nuclear internal transcribed spacer I locus (ITS1) (Fontaneto, 2014). Using both these markers, solid evidence for mitonuclear phylogenetic concordance was found in the cryptic species complex of the marine rotifer *Brachionus plicatilis* Müller, 1786 (Gómez, Serra, Carvalho, & Lunt, 2002; Mills et al., 2017). In striking contrast, using the same markers and microsatellite genotyping, widespread mitonuclear phylogenetic discordance was demonstrated in the cryptic species complex of the freshwater rotifer *B. calyciflorus* Pallas, 1776 (Papakostas et al., 2016). Morphometric analysis further revealed that such discordance may have profound implications at correctly assessing species boundaries and estimating levels of morphological plasticity or stasis (Papakostas et al., 2016). As such, species inference could be non-trivial if mitonuclear discordance at different levels is a rampant phenomenon in rotifers, particularly as the taxonomy of many rotifer taxa is still unclear (Wallace, 2006), and most rotifer species show a large phenotypic polymorphism (Segers & De Smet, 2008).

Little is known about the potential effect of hybridization, HGT or ILS on monogonont rotifer species delimitations, except for the two previously mentioned contrasting cases in the genus *Brachionus*. In *B. plicatilis* cryptic species, species boundaries are clearly maintained by reproductive isolation in the field (Gómez & Serra, 1995), even though potentially not under laboratory conditions (Suatoni et al., 2006). In *B. calyciflorus* cryptic species, hybrids have been found both in the wild and in laboratory conditions (Papakostas et al., 2016). These completely different scenarios in two species complexes of the same genus make any inference on other rotifer species or genera rather speculative. We can already rule out a taxonomic bias in mitonuclear discordance, given that *B. calyciflorus* and *B. plicatilis* species complexes belong to the same genus. In other genera, intermediate morphological forms between nominal species, proposed to be the result of introgressive hybridization, have been noted in the rotifers *Polyarthra* sp. and *Conochilus* sp. already several years ago (Pejler, 1956). Interspecific gene flow in rotifers was also recently found in the asexual bdelloid *Adineta vaga* (Davis, 1873) (Debortoli et al., 2016). For *A. vaga*, its cause was attributed to HGT, as no gene flow is possible for the strictly parthenogenetic bdelloid rotifers (Debortoli et al., 2016). Altogether, investigating the porosity of the rotifer genomes to interspecific gene flow is still in its infancy. Thus, the generality and the

level of phylogenetic discordance in the DNA taxonomy of rotifers between mitochondrial (mtDNA) and nuclear (nuDNA) markers are still unclear.

In this study, we explored the prevalence of mitonuclear discordance in the rotifer species complexes *Keratella cochlearis* (Gosse, 1851), *Polyarthra dolichoptera* (Idelson, 1925) and *Synchaeta pectinata* (Ehrenberg, 1832). These species are positioned at varying phylogenetic distances from the *Brachionus* sp. rotifers within Monogononta, which is the largest major rotiferan clade (Sorensen & Giribet, 2006) and is characterized by cyclical parthenogenesis. While *K. cochlearis* represents a closely related clade within the same family Brachionidae, *P. dolichoptera* and *S. pectinata* represent clades within the distinct family Synchaetidae. Previous studies have delimited species in these complexes using solely the COI gene: eight putative species were recognized in the *K. cochlearis* complex (Cieplinski, Weisse, & Obertegger, 2017), twelve putative species in the *P. dolichoptera* complex (Obertegger, Flaim, & Fontaneto, 2014), and five putative species in the *S. pectinata* complex (Obertegger, Fontaneto, & Flaim, 2012). Notably, in the *K. cochlearis* species complex, taxa were partially supported by differences in lorica morphology (Cieplinski, et al., 2017), and the pattern of occurrence of different taxa of the *P. dolichoptera* species complex was predicted to some degree by environmental conditions (Obertegger et al., 2014), thus giving support to the COI-based delimitations.

Apart from describing mitonuclear discordance, we investigated the underlying mechanisms leading to such potential discrepancies. We aimed at distinguishing hybridization-from ILS-driven mitonuclear discordance as the most plausible explanation for any observed mitonuclear discordance. We also considered unresolved polytomies as the most parsimonious explanation for any discordance. To discriminate between hybridization and ILS, we employed a statistical framework that uses the multispecies coalescent (Joly, 2012; Joly, McLenachan, & Lockhart, 2009). This approach has been previously validated in cyclical parthenogenetic rotifers by comparing results in hybridizing and non-hybridizing *Brachionus* sp. cryptic species (Papakostas et al., 2016). We employed the same mitochondrial and nuclear markers, namely COI and ITS1, as used by several studies such as Gómez et al. (2002), Papakostas et al. (2016) and Mills et al. (2017). Nevertheless, detecting hybridization out of gene genealogies can be especially challenging (Holder, Anderson, & Holloway, 2001). Gene conversion, which is typical for multicopy markers like the ITS1, and facultative sexual reproduction, like in the studied cyclical parthenogenetic rotifers, both have unaccountable effects in the standard multispecies coalescent model (Hartfield, Wright, & Agrawal, 2016). In conclusion, we discuss the frequency and causes of mitonuclear discordance in the monogonont rotifer taxa for which data are currently available.

2 | MATERIALS AND METHODS

2.1 | DNA extraction and sequencing

To correctly assess mitonuclear discordance, COI and ITS1 were sequenced from the same animals. We selected animals of *K. cochlearis*, *P. dolichoptera* and *S. pectinata* from previous studies in which we sequenced the COI marker and delimited species (Cieplinski et al., 2017; Obertegger et al., 2012, 2014). Sequenced specimens came from the Trentino–South Tyrol area in northern Italy, which is certainly more restricted than the worldwide sampling distribution of the *B. plicatilis* study (Mills et al., 2017). Nevertheless, the reported mitonuclear discordance in *B. calyciflorus* was inferred using samples from a comparably restricted area in the Netherlands (Papakostas et al., 2016), which makes the results of our study relevant. Given that we do not provide conclusive evidence on species identity but only hypotheses on these taxa, we will refer to delimited species from DNA taxonomy as evolutionary significant units of diversity (ESUs), and we will distinguish between COI-ESUs and ITS1-ESUs. For *K. cochlearis*, we selected 47 animals that covered 35 COI haplotypes and six COI-ESUs out of 57 COI haplotypes and eight COI-ESUs described in Cieplinski et al. (2017); for *P. dolichoptera*, the 27 selected animals covered 15 COI haplotypes and 10 COI-ESUs out of 53 COI haplotypes and 12 COI-ESUs described in Obertegger et al. (2014); for *S. pectinata*, the 37 selected animals covered 11 COI haplotypes and four COI-ESUs out of 16 COI haplotypes and five COI-ESUs described in Obertegger et al. (2012). The correspondence between COI-ESUs names used here and those of the previous papers is reported in Tables S1–S3.

To increase the low COI-ESU number in *S. pectinata*, we also sequenced six additional animals for COI and ITS1, to obtain a total of 43 animals sequenced for both markers. For these samples, DNA was extracted from single live individuals with 35 µl of Chelex (InstaGene Matrix, Bio-Rad, Hercules, CA, USA). A part of the COI region was then PCR-amplified using the primers LCO1490 (5′-GGTCAA CAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAA ACTTCAGGGTGACCAAAAAATCA- 3′) (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). PCR conditions for COI comprised initial denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 48°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 7 min.

The complete ITS1 region for all specimens was PCR-amplified using the primers III (5′-CACACCGCCCGTCGCT ACTACCGATTG-3′) and VIII (5′-ACCGCCCGTCGCTAC TACCGATTG-3′) (Palumbi, 2006). PCR conditions for ITS1 comprised initial denaturation at 98°C for 3 min, followed by 40 cycles at 98°C for 15 s, 50°C for 20 s and 72°C for 1 min and a final extension step at 72°C for 3 min. PCR products

were sequenced in-house using the same PCR primer with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Monza, Italy), according to the manufacturer's protocols and recommendations. After purification using the Agencourt CleanSEQ kit (Beckman Coulter, Milan, Italy), products were run on an Automated Capillary Electrophoresis Sequencer 3730XL DNA Analyser (Applied Biosystems).

2.2 | Phylogenetic reconstructions

Phylogenetic reconstructions and ESU delimitation based on COI were obtained from previous studies (Cieplinski et al., 2017; Obertegger et al., 2012, 2014) on the same individuals used here. The additional six new *S. pectinata* COI sequences were aligned using ChromasPro (Technelysium Pty. Ltd.) and checked for congruence with the haplotypes of Obertegger et al. (2012).

ITS1 sequencing reads were trimmed to only contain the ITS1 locus and then aligned using the *mlocarna* function of the LocARNA v.1.8.7 software with default settings (Will, Joshi, Hofacker, Stadler, & Backofen, 2012). LocARNA aligns non-coding RNAs by considering both sequence and secondary structure similarities, which may be especially relevant for correctly aligning ITS1 sequences (Coleman, 2015; Wolf, 2015). In the case of ITS1, fitting an outgroup proved non-trivial. Thus, as this study aims at ingroup relationships, we omitted the use of an outgroup and used the mid-point rooting method for visualization purposes as implemented in FigTree v.1.4.3 (available from: http://tree.bio.ed.ac.uk/software/figtree/—last accessed 1 June 2017).

Upon alignment, unique sequences, hereafter called haplotypes, were recognized per taxon and per marker using the program DNAsp v.5 (Librado & Rozas, 2009); indels were considered as different characters for ITS1, and thus, different haplotypes may have zero uncorrected genetic distances between them. Phylogenetic relationships between haplotypes were based, for COI, on best-fit models of nucleotide substitution as used in previous studies on the complexes (Cieplinski et al., 2017; Obertegger et al., 2014, 2012), while for ITS1, according to the Bayesian information criterion (BIC; Schwarz, 1978) using jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012). With jModelTest 2, 88 models and 11 substitution schemes were tested on maximum-likelihood topologies obtained with the subtree pruning and regrafting algorithm implemented in PhyML v.3.0 (Guindon et al., 2010).

By applying the appropriate models, we then reconstructed the phylogenetic relationships of the studied haplotypes according to maximum-likelihood (ML) and Bayesian inference (BI) approaches. ML phylogenies were obtained with the program PhyML 3.0 (Guindon & Gascuel, 2003). Node support values in ML phylogenies were obtained by the approximate log-likelihood ratio test. BI phylogenies were obtained with BEAST v1.8.3 (Drummond, Suchard, Xie, & Rambaut, 2012) under a log-normal relaxed (uncorrelated) clock following Monaghan et al. (2009) and Wertheim, Sanderson, Worobey, and Bjork (2009). BEAST was run for 100 million generations, sampling every 10,000 generations. Convergence was assessed with Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014) by requiring effective sample size (ESS) values above 200 for all parameters. TreeAnnotator v.1.8.3 (part of the BEAST package) was used to calculate node support estimates after discarding the first 20% of the trees as burn-in, while keeping the node heights of the highest log clade credibility tree.

2.3 | Species delimitations

In the absence of a single best DNA taxonomy method to delimit species (Carstens, Pelletier, Reid, & Satler, 2013; Dellicour & Flot, 2015; Fontaneto, Flot, & Tang, 2015), we considered the combined outcome of four delimitation methods that corresponded to three kinds of approaches (Flot, 2015): (a) the generalized mixed Yule–coalescent model (GMYC: Pons et al., 2006; Fujisawa & Barraclough, 2013) and the Poisson Tree Process (PTP: Zhang, Kapli, Pavlidis, & Stamatakis, 2013), which are both tree-based approaches; (b) the automatic barcode gap discovery method (ABGD: Puillandre, Lambert, Brouillet, & Achaz, 2012), which is a distance-based approach; and (c) the haploweb method (Flot, Couloux, & Tillier, 2010), which is an allele-sharing approach. Haploweb depends on heterozygosities, detectable as double peaks in the chromatograms, and therefore was applicable only to the ITS1 datasets. In all ITS1 cases, species were delimited without considering any outgroup, and mid-point rooting was used in phylogenetic trees. GMYC was applied according to the single threshold method to the summary trees from the BEAST runs using the *gmyc* function of the R package "splits" (Ezard, Fujisawa, & Barraclough, 2009). PTP was applied to the ML trees using the bPTP server with default settings (http://species.h-its.org—last accessed 1 June 2017). ABGD was applied directly to the haplotype alignments using the ABGD web server with default settings (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html—last accessed 1 June 2017). Haploweb delimitation was performed as described in Flot et al. (2010). Briefly, ITS1 haplotypes identified from the chromatographs were used to construct a median-joining network with the program Network v.5.0.0.1 (available online at http://www.fluxus-engineering.com/sharenet_rn.htm—last accessed 1 June 2017). Haplotype heterozygosities were used to determine fields of recombination of putative cryptic species status (Doyle, 1995; Flot et al., 2010). Heterozygosities were recognized as double peaks in the chromatograms using FinchTV 1.4 (http://www.geospiza.com/ftvdlinfo.

html). Phasing of heterozygote sequences was trivial as no length-variant heterozygotes were observed.

In Obertegger et al. (2014), *Polyarthra* ESU delimitation was performed only based on GMYC. We, thus, here performed also PTP and ABGD ESU delimitations on the same data set.

2.4 | Phylogenetic discordance and tests for signatures of hybridization

To infer phylogenetic discordance, our first step was to decide on COI-ESUs and ITS1-ESUs. Given there is currently no systematic nomenclature for referring to those delimited ESUs, we henceforth referred to COI-ESUs using a sequential Latin numeric identifier, "I," "II" etc., and to ITS1-ESUs using ordered lowercase Greek letters, "α," "β," etc. (Tables S1–S3). Because different delimitation methods may yield different results, for example, they may oversplit or overlump taxa (Dellicour & Flot, 2015), we weighted each method equally and considered the most conservative outcome in final species delimitation.

To assess the level of mitonuclear discordance in our data sets, we considered four steps each corresponding to somewhat different types of discordance. First, we assessed whether COI-ESUs and ITS1-ESUs obtained from the same set of animals did not match. Second, within the previously identified cases, we searched for the extreme cases when animals from different COI-ESUs shared even the same ITS1 sequence. Third, for cases in which fewer COI-ESUs were observed compared to ITS1-ESUs, we checked whether the COI-ESUs nested within each ITS1-ESU revealed a monophyletic group of COI-ESUs, or fourth, whether the COI-ESUs nested within an ITS1-ESU formed non-monophyletic groups. We did not check for phylogenetic incongruences between COI and ITS1 within the same taxonomic units, given that this would simply be evidence of gene flow within species with no effect on DNA taxonomy.

Additionally, to investigate the plausibility of hybridization or ILS as drivers of any observed mitonuclear discordance across delimited ESUs, we employed the statistical framework of Joly et al. (2009) implemented in JML v.1.3.0 (Joly, 2012) as described in Papakostas et al. (2016).

3 | RESULTS

3.1 | Species delimitations

Upon ITS alignment available at Mendeley https://doi.org/10.17632/h6b76xj7wh.1), we distinguished 10 ITS1 haplotypes in *K. cochlearis*, eight ITS1 haplotypes in *P. dolichoptera,* and four ITS1 haplotypes in *S. pectinata*. Heterozygotes with single nucleotide polymorphisms in ITS1 were found in 19 specimens in *K. cochlearis*, whereas no heterozygotes were found in *P. dolichoptera* and *S. pectinata*. (Table S1) The best-fit models of nucleotide substitution employed for the phylogenetic reconstructions and in JML tests were for COI, HKY + I + G, HKY + I + G and GTR + I + G, and for ITS1, HKY, F81 + I and F81 in *K. cochlearis, P. dolichoptera* and *S. pectinata,* respectively. The additional *S. pectinata* sequences belonged to one published haplotype of Obertegger et al. (2014), and thus, we finally covered 12 COI haplotypes and four COI-ESUs of Obertegger et al. (2014).

For ITS1 of all three species complexes, the GMYC method did not produce delimitations significantly more likely than the null model of just one ITS1-ESU. For *K. cochlearis* and *S. pectinata*, PTP and ABGD indicated the same ITS1-ESUs, while for *P. dolichoptera,* PTP indicated more ITS-ESUs than ABGD. Haploweb indicated more ITS1-ESUs than PTP and ABGD for *K. cochlearis* and *P. dolichoptera* and the same for *S. pectinata* (Figures 1–3; Figs. S1–S3). We thus investigated phylogenetic discordance in the most conservative way possible and focused on two ITS1-ESUs for *K. cochlearis* and *S. pectinata* and on four ITS1-ESUs for *P. dolichoptera*.

3.2 | Mitonuclear discordance

COI-based ESUs were more numerous than ITS1-ESUs (Figures 1–3). Yet, most of the COI-ESUs were nested within ITS1-ESUs monophyletic clades, and thus occurrence of actual discordance was rather low, due to single cases (Table 1). In comparison with previously published results, all the three analysed species complexes had intermediate levels of mitonuclear discordance, between the cases of *B. plicatilis* with perfect congruence between COI-ESUs and ITS1-ESUs and of *B. calyciflorus* with a high level of discordance (Table 1).

Mitonuclear discordance in delimitation of ESUs was thus found in all three species complexes; yet in *K. cochlearis*, the COI-ESUs were nested within the monophyletic ITS1-ESUs (Figure 1, Table 1), making the incongruence potentially only a matter of different phylogenetic resolution in the two markers. In *P. dolichoptera* and *S. pectinata,* the discrepancy was mirrored also in discordance at deeper nodes (Figures 2 and 3; Table 1). The observed mitonuclear discrepancies were tested for signatures of hybridization as a major driving process, but all p-values from JML tests were higher than 0.05, and thus, hybridization could be ruled out, and incomplete lineage sorting was suggested as the most likely cause of the phylogenetic discordance between COI and ITS1 in species delimitation by DNA taxonomy.

4 | DISCUSSION

Evidence for mitonuclear discordance was found in all three species complexes, even though at different levels. Regarding

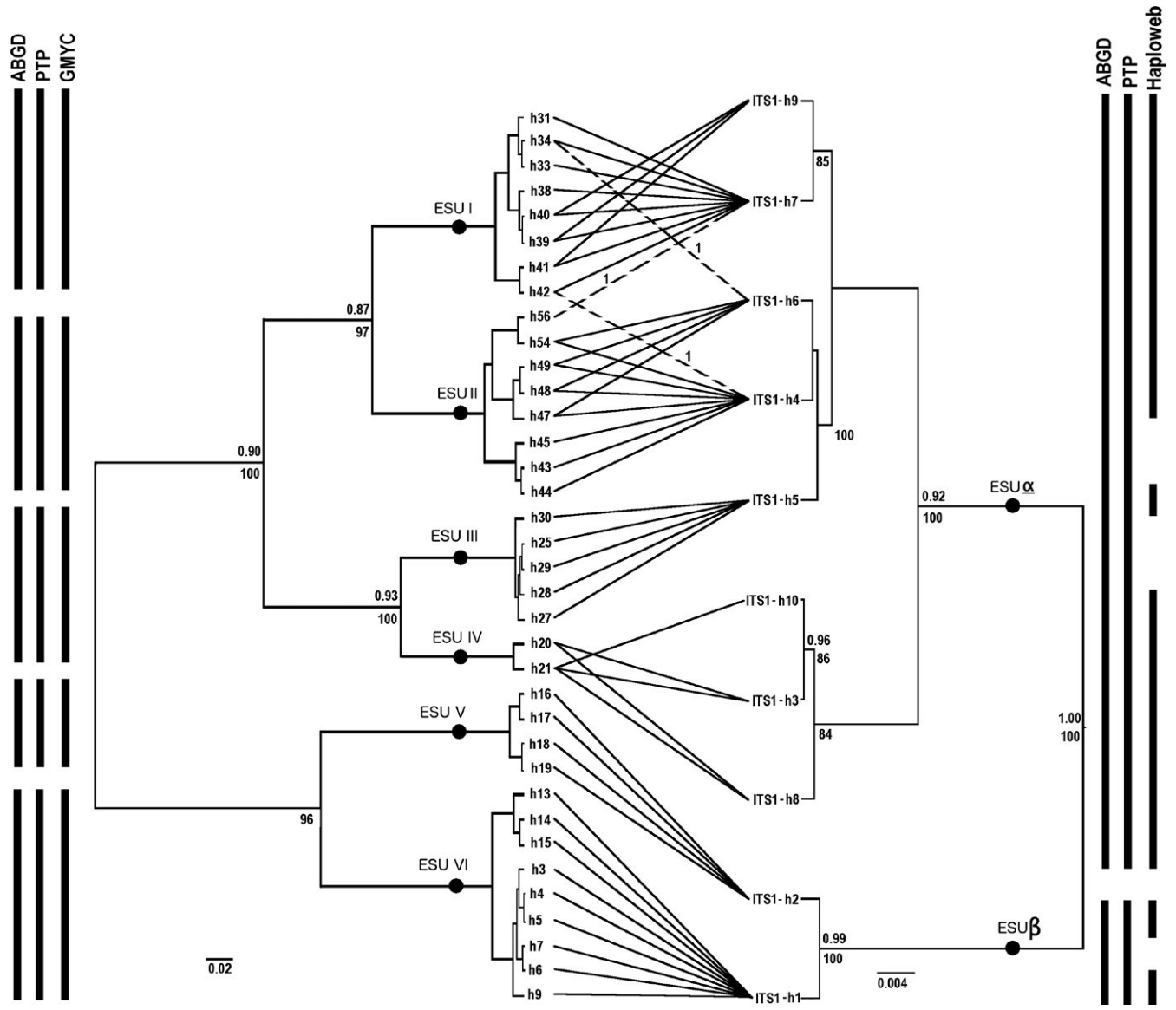

FIGURE 1 Tanglegram for *Keratella cochlearis* of summarized Bayesian phylograms illustrating the correspondence of distinct COI (left) with ITS1 (right) haplotype combinations found in samples sequenced to both markers. Dashed lines indicate instances of animals (number of animals on the line) from different COI-ESUs sharing the same ITS1: the single outlier individuals are reported (e.g., COI-ESU I h34), sharing the same ITS1 haplotype (ITS1-h6) with other individuals that are identified as a different COI-ESU (COI-ESU II). Vertical bars at the side of each tree represent COI-ESUs (left) and ITS1-ESUs (right) obtained with each designated species delimitation method. Agreed ESUs are denoted as black dots. Node support is shown with values only above 75% bootstrap support and above 0.75 posterior probability. Scale bars show the number of expected nucleotide changes per site

the details of DNA taxonomy, the ITS1 phylogeny always indicated fewer ESUs than the COI phylogeny in the taxa considered; yet, this discrepancy was expected due to lower variability in ITS1 than in COI in rotifers (Mills et al., 2017; Papakostas et al., 2016). One problem in DNA taxonomy in the three species complexes is therefore due to a different resolution between COI and ITS1. Such difference is less strong than in comparisons between COI and 18S typically in microscopic organisms (Kimpel, Gockel, Gerlach, & Bininda Emonds, 2015; Tang et al., 2012), although 18S is commonly less variable than ITS1 (Mills et al., 2017; Papakostas et al., 2016; Tang et al., 2012). Thus, the poor performance of GMYC based on ITS phylogenies might have been related to the low number of ITS1 haplotypes and due to the relatively low variability in this marker. It is known that low haplotype diversity can hinder GMYC from fitting-in interspecific branching rates (Dellicour & Flot, 2015; Talavera, Dinca, & Vila, 2013). The same problem may occurred also for haploweb; simulations have showed that oversplitting of ESUs with haploweb may happen in the presence of low number of heterozygous individuals (Dellicour & Flot, 2015). We suggest that this was the case in our study, especially because

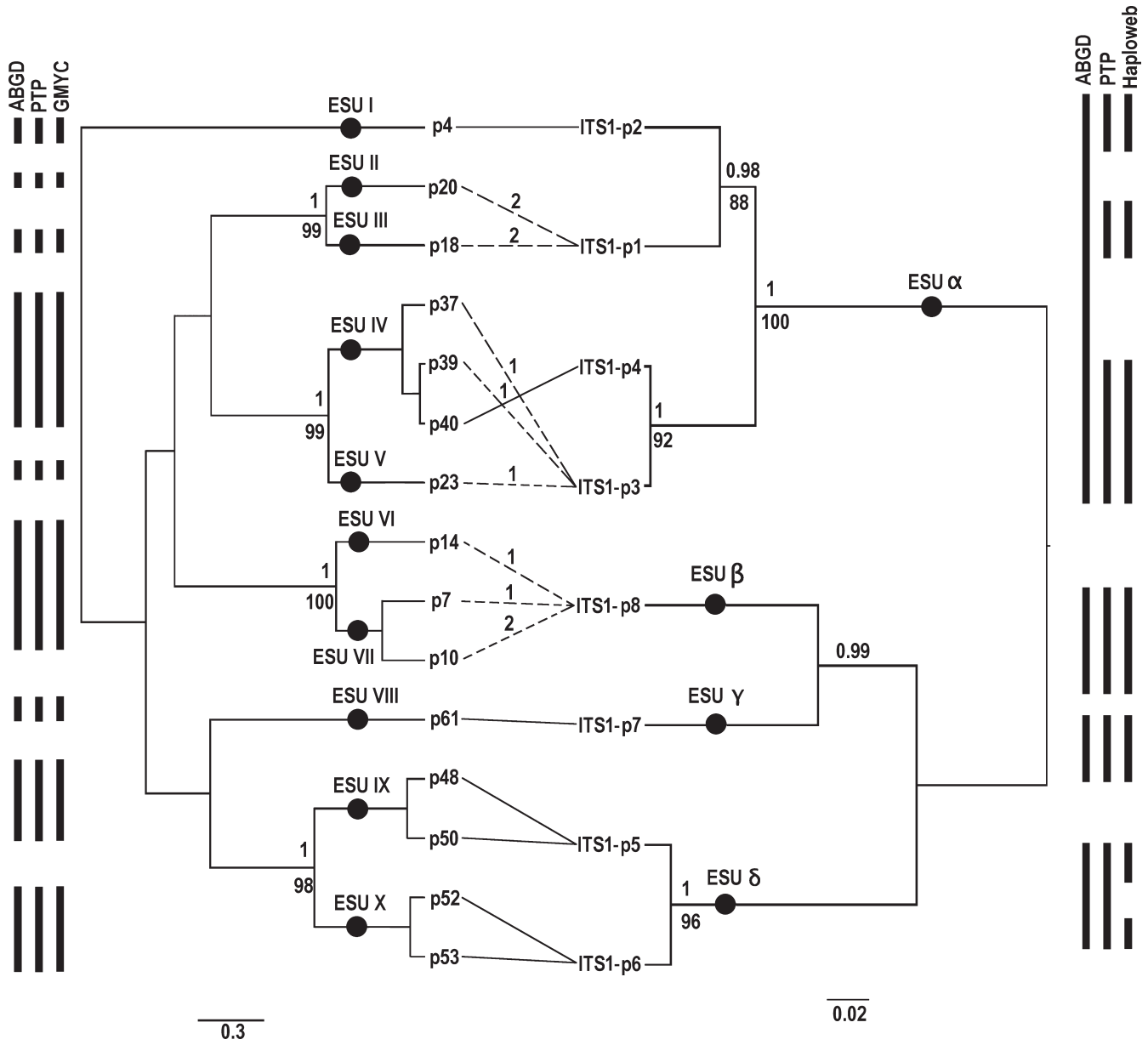

FIGURE 2 Tanglegram for *Polyarthra dolichoptera* of summarized Bayesian phylograms illustrating the correspondence of distinct COI (left) with ITS1 (right) haplotype combinations found in samples sequenced to both markers. Dashed lines indicate instances of animals (number of animals on the line) from different COI-ESUs sharing the same ITS1. Vertical bars at the side of each tree represent COI-ESUs (left) and ITS1-ESUs (right) obtained with each designated species delimitation method. Deduced ESUs are denoted as a black dot. Node support is shown with values only above 75% bootstrap support and above 0.75 posterior probability. Scale bars show the number of expected nucleotide changes per site

no heterozygotes at all were found in *P. dolichoptera* and *S. pectinata*, and very few heterozygotes were found in *K. cochlearis*.

Interspecific gene flow could be potentially present in rotifers (Debortoli et al., 2016; Papakostas et al., 2016), and this could be the cause of the discrepancies between COI-based and ITS1-based reconstructions, providing ITS1-ESUs including non-monophyletic groups of COI-ESUs; yet, we did not find any clear evidence of hybridization in any of the three cryptic species complexes of monogononts, and the phylogenetic discordance could be simply driven by ILS or by low support values of deeper dichotomies (i.e., the discrepancies in multiple COI-ESUs polyphyletic within ITS1-ESUs could be resolved considering basal dichotomies as polytomies). The missing evidence of hybridization in the three species complexes cannot rule it out completely due to the relatively low sample size of our data sets to search for rare events. These and other taxa should be further investigated in larger sample sizes, and using more genetic markers, before we fully exclude the possibility of hybridization as a general process in rotifers. Decisions on species boundaries should not be limited to molecular

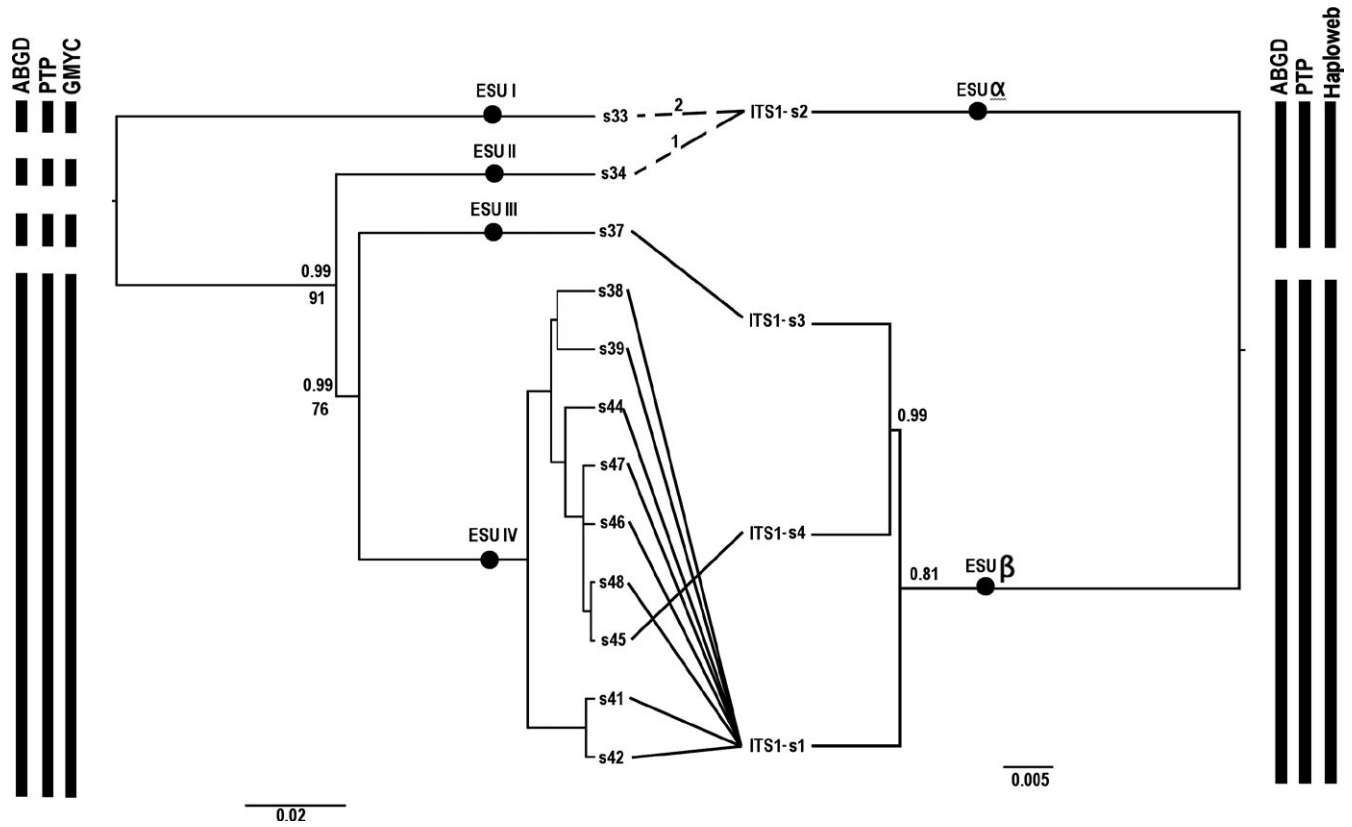

FIGURE 3 Tanglegram for *Synchaeta pectinata* of summarized Bayesian phylograms illustrating the correspondence of distinct COI (left) with ITS1 (right) haplotype combinations found in samples sequenced to both markers. Dashed lines indicate instances of animals (number of animals on the line) from different COI-ESUs sharing the same ITS1. Vertical bars at the side of each tree represent COI-ESUs (left) and ITS1-ESUs (right) obtained with each designated species delimitation method. Agreed ESUs are denoted as black dots. Node support is shown with values only above 75% bootstrap support and above 0.75 posterior probability. Scale bars show the number of expected nucleotide changes per site

TABLE 1 Different levels of mitonuclear discordance between COI-ESUs and ITS1-ESUs

Species complex	ITS1-ESUs matching COI-ESUs	Different COI-ESUs with the same ITS1	COI-ESUs monophyletic within ITS1-ESUs	COI-ESUs non-monophyletic within ITS1-ESUs	Source
Brachionus calyciflorus	no, fewer ESUs in ITS1	several	no	yes	Papakostas et al. (2016)
B. plicatilis	yes	no	NA	NA	Mills et al. (2017)
Keratella cochlearis	no, fewer ESUs in ITS1	ESU I and ESU II with ITS1-h 4, 6 and 7	yes	no	this study
Polyarthra dolichoptera	no, fewer ESUs in ITS1	ESU II and ESU III with ITS1-p1 ESU IV and ESU V with ITS1-p3 ESU VI and ESU VII with ITS1-p8	yes (except for the case of ESU α)	yes (ESU α with ESU I and ESU II to ESU V)	this study
Synchaeta pectinata	no, fewer ESUs in ITS1	ESU I and ESU II with ITS1-s2	yes (except for the case of ESU α)	yes (ESU α with ESU I and ESU II)	this study

For the four cases described in this study, refer to Figures 1–3 for symbols and acronyms; NA means not applicable.

evidence only. For instance, cryptic species barriers in the *B. plicatilis* complex were also largely supported by interspecific mating experiments (Suatoni et al., 2006) as well as by morphological and ecological features (Ciros-Pérez, Gómez, & Serra, 2001; Hwang, Dahms, Park, & Lee, 2013; Michaloudi et al., 2017). It is also known that in the presence of mitochondrial introgression, species delineation can overestimate or underestimate ecological divergence

(Wielstra & Arntzen, 2014) or levels of morphological variation (Papakostas et al., 2016). In the same spirit, mitochondrial COI-based *B. calyciflorus* species delimitation was found to be a worse predictor of ecological differentiation and morphological variation compared to nuclear ITS1-based delimitation (Papakostas et al., 2016). We anticipate future research to address questions on how hybridization and introgression may impact the morphology and the ecology of rotifer species, particularly as monogonont rotifers provide a suitable model to also investigate results in a comparative phylogenetic context.

When multiple loci are investigated in DNA taxonomy, delineation of species becomes more accurate even though a certain amount of incongruence in delineation is expected (Fonseca, Fontaneto, & Di Domenico, 2017). Furthermore, polyphyly in mitochondrial and nuclear gene trees can further complicate species delimitations, and thus, a cross-check by adding morphology and ecology as a non-DNA-based source of information is recommended (Schlick-Steiner et al., 2010). For *K. cochlearis,* only COI-ESU I and COI-ESU VI could be differentiated based on morphology (Cieplinski et al., 2017). For *S. pectinata,* a different preference for total phosphorus was found for COI-ESUs I and III versus COI-ESU IV (Obertegger et al., 2012), and for *P. dolichoptera,* COI-ESU III was considered a species occurring throughout the year, while COI-ESU IX a winter/spring species (Obertegger et al., 2014). These ecological and morphological differences were reflected in the ITS1 phylogenies for *P. dolichoptera* and for *K. cochlearis* while not for *S. pectinata*.

In summary, all the plausible scenarios of mitonuclear discordance can be observed in the DNA taxonomy of monogonont rotifers, ranging from complete congruence between mitochondrial and nuclear markers (as in the case of *B. plicatilis*), through different intermediate levels of discordance, mostly due to differential resolution from different markers (*K. cochlearis*, *P. dolichoptera, S. pectinata*), to complete discordance (*B. calyciflorus*). Thus, in rotifers, DNA-based taxonomy based on one single locus could still be partially valid, but in most cases, additional markers and additional information from ecology and morphology with a large sample size are advisable. Given the importance of molecular phylogenies for rotifer cryptic species recognition (e.g., Fontaneto et al., 2015), we thus consider that any shortcomings associated with phylogenetic conflict may be crucial at correctly assessing rotifer biodiversity, species boundaries and guiding future research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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ARTICLE IV

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ORIGINAL ARTICLE

Filming of zooplankton: a case study of rotifer males and *Daphnia magna*

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ABSTRACT

Filming live organisms can give new insights into the hidden life of plankton. Accessibly priced digital cameras are now available for a large range of users. Here, we demonstrate the technical setup and workflow of using a single-lens reflex (DSLR) camera to film the behaviour of males of two rotifer species, Brachionus angularis *Gosse (1851) and* Keratella cochlearis *Gosse (1851), and of the cladoceran* Daphnia magna *Straus (1820). Rotifers are cyclical parthenogens that produce males only under certain environmental conditions. Thus, knowledge on rotifer males is still limited because of their ephemeral nature and because they are often smaller than females. We filmed males of* B. angularis *and* K. cochlearis *with a DSLR camera connected to a compound microscope to better understand their morphology and behaviour in comparison to conspecific females. While written descriptions have their scientific value, seeing is complementary because everyone can verify what has been described. We made our videos publicly accessible through links connected to the paper. Our videos are, to our best knowledge, the first on males of* B. angularis *and* K. cochlearis*. Furthermore, we filmed the behavioural response of* D. magna *to ultraviolet (UV) radiation with a macro lens attached to the DSLR camera. Approaches like this are valuable tools in environmental teaching. To see live organisms with one's own eyes may contribute to raising public awareness about the value of water resources and their hidden communities. In summary, filming can be a valuable tool to ignite scientific discussion, but the videos need an open-access platform where they can be referenced in a topic-related order.*

Key words: Video-microscopy; males; Lake Tovel; Brachionus angularis*;* Keratella cochlearis*; DSLR.*

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INTRODUCTION

Since the pioneering work of Antonie van Leeuwenhoek (1632-1723) in developing microscopic biconvex lenses (Ford, 1982) microscopy has become a fundamental method for studying microorganisms. Microscopy has made significant advances, and by techniques such as scanning electron and transmission electron microscopy amazing details on morphological structures of organisms can be obtained. However, these techniques require fixed samples limiting the observation of live organisms. In fact, behaviour and morphology of microscopic organisms are currently described by text, schematic representations, and images.

Filming live organisms can give new insights into the hidden life of plankton. In the past decades, a few researchers (Gilbert, 1963; Coulon *et al.*, 1983) recorded rotifers on video cassette support to study behaviour such as mating, speed, trajectory, and predation. However, this technique did not find a vast application probably because of the restricted possibility to edit the filmed material, difficulty in data sharing with a vast audience, and the physical space needed for storing the videotapes. Recently, digital single lens reflex (DSLR) digital single-lens translucent (DSLT), and electronic viewfinder interchangeable lens (EVIL) cameras have become popular among amateurs and professionals alike for photo shooting and filming; however, even though such digital cameras can be easily attached to a microscope (Fig. 1), researchers seldom use these cameras to film microscopic life and produce a video. A video gives the possibility to observe repeatedly the same scene by simply freezing or decelerating certain parts (*i.e.*, frames). This allows observing in detail the behaviour and morphological structures of organisms that are generally examined in a fixed state. In fact, the traditional faunological approach of sampling planktonic organisms followed by fixation is still the most common one. Fixing plankton such as rotifers often leads to changing morphology because generally rotifers retract the corona and the foot when exposed to a preservative, and illoricate species especially often become sphere-like indistinct objects (Obertegger *et al.*, 2006). This morphological distortion also holds true for male rotifers possessing a weak to no lorica (Ricci and Melone, 1998).

Rotifer males are rarely investigated because they only appear for a restricted period during the year and populations mainly reproduce parthenogenetically (Fontaneto and DeSmet, 2015). While males of Bdelloidea have never been observed, rotifers of the subclass Monogononta show sexual dimorphism (Wallace *et al.*, 2006). Rotifer males have: i) a rudimentary digestive apparatus (Pontin, 1978) because they do not feed; ii) are considerably smaller than females (Ricci and Melone, 1998); iii) have a shorter live-span than females (Gilbert, 1963); and

iv) show a weak to marked resemblance to females (Fontaneto and DeSmet, 2015).

Here, we show how to easily use a DSLR camera to observe live zooplankton. We underline the utility of sharing videos within the scientific community to display and discuss the behaviour and morphology of microscopic organisms captured in videos. Open-access platforms, such as the well-known sites YouTube or DailyMotion may be suitable to share observations obtained in the laboratory with the scientific and lay community. We focused on rotifer males of *Brachionus angularis* and *Keratella cochlearis* to demonstrate the technical setup and workflow. Apart from descriptions and drawings of morphology and behaviour of rotifer males (Gilbert, 1963; Sudzuki, 1964; Pontin, 1978; Dumont *et al.*, 2006; Fontaneto and DeSmet, 2015), there are, to the best of our knowledge, very few videos on rotifer males (see https://www.youtube.com/watch?v=F61cHnGih54). Our videos, which are accessible on YouTube to a vast audience, apart being the first for males of these species, also show details on morphology, swimming, and interaction of males with females. Furthermore, we filmed the behaviour of young individuals of *Daphnia magna* in response to ultraviolet radiation (UVA; 320-400 nm). Cladocera are known to avoid UVA radiation by actively swimming in the opposite direction (Hylander *et al.*, 2014). The small experimental setup we describe can be readily used in environmental teaching and used for several purposes such as explaining and promoting scientific research.

METHODS

Brachionus angularis (Gosse, 1851) and *K. cochlearis* (Gosse, 1851) were isolated from Lake Tovel (Italy; 46°15'N 10°56'E) and cultivated in petri dishes with WMC culture medium (Guillard and Lorenzen, 1972) under a 14:10 light/dark cycle at 13.5°C. Cultures were fed weekly with *Chlorella vulgaris* (Scandinavian Culture Collection of Algae and Protozoa strain K-1801).

Males of *B. angularis* and *K. cochlearis*, respectively, were placed together with conspecific females using a

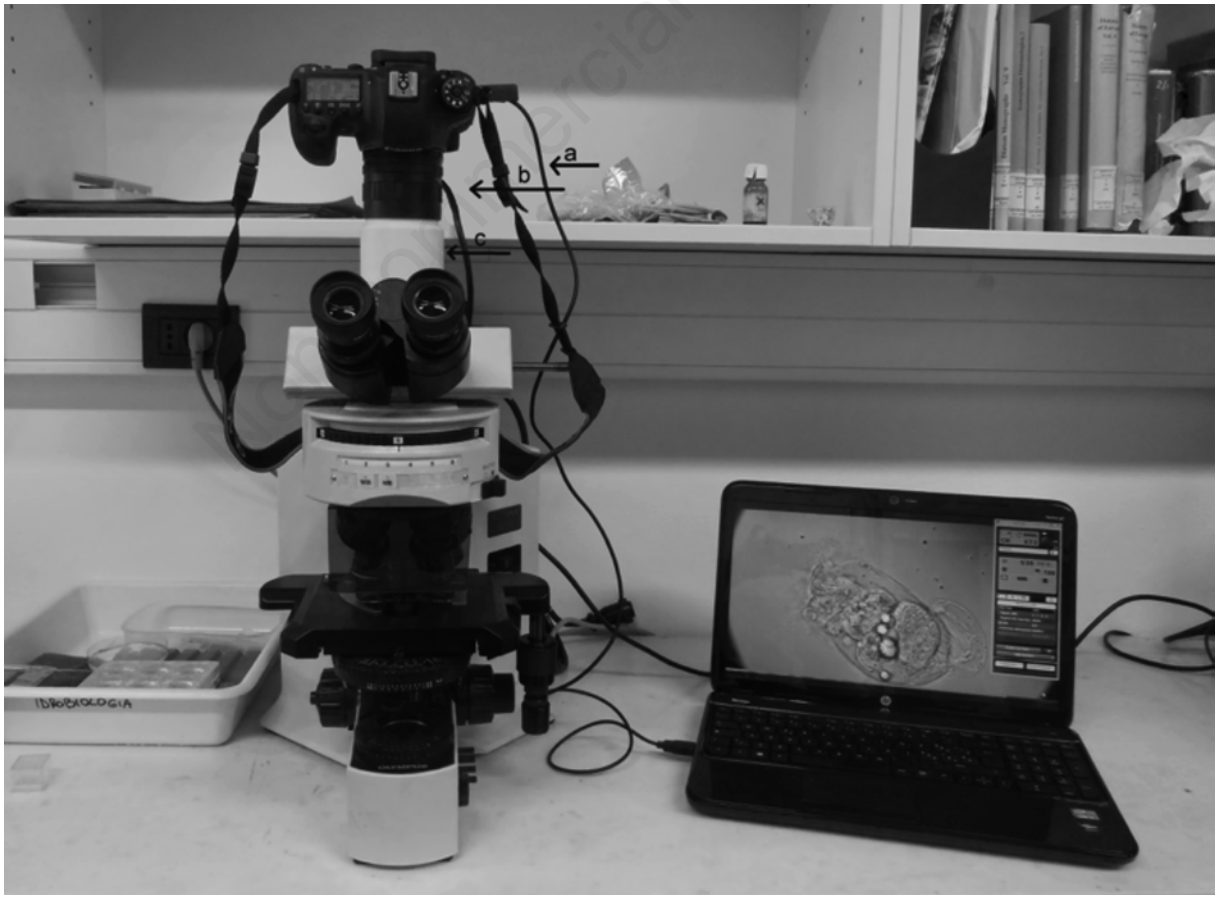

Fig. 1. Microscope with attached DSLR camera connected to the computer via USB cable (a) for live-preview filming; please note that the camera's objective lense was removed and that the camera was connected by an extension tube (b) to the microscope. A plastic adaptor (c) held the extension tube in place.

glass pipette on an *ad hoc* swimming chamber (Fig. 2; description is given below), and their behaviour was filmed with a DSLR camera (Canon 6D full-frame digital camera shooting at 25 frames per second) connected to an Olympus BX-51 compound microscope. We would like to underline that any less expensive DSLR camera can also be used. Instead of the official Olympus camera adapters, an extension tube set was used to couple the camera to the microscope (Fig. 1). Here we assembled a 51 mm unbranded generic extension tube by screwing together three threaded pieces (7, 14, 28 mm), and fixed the tube to the microscope by a plastic cylinder (Fig. 1). Longer tube length gives higher resolution but reduces the field diameter. To ease the workflow of filming, we used the Canon Utility software to connect the camera to a computer to remotely control the settings and to view in live-preview mode on the computer monitor (Fig. 1). The first 30 seconds of the video on *B. angularis* (video 1) were filmed under a stereomicroscope (Wild Macroscope M420) and the remaining time under a compound microscope (Olympus BX-51) with differential interference contrast (DIC) light settings to gain adequate contrast. In video 2 (video 2), filmed using the compound microscope, the first 1:06 minutes show a live female of *B. angularis* in the swimming chamber while afterwards the female is on a glass slide. Video 3 (video 3) shows *K. cochlearis* with the same setup as video 1. When filming the swimming chamber, we used the 20x/0.5 UPlanFI Olympus objective lens, and, when we used glass slides in combination with a cover slip we used the 40x/0.75 UPlanFI and the 60x/0.90 UPlanAp Olympus objective lenses (as indicated in the video).

The swimming chamber (Fig. 2) was built according to Coulon's *et al.* (1983) general design. We used a glass microscope slide as the base of the swimming chamber, upon which four glass coverslips were glued with cyanoacrylate glue to form a chamber (2×2×0.25 mm) with a total volume of approximately 1 mm^3 (Fig. 2). The swimming chamber gave the necessary three-dimensional space for normal swimming behaviour such as making flips, revolutions and loops, while at the same time kept the specimen within the field of vision. In filling the chamber with specimens and culture medium, we tried to prevent the formation of a water meniscus, which induces chromatic aberrations and perspective distortions, by adding as little water as possible; no cover slip was used. Unfortunately, rotifer males are quite delicate and a compromise had to be found between the amount of water added and survival of males. Because our focus was on filming male – female interactions, an inferior image quality was acceptable considering we were interested in gross scale morphological details. In fact, the height of the chamber was too thick for perfect image quality with respect to the size of the rotifer males and females of *K. cochlearis* and *B. angularis* (maximum length 200 µm), and thus morphological details were obtained by placing specimens on a glass slide.

Daphnia magna neonates (≤24 hours old) were obtained from a laboratory culture and exposed to UVA (14.6±4.5 W m^{-2}) radiation produced by UVA LEDs (3 V, 20 mA). UVA radiation was measured with a DeltaOhm-HD2102.1 radiometer. Three UV radiation transparent cuvettes were used as experimental chambers (Fig. 3). Each cuvette, with 5 mL of culture medium, received five neonate *Daphnia* specimens. The neonates were exposed to five cycles of 15 seconds (") of UVA radiation interrupted by 60" of visible light. The experiment was filmed (video 4) with a 180 mm macro lens.

Video editing

Several video editors exist; some are released with proprietary licence (*e.g.*, Adobe Premiere, Sony Vegas), whereas others are released as freeware (*e.g.*, Lightworks). To produce the final video, we edited the raw material using basic operations such as: i) pan/crop to move and zoom to the point of interest; ii) text overlay to indicate specific morphological characters; iii) time stretches

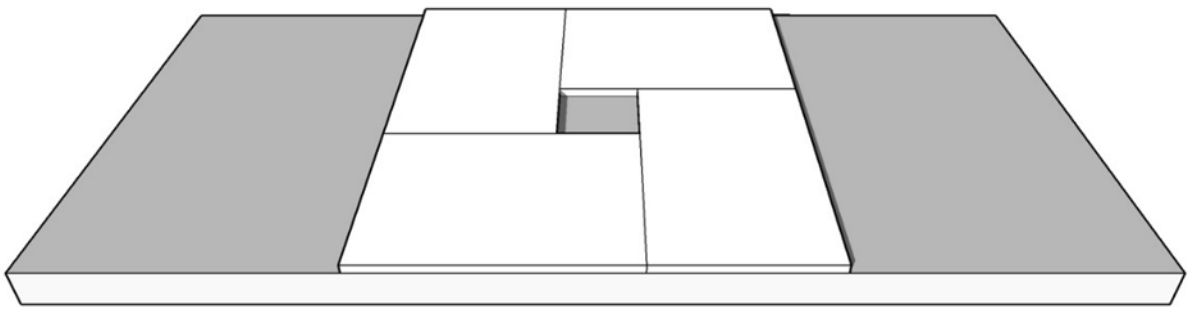

Fig. 2. Swimming chamber; note the alternate displacement of the coverslips.

to slow down or accelerate un-interesting frames/scenes; iv) fading controls to obtain a smoother transition between different scenes; v) brightness/contrast controls to maximise the sharpness of the images.

RESULTS AND DISCUSSION

The videos confirmed most of the characters described in dedicated keys or zoology texts (Sudzuki, 1964; Pontin, 1978; Koste, 1978; Fontaneto and DeSmet, 2015) on rotifer males.

Brachionus angularis (video 1)

The initial sequence (time 4-30") shows the swimming activity of *B. angularis*. Males swim considerably faster (27") and are smaller (50") than females. An overview of the three-dimensional structure of the male can be seen by observing the rotating male (55"). We tentatively suggest that we can see moving spermatozoa (1'14") in the posterior part of the body. According to Sudzuki (1964), the foot in *B. angularis* is retractable while Pontin (1978) is not clear about this. Our film clearly shows the ability of a *B. angularis* male to move its foot in and out from the delicate lorica (1'30"), partially exposing the non-turgid penis (2'05"). Our videos also offer the opportunity to observe (1'43") a brownish spot located over the prostatic gland in posterior dorsal position in a living male that we link to *excretion* granules as described by Fontaneto and De Smet (2015). Furthermore, sensory bristles (1'52") can be seen, which are probably used in female recognition and sensing of the environment (Schmidt-Rhaesa and Kükenthal, 2015).

Males and females were put together several times but we were not able to observe any mating behaviour, except for one occasion (2'15"). This difficulty in observing mating behaviour is in contrast to Gilbert (1963) and Gómez and Serra (1995) who describe an immediate mating between males and mictic or amictic females. However, Gilbert (1963) states that young males are more successful than old ones. Probably, we used older males for our filming of male-female interactions or we missed mating because of the time necessary to adjust the film settings. For the only interaction between a male and a female (2'15"), we hypothesise that we observed the last sequence of the mating behaviour as described in Gilbert (1963): the male drags behind the female. Our video shows (2'30") that the sagittal plane of the female was not in the same direction of the movement, indicating that the male was responsible

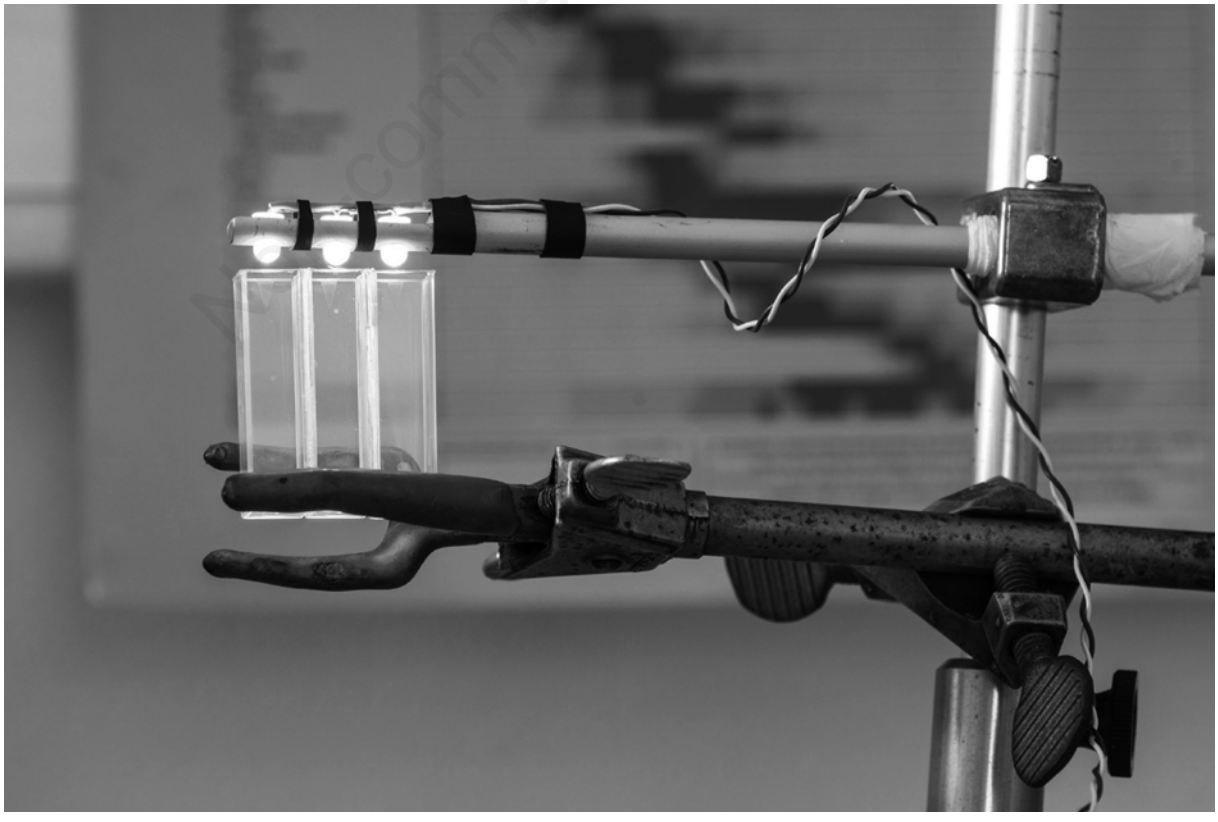

Fig. 3. *Daphnia magna* film setup: the three cuvettes are exposed to UVA radiation from above.

for the swimming direction by dragging behind the female. Furthermore, the video shows a thread-like structure linking male and female. Gómez and Serra (1995) report on a *fine thread* between the mating pair, but the nature of this structure is unclear. In any case, further videos are needed to give more details on the complex mating behaviour of *K. cochlearis*.

We also filmed a live female of *B. angularis* (video 2) in the swimming chamber (1'06") and afterwards on a glass slide. In filming the female, we changed the DIC settings to find the best contrast for certain structures such as trophi. We kept this sequence in the video to show how the quality of the video changes accordingly to the shift of the Normaski prism. In the video, we highlighted several morphological features otherwise difficult or impossible to observe in a fixed state. The dynamic movements of the corona, trophi, intestine, and foot might not only fascinate rotiferologists but also the public. In our video, not only the foot and the malleate trophi but also the muscles that control the foot can be seen in action. Recently, the fine-scale movement of the trophi has attracted attention (Hochberg *et al.*, 2015), and videos such as ours can be the first step to investigate *in vivo* trophi mechanics. Furthermore, the three-dimensional structure of the corona and the body of *B. angularis* can be more easily grasped by our video than by photos or drawings. Towards the end of the video, we can see the dying rotifer female that starts to lose the cilia of the corona.

Keratella cochlearis (video 3)

The initial sequence (1-26") gives an overview on sexual dimorphism in *K. cochlearis* and shows that, similarly to *B. angularis*, males are smaller (approximately 50 µm) and swim faster than females. As shown in Voigt and Koste (1978), males lack spines and have a permanently exposed foot (27-50"). Interestingly, the body of a *K. cochlearis* male lacks the brownish granules seen in *B. angularis*. The rotation of the male (44-55") gave an impression of the three-dimensional structure. Our video clearly shows that also males of *K. cochlearis* are able to retract their foot (1'12") and possess cilia on the foot (1'40"). Noteworthy is the constriction under the head (1'25") that might be a sign of stress under laboratory conditions.

Daphnia magna response to UVA (video 4)

The video starts by showing three cuvettes with *D. magna* neonates exposed to normal light. Individuals swam stochastically and occupied the entire space. Subsequently, by exposing the upper part of the cuvette to UVA radiation (1'00"), individuals swam to the bottom of the cuvette seeking refuge from UVA radiation. After switching off the UVA radiation (1'15"), the neonates again showed normal swimming behaviour, reaching the middle-high portion of the cuvette after a few seconds. The cycle of switching on and off UVA radiation was then repeated four times. This simple setup showed in a straightforward way how zooplankton escapes harmful UVA radiation. In order to do so, zooplankton must sense the threat (*e.g.*, see Smith and Macagno, 1990), and this topic can be easily discussed in the classroom or with the public by using a simple setup as the one shown.

CONCLUSIONS

The observation of microorganisms is still as fascinating now as it was at the time of Antonie van Leeuwenhoek, when he was visited both by curious and sceptics (Ford 1982). Filming microinvertebrates with a DSLR camera gave us the opportunity to see what others described in writing. Our methodological setup does not require specific technical skills and can be easily extended to filming other microorganisms such as algae or protists. In any case, the filming of moving organisms poses some challenges and requires some testing to find the right settings. While schematic drawings and text have their place in a scientific context, seeing is complementary to describing. Nowadays, we can easily disseminate videos on open-access platforms such as YouTube, DailyMotion, vimeo.com or ZippCast that try to serve the preferences of their users, and thus can reach a vast audience. From a scientific point of view, it would be advisable to find a dedicated database such as http://rotifera.hausdernatur.at/ that could review and host subject-specific links to videos to guarantee their scientific standard and facilitate their dissemination. A dedicated platform could also encourage discussion between researchers on their observations. From a citizen science perspective, filming allows showing an interested public the fascinating life of microorganisms that is generally accessible only to researchers. The ecological importance of plankton is especially neglected by the public, and videos such as these could attract attention and raise public awareness.

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VIDEO LINKS

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