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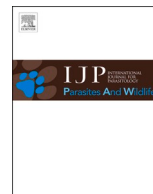
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## Wide geographic distribution of overlooked parasites: Rare Microsporidia in *Gammarus balcanicus*, a species complex with a high rate of endemism

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### ABSTRACT

Parasites and other symbionts deeply influence host organisms, and no living organism can be considered to have evolved independent of its symbionts. The first step towards understanding symbiotic influences upon host organisms is a strong supporting knowledge of parasite/symbiont diversity. Parasites of freshwater amphipods are diverse, with Microsporidia being a major group. These intracellular parasites impact gammarid fitness in different ways, ranging from reduced fitness to increased fecundity. Many Microsporidia have been recorded using molecular data, with multiple taxa pending formal taxonomic description. While some parasites are common, others are known only through sporadic records of single infections. In this study, we focus on rare/ sporadic microsporidian infections within *Gammarus balcanicus*, a host species complex with a high level of endemism. In addition to enriching our knowledge on Microsporidia parasite diversity in amphipod hosts, we test whether these symbionts are specific to *G. balcanicus* or if they are the same taxa infecting other gammarid species. Of 2231 hosts from 87 sites, we catalogued 29 sequences of “rare” Microsporidia clustering into 19 haplogroups. These haplogroups cluster into 11 lineages: four pre-described taxa (*Cucumispora roeselum*, *C. ornata*, *C. dikerogammari* and *Enterocytozpora artemiae*) and seven ‘Molecular Operational Taxonomic Units’, which are known from previously published studies to infect other European amphipod species. Our study significantly widens the geographic range of these Microsporidia and expands the known spectrum of hosts infected. Our results suggest that these parasites are ancient infections of European gammarids. For some host-parasite systems, we hypothesize that the common parasite ancestors that infected the hosts’ common ancestors, diversified alongside host diversification. For others, we observe Microsporidia taxa with wide host ranges that do not follow host phylogeny.

### 1. Introduction

Parasites and other symbionts deeply influence host organisms via behavioural change (Hughes et al., 2012), ecological parameters (Hudson et al., 2002; Thomas et al., 2005) and generally, host evolutionary processes (O’Neill et al., 1997; Poulin, 2007). The concepts surrounding ‘extended phenotype’ (Dawkins, 2016) and ‘holobiont’ (Simon et al., 2019) state that no living organism evolves in total independence from its symbionts. The first step towards understanding the influence of parasites/symbionts upon any host organism is a knowledge of their diversity. Unfortunately, parasite diversity is often neglected or overlooked (Besansky et al., 2003), despite being estimated to constitute 40%–75% of known biodiversity (reviewed in Dobson et al., 2008). This

is especially true for parasites of host organisms that are neither model species or species of economic importance. In a recent review, Bojko and Ovcharenko (2019) stressed that amphipod crustaceans, despite including more than 10,000 described species, are overlooked with reference to the impacts and diversity of their parasitic communities. This deficiency is unfortunate because amphipods are key organisms in aquatic environments (Degani et al., 1987; Kelly et al., 2002; Constable and Birkby, 2016). Their parasites are known to alter several traits (e.g., predation rate, shredding efficiency, tolerance for pollutants), impacting an amphipods’ role in ecosystem functioning (MacNeil et al., 2003; Gismondi et al., 2012a; Labaude et al., 2017). Amphipods may also be important carriers of emerging diseases, especially as some species are invasive and are shown to carry parasites from native to invasive ranges

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(Wattier et al., 2007; Bacela-Spychalska et al., 2012; Bojko et al., 2019, 2020).

The Microsporidia are a major parasite group of amphipod crustaceans, in particular freshwater gammarids (Terry et al., 2004; Krebs et al., 2010; Madyarova et al., 2015; Bacela-Spychalska et al., 2018; Dimova et al., 2018; Ironside and Wilkinson, 2018; Quiles et al., 2019; Drozdova et al., 2020; Park et al., 2020). Microsporidia are obligate unicellular endoparasites belonging to an extremely ancient and phylogenetically diverse phylum close to the Fungi (Capella-Gutiérrez et al., 2012). They are ubiquitous parasites of vertebrate and invertebrate hosts and are characterized by a complex history of host-parasite associations (Keeling and Fast, 2002). In amphipods, while many divergent phylogenetic lineages have been identified based on SSU rDNA sequences, out of 13 formally described genera only five have been defined based on both molecular and morphological characteristics (Bojko and Ovcharenko, 2019). The four genera, i.e. *Nosema* (Naegeli, 1857), *Pleistophora* (Terry et al., 2003), *Dictyocoela* (Terry et al., 2004) and *Cucumispora* (Ovcharenko et al., 2010) are detected, often with high prevalence, in many gammaridean species across Eurasia (Terry et al., 2004; Krebs et al., 2010; Grabner et al., 2015; Dimova et al., 2018), North America (Galbreath Slothouber et al., 2004; Winters and Faisal, 2014) and Oceania (Park et al., 2020). They infect a wide range of species within this group, especially *Gammarus* spp. (Bacela-Spychalska et al., 2018; Bojko and Ovcharenko, 2019). Their life-history traits and life-cycle are variable. In two hosts, *Gammarus duebeni* and *G. roeselii*, some *Dictyocoela* species and *Nosema granulosis* are vertically transmitted: they infect oocytes and are transmitted to most embryos (Terry et al., 1999; Haine et al., 2007; Dubuffet et al., 2013). These Microsporidia induce sex-ratio distortion in their host populations, also by male hosts becoming functional females (Haine et al., 2004; Rodgers-Gray et al., 2004; Terry et al., 2004; Jahnke et al., 2013). Other well-described gammarid-infecting Microsporidia are pathogens and utilise horizontal transmission. *Cucumispora* spp. kill their amphipod hosts and are transmitted mainly through scavenging and cannibalism (Ovcharenko et al., 2010; Bacela-Spychalska et al., 2012; Bojko et al., 2019). Other Microsporidia are responsible for disrupting host responses to heavy metal contaminants (e.g. Gismondi et al., 2012a; 2012b). Such data outline the role of Microsporidia as impactful parasites of gammarid hosts.

In Europe, surveys (mainly based on partial SSU rDNA sequences) of amphipod microsporidian diversity have been performed either at the local scale – sometimes as part of a host community analysis – or at a larger geographic scale, focusing on a single host species across its entire range. Both types of surveys, assuming that sampling effort is large, have highlighted the presence of new and rare parasite lineages (or haplotypes within lineages) (Terry et al., 2004; Krebs et al., 2010; Grabner et al., 2015; Quiles et al., 2019). This suggests that the number of microsporidian species infecting gammarids remains unknown and understudied. Furthermore, most of these “rare” lineages have not been formally described (see for example the analyses of Grabner et al., 2015; Bojko et al., 2017; Quiles et al., 2019), and are presented as sporadic records diluted in large amounts of data dealing with more common Microsporidia (namely *Nosema granulosis* and *Dictyocoela* spp.). GenBank is currently harboring plenty of sequences registered under ‘holding’ names such as *Microsporidium* sp. followed by a given code (e.g. *Microsporidium* sp. 505, *Microsporidium* sp. M1). Microsporidian biology (life cycle, degree of virulence, host range, etc.) also remains unknown for most PCR-identified isolates.

*Gammarus balcanicus* (Amphipoda) is an interesting host to understand the evolutionary history of rare microsporidian lineages. It is mostly restricted to mountainous areas, ranging from the eastern Carpathians and Balkan Peninsula, to the eastern Alps. This area is known to be a biodiversity hotspot with high level of endemism (Médail and Diadema, 2009). This is also the case for many endemic freshwater gammarids (Grabowski et al., 2017; Csapó et al., 2020; Wattier et al., 2020). Within the *G. balcanicus* morphospecies, at least 50 divergent

MOTUs (Molecular Operational Taxonomic Units) have been identified (Mamos et al., 2016), most of them being locally endemic, dating back even to 15 Mya. *Gammarus balcanicus* is in relative isolation from other European gammarids, since it rarely co-occurs with other gammarids (Copilaş-Ciocianu et al., 2014). During a survey of Microsporidia in the *G. balcanicus* complex over the entire host geographic range, we identified several lineages outside of the commonly identified *N. granulosis* and *Dictyocoela* taxa (Quiles et al., 2020). These infections were considered “rare” (i.e. present in few individuals and/or at few sites). We provide an analysis of these “rare” infections and consider the following questions: i) Are we able to identify new microsporidian MOTUs from amphipod hosts? ii) Could novelties in the Microsporidia assemblage of *G. balcanicus* be favoured by host diversification and endemism? And alternatively, iii) are these new sequences part of pre-identified “rare” species-level-taxa infecting other gammarid species, rediscovering old associations but extending their known geographic and host ranges?

## 2. Material and methods

### 2.1. Sampling and total DNA extraction

Samples used in the present study correspond to the *G. balcanicus* samples used by Mamos et al. (2016) and Quiles et al. (2020). In short, *G. balcanicus* individuals were collected at 87 sites in 13 countries during several sampling campaigns between 2004 and 2016. This sampling covered the entire distribution range of this morphospecies in Europe (Fig. 1, Table S1). Samples were collected using hand nets and kick-sampling methods. All individuals were immediately fixed in 96% ethanol at the sampling site and stored at room temperature after returning to the laboratory. Amphipods were identified to the morphospecies level using morphological characters described by Karaman and Pinkster (1977). All the specimens are stored at the Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Poland.

A total of 2231 host individuals were dissected under a stereomicroscope and a tissue biopsy (approximately 2 mm<sup>3</sup> including muscles and gonads) was taken from the 6th and 7th thoracic segments of each specimen. Since Microsporidia are intracellular parasites, both parasite and host DNA were co-extracted. DNA extraction was performed on individuals, using either a standard phenol-chloroform protocol (Hillis et al., 1996) or ‘Biobasic EZ-10 96 Well Plate Genomic DNA Isolation Kit for Animal Samples’, and eluted in 100 µl of TE (pH 8) buffer. The DNA samples were kept at 4 °C until amplification and subsequently at –20 °C for long-term storage.

### 2.2. Molecular screening for Microsporidia

Following the strategy proposed by Quiles et al. (2019), all the 2231 individuals were PCR screened for the presence of Microsporidia using the Microsporidia-specific primers V1f (forward) (5'-CAC CAG GTT GAT TCT GCC TGA C-3') and UNIr (reverse) (5'-TCA GGC TCC CTC TCC GGA AT-3'), targeting the 5' part of the small ribosomal subunit gene (SSU rDNA). This diagnostic fragment is short (c. 350 bp long), maximizing the ability to detect the presence of Microsporidia even in the case of low infection intensity or partial degradation of the DNA. A negative (water) and a positive (*Dictyocoela roeselium* DNA) PCR control was used. The PCR conditions and visualization of PCR products were as described by Quiles et al. (2019).

Following Quiles et al. (2019), individuals positively PCR-diagnosed for Microsporidia infections were again tentatively amplified using V1f, but either using HG4r (5'-GCG GCT TAA TTT GAC TCAA C-3') or 530r (5'-CCG CGG CTG CTG GCA C-3') as reverse primers, targeting a ca. 800 and 530 bp long fragment for sequencing, respectively. When unsuccessful, the V1f-UNIr fragment was used for sequencing. Even if this fragment is short (ca. 350bp long) it contains enough phylogenetic information to assign sequences to a species-level taxon without any



Fig. 1. *Gammarus balcanicus* sampling sites. Sites are identified by black dots with numbers as in Table S1 (87). See Additional Table S1 for details (e.g. sampling sites, GPS coordinates). Countries identified with ISO code. Map created by authors using Qgis 2.18.4 (QGIS Development Team 2009).

ambiguity (Quiles et al., 2019, Table S2). Prior to sequencing, the PCR products were cleaned using the EXO-FastAP method (Thermo Scientific). PCR products were purified and sequenced directly with the BigDye technology by Genewiz, Inc., UK, using the V1f primer. Using Geneious 10.2. (Kearse et al., 2012), raw sequences were edited, trimmed and confirmed as microsporidian sequences using the BlastN search tool available in GenBank/NCBI (Madden, 2003).

### 2.3. Sequence dataset and phylogeny reconstruction for *Microsporidia*

Four types of microsporidian SSU rDNA sequences were included in our dataset: (i) sequences newly produced from our collection of infected *G. balcanicus*; (ii) published sequences of Microsporidia infecting European freshwater or brackish water amphipods, representing parasite diversity and divergence; (iii) published sequences for Microsporidia infecting other taxa closely related to newly produced sequences, prioritizing freshwater or brackish water invertebrates; (iv) published sequences representative of the five Microsporidia clades (Clades I–V), as determined in the integrative phylogenies presented in literature (Vossbrinck and Debrunner-Vossbrinck, 2005; Williams et al., 2018). Detailed information on the sequences used (e.g. GenBank accession numbers, host name, assignment to a fully described species or MOTU) are presented in Table S2. Sequences were aligned using MAFFT7.388 software (Katoh and Standley, 2013), incorporated in Geneious 10.2.2, with the E-IIONS-I algorithm using the legacy gap penalty option.

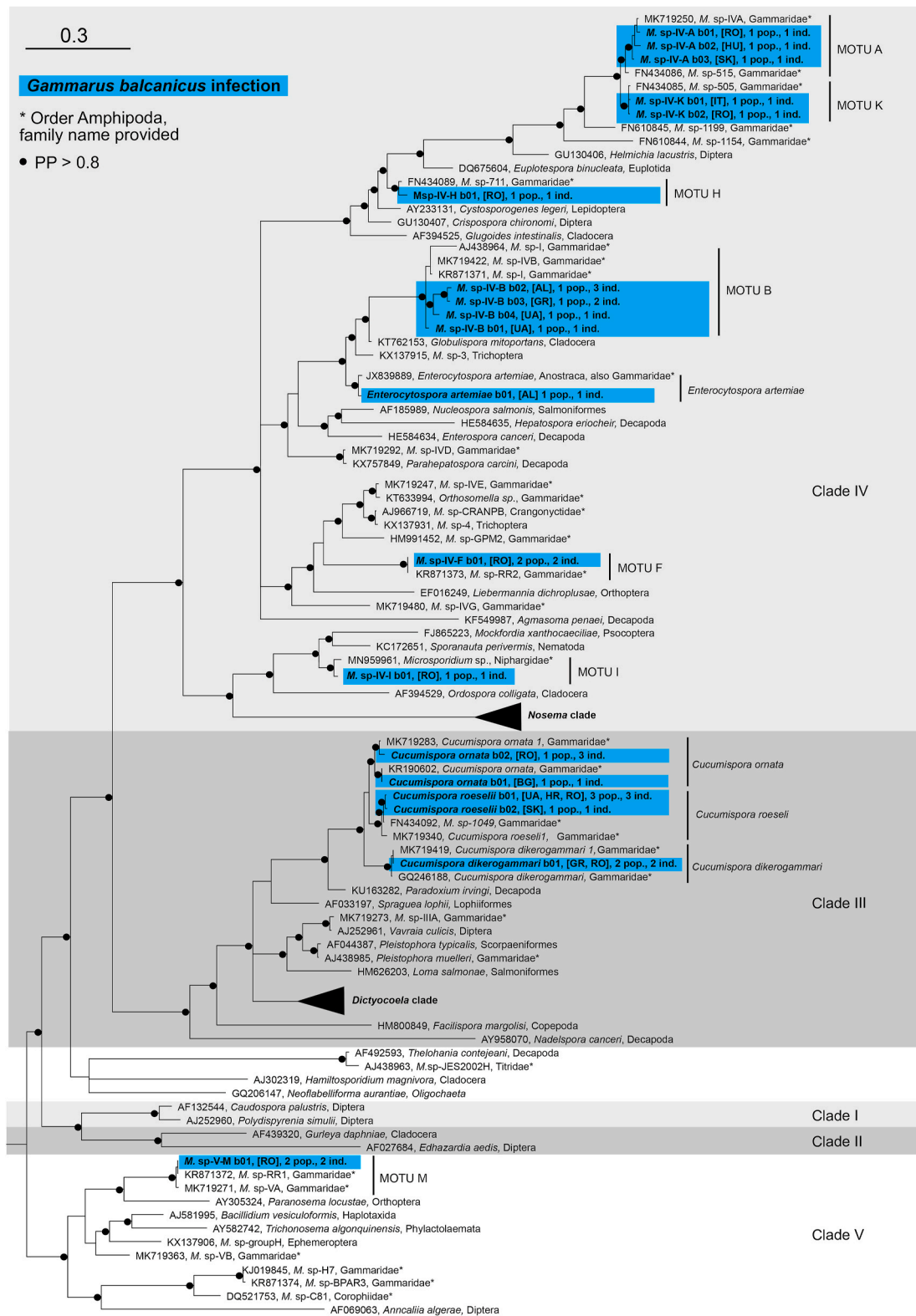
Our dataset contained sequences of different lengths, both among the newly produced sequences and the published ones (Table S2). As some sequences were relatively short, reducing the full dataset to a common size would allow, on the one hand, defining haplotypes but, on the other hand, would potentially reduce phylogenetic information content. Therefore, following the strategy defined by Quiles et al. (2019), we assigned each sequence to a haplogroup. Sequences belonging to distinct haplogroups possessed at least one variable site in the shared part of the

two aligned sequences, therefore generating diagnostic features. The longest sequence of each haplogroup was then used for the phylogeny reconstruction and, for sake of clarity, only these sequences were shown on the tree (Table S2, Fig. 2). This rule applied to any sequence in our data set, with one exception: if the longest sequence for a given haplogroup was derived from the literature but a new *G. balcanicus* also belonged to this haplogroup, both were shown on the tree in order to highlight all the diversity found in *G. balcanicus*. For newly produced sequences, we conservatively used provisional names, e.g. Microsporidium sp. (hereafter abbreviated M. sp) followed by: the clade number (from I to V) *sensu* Vossbrinck and Debrunner-Vossbrinck (2005), MOTU identification by capital letter (as in Fig. 2), letter “b” for *balcanicus* and a number identifying the haplogroup (e.g. M. sp-IV-A b01). Conversely, our newly produced sequences of parasites assigned to a fully described species were labelled by generic and specific names, and using “b” for *balcanicus* followed by a number identifying the haplogroup (e.g. *Cucumispora ornata* b01, *Cucumispora ornata* b02).

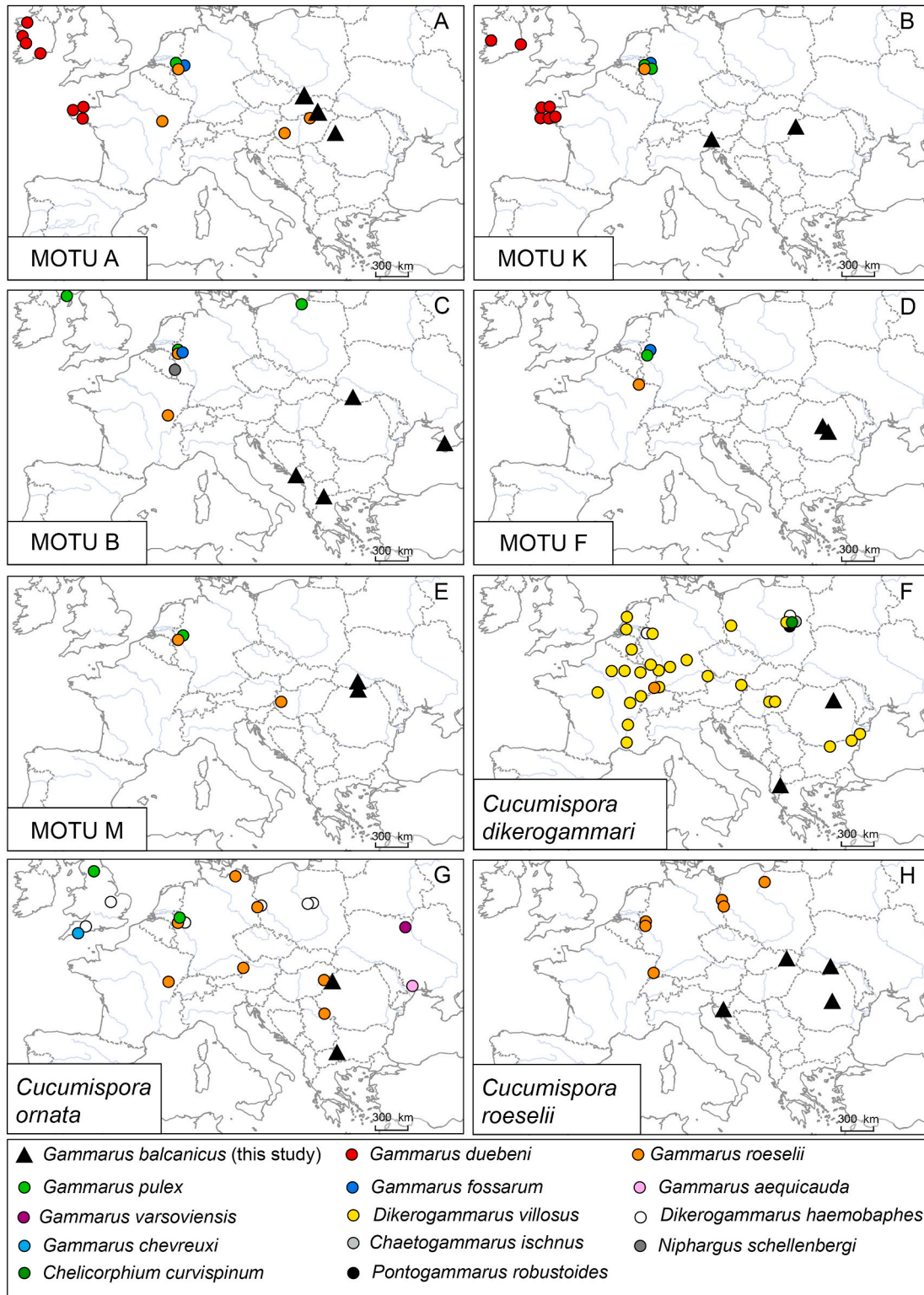
Bayesian phylogeny reconstructions were performed with MrBayes (Huelsenbeck and Ronquist, 2001) in Geneious 10.2.2. The best-fitting model of nucleotide substitution was selected with JModelTest-2.1.10 (Durriba et al., 2012) as being the General Time Reversible (GTR) model with gamma-distributed rate heterogeneity (G) and a proportion of invariable sites (I). Four heated chains, each 1,100,000 iterations long, sampled every 200 iterations, were run. The runs reached satisfactory effective sampling sizes (ESS > 200). The 50% majority-rule consensus tree was constructed after the removal of 10% ‘burn-in’ trees. We used *Basidiobolus ranarum* (GenBank: AY635841) as the outgroup, following Vossbrinck and Debrunner-Vossbrinck (2005).

We identified our sequences to species level when they grouped together with type sequences of fully described Microsporidia in well-defined, supported clade (see results). In cases where our sequences were grouped with undescribed Microsporidia, we referred such groups of sequences as to Molecular Operational Taxonomy Units (MOTUs).

The sites reported on Figs. 1 and 3 were plotted on a map using QGIS



**Fig. 2.** Bayesian phylogenetic reconstruction of Microsporidia based on partial small ribosomal subunit rDNA alignment (Supplementary data 1). Labels in bold and in blue frames are parasites of *Gammarus balcanicus* found in the present study. These labels show the name of the parasite (in case of described species) or in the case of undescribed taxa the name consist of: *M. sp* (= *Microsporidium sp.*) followed by clade number *sensu* Vossbrinck and Debrunner-Vossbrinck (2005), MOTU, haplogroup number (e.g. b01, b02), then the country where it was found (two letter ISO code, see Table S1), the number of infected populations (=pop.), and the total number of infected individuals (=ind.). Labels with accession numbers are parasite sequences taken from GenBank. These labels show the accession number, the parasite name given in the associated publication, the order of the host (except for amphipod hosts where the family is provided). Microsporidia clade numbers are as in Vossbrinck and Debrunner-Vossbrinck (2005). Branches are collapsed for the two genera *Nosema* and *Dictyocoela* (triangle sizes not reflecting actual size). Abbreviation: PP, Bayesian posterior probability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Geographic distribution of the main rare Microsporidia infecting *Gammarus balcanicus*, showing their occurrence in other gammarid species over Europe. Each map (A–H) refers to the parasite taxa presented in the bottom-right inset. The host and geographic range of the Microsporidia based on this study and 1) literature data: Terry et al. (2004); Wattier et al. (2007); Krebs et al. (2010); Ovcharenko et al. (2010); Bacela-Spychalska et al. (2012); Rode et al. (2013); Grabner et al., 2014; 2015; 2017; Bojko et al. (2015); 2017; 2018; Weigand et al. (2016); Quiles et al. (2019); 2) Gen Bank sequences: MT645708 (Chen, Y. and Jiang, H. direct submission); KP699690 (Bacela-Spychalska, K. direct submission) and 3) Bacela and Ovcharenko, unpublished data.

2.18.4 (QGIS Development Team, 2016).

### 3. Results

The length of the 29 partial SSU sequences we produced throughout this study ranged from 140 bp to 816 bp (Table S2). These 29 sequences clustered into 19 haplogroups, which themselves clustered into 11 lineages (four formally described taxa and seven MOTUs, Table S2). Among the five clades defined in the phylogeny of the Microsporidia by Vossbrinck and Debrunner-Vossbrinck (2005) these sequences fit to 3 clades (III, IV and V) (Fig. 2).

Seven of the lineages found in *G. balcanicus* can be assigned to MOTUs already found in other European gammarids, but not formally described (MOTUs A, B, F, H, I, K, and M, Fig. 2). The three haplogroups **M. sp-IV-A b01-b03** were found, each within a single individual and site (Fig. 2, Table S1-2) in Hungary, Romania and Slovakia, respectively (Fig. 3A, Table S1-2). They were part of MOTU A. Sequences from the literature belonging to MOTU A, are *M. sp-515* infecting Irish *Gammarus duebeni celticus* (Krebs et al., 2010), those infecting *G. pulex*, *G. fossarum* and *G. roeselii* in Germany (Grabner, 2017; Grabner et al., 2015) and *M. sp-IV-A r01* infecting *G. roeselii* in France and Hungary (Quiles et al., 2019) (Figs. 2 and 3A, Table S1-2).

The two haplogroups **M. sp-IV-K b01 and b02** (Fig. 2, Table S1-S2) were found each in a single individual from Italy and Romania (Fig. 3B, Table S1-S2). They were part of the same MOTU K as *M. sp-505* infecting Irish and French populations of *G. duebeni celticus* (Krebs et al., 2010) but also *G. pulex*, *G. fossarum* and *G. roeselii* in Germany (Fig. 3B, Table S2) (Grabner, 2017; Grabner et al., 2015).

**Microsporidium sp-IV-H b01** (Fig. 2, Table S1) was found in an individual from Romania (Table S1-S2) and is part of MOTU H together with *M. sp-711* previously found in one *G. duebeni duebeni* individual reported from France (Krebs et al., 2010).

The four haplogroups **M. sp-IV-B b01-b04** (Fig. 2, Table S1-S2) were found from seven individuals at four sites across Albania, Greece and Ukraine (Fig. 3C, Table S1-S2). They belong to MOTU B, including *M. sp-I* and *M. sp-IV-B*, which are Microsporidia detected from *G. roeselii* at sites in Germany (Grabner, 2017; Grabner et al., 2015) and in one French locality (Quiles et al., 2019) (Fig. 3C). Additional phylogenetically related sequences being part of MOTU B were detected in *G. pulex* in Germany (Grabner et al., 2015) and Scotland (Terry et al., 2004), but also in *Niphargus schellenbergi* in Luxembourg (Weigand et al., 2016) (Fig. 3C, Table S2).

**Microsporidium. sp-IV-F b01** haplogroup (Fig. 2, Table S1) was associated with two individuals from two sites in Romania (Fig. 3D, Table S1). This sequence was identical to *M. sp-RR2* found in the Ruhr region of Germany from *G. pulex*, *G. fossarum* and *G. roeselii* (Grabner et al., 2015) and *M. sp-IV-F* from *G. roeselii* from two French localities (Quiles et al., 2019), all part of MOTU F.

A single haplogroup of **M. sp-IV-I b01** (Fig. 2) infected one individual *G. balcanicus* from Romania. Its closest relative was a Microsporidium sp. infecting the subterranean amphipod *Niphargus aquilex* in Germany (Grabner et al., 2020). These two sequences formed MOTU I.

Finally, **M. sp-V-M b01**, the only haplogroup in Clade V, was collected from two *G. balcanicus* at two sites in Romania. Sequences from the literature that formed MOTU M together with this haplogroup were *M. sp-RR1* from several *G. pulex* individuals and one *G. roeselii* individual from Germany (Grabner et al., 2015) and *M. sp-V-A* found in one *G. roeselii* individual from Hungary (Quiles et al., 2019). Due to the short length of our sequences, we were not able to assign them to either *M. sp-RR1* or *M. sp-V-A* (Fig. 2).

The remaining rare infections in *G. balcanicus* were assigned to fully described taxa, including three *Cucumispora* sp.: *C. dikerogammari* (Ovcharenko et al., 2010), *C. roeselii* (Bojko et al., 2017) and *C. ornata* (Bojko et al., 2015), and one being *Enterocytozpora artemiae* (Rode et al., 2013). The haplogroup belonging to *C. dikerogammari* (*C. dikerogammari* b01, Fig. 2, Table S1) was found infecting two

individuals from two sites, in Greece and Romania (Fig. 3F). This haplogroup showed identical sequences to *C. dikerogammari* initially identified from *Dikerogammarus villosus* (Wattier et al., 2007; Ovcharenko et al., 2010). More precisely, it shows 100% pairwise identity to *C. dikerogammari* r01 found in *G. roeselii* in France (Quiles et al., 2019). *Cucumispora dikerogammari* is frequent and widespread in *D. villosus* across Europe (Wattier et al., 2007; Ovcharenko et al., 2010), but it is also known to infect *D. haemobaphes*, notably in Germany (Fig. 3F). Two haplogroups were identified as *C. roeselii* (*C. roeselii* b01 and b02, Fig. 2, Table S1), infecting four individuals in total, each associated with a single site from Slovakia, Hungary, Romania and Austria (Fig. 3H). These two haplogroups were phylogenetically very close, but not identical, to the lineage of *C. roeselii* used for the full description of the species (Bojko et al., 2017). *Cucumispora roeselii* was also previously found to infect *G. roeselii* in Poland (Bojko et al., 2017) and Germany (Grabner, 2017; Quiles et al., 2019) (Fig. 3H Table S2). Finally, two haplogroups were assigned to *C. ornata* (*C. ornata* b01 and b02, Fig. 2), one being present in three individuals from a site in Hungary, the other one in one individual from one site in Bulgaria (Fig. 3G). *Cucumispora ornata* was also found in a variety of gammarid taxa all over Europe (e.g. *G. roeselii*, *G. fossarum*, *G. varsoviensis*, *G. aequicauda*, *D. haemobaphes* (Fig. 3G, Table S2, Quiles et al., 2019).

One haplogroup found in two *G. balcanicus* individuals was ascribed to *Enterocytozpora artemiae* (*Enterocytozpora artemiae* b01) (Fig. 2). It was present in one individual from Albania (Fig. 2). This sequence is closely related to Microsporidia infecting *G. roeselii* from Greece (Quiles et al., 2019), which showed 100% pairwise identity to the *Enterocytozpora artemiae* sequence from *Artemia franciscana* from France, USA and Israel (Rode et al., 2013).

### 4. Discussion

All the rare Microsporidia found in *G. balcanicus* belong to MOTUs or formally described species predetermined to infect other European amphipods. Our results are therefore in contrast with the recent Microsporidian diversity study of another host over its entire range, *G. roeselii* (Quiles et al., 2019). In the latter, we found four rare MOTUs not found previously in amphipods, but these were relatively similar to parasites of other aquatic invertebrates (Ephemeroptera, Diptera, Decapoda, and Anostraca) (Quiles et al., 2019). Unlike *G. balcanicus*, which is not an expanding species, *G. roeselii* is a recent colonizer of North and Western Europe (Csapo et al., 2020) and part of its parasite diversity, specifically considering rare Microsporidia, was probably a result of parasite acquisition from local fauna (Quiles et al., 2019). Among the 11 microsporidian lineages detected in *G. balcanicus*, seven corresponded to MOTUs sporadically detected in other studies. They represent a neglected parasitic assemblage in most previous studies (Terry et al., 2004; Krebs et al., 2010; Grabner et al., 2015; Grabner, 2017). Their detection is challenging, and understanding their diversity is only possible when the investigations are intensive in terms of the number of individuals sampled, regardless of the geographical distribution: in large scale studies (Krebs et al., 2010; Quiles et al., 2019) or in very local studies (Grabner et al., 2015). In addition, their biology remains largely unknown since most of these Microsporidia have only been detected via a molecular census, such as the method used in this study. Nonetheless, our extensive investigation of *G. balcanicus* did not find any new parasite MOTUs, which could mean that we are approaching the maximal number of Microsporidia groups infecting the genus *Gammarus*. Only further extensive studies can confirm this assumption.

Nevertheless, our census in *G. balcanicus* considerably expands the geographic range of most of the Microsporidia MOTUs (Fig. 3). This is the case for the sister MOTUs A and K (Fig. 3A and B), for MOTU B (Fig. 3C), F (Fig. 3D), M (Fig. 3E) and *Cucumispora roeselii* (Fig. 3H). Most of these parasites were initially detected in north-western or north-central Europe and were first thought to be restricted to a single host or

local site. For example, M. sp-515 and -505 respectively are part of the MOTUs A and K as our M. sp-IV-A and M. sp-IV-K haplogroups, which were initially detected in several populations of *G. duebeni celticus* (Krebs et al., 2010). Other members of the same MOTUs were later found in *G. fossarum* and *G. pulex* (Grabner et al., 2015; Grabner, 2017), and in *G. roeselii* (Quiles et al., 2019), progressively extending the host range. Our study expands this geographic extent to central Europe. It is also notable that, within MOTU A, we found sequences previously associated to individuals outside of Europe, i.e. from Baikal region, infecting two more host species (*Gmelinoides fasciatus* and *Linevichella vortex*) (FJ820190, FJ820188, FJ820191, FJ820187, FJ756007, FJ756006), which further extend its distribution and host range.

A similar pattern was found for MOTU B. It was first detected in *Gammarus pulex* in the UK (Terry et al., 2004) and then in north-western Europe in *G. roeselii* and *G. pulex* (M. sp-I parasites) (Grabner et al., 2015; Quiles et al., 2019), but also in *Niphargus schellenbergi* and in *G. fossarum* (Weigand et al., 2016). Here we show that the M. sp-IV-B b01-b04 haplogroups from *G. balcanicus*, which are part of the MOTU B, occurred in individuals from Greece, Albania and Ukraine, thus widely expanding its geographic distribution eastward. Further, MOTUs A and B now harbor a dozen of haplogroups, all infecting amphipod hosts. These MOTUs may therefore represent microsporidian groups specialised to these hosts, like the *Cucumispora* and *Dictyocoela* clades (Fig. 2) (Bojko et al., 2017; Bacela-Spychalska et al., 2018), and deserve further study to understand their biological impacts and evolutionary history.

Range extensions were also found for MOTUs M and F. They were initially found in a small area of the German Ruhr region (Grabner et al., 2015) and in one French *G. roeselii* site (Quiles et al., 2019). The haplogroups now evidenced in *G. balcanicus* (M. sp.-V-M b01-b02 and M. sp.-IV-F b01) considerably extends the geographic range of these parasite MOTUs eastward. MOTU H represents an extreme case of range extension. The single haplogroup of M. sp-IV-H b01 from Romania is similar (98.9%) to M. sp-711, which was previously found in *G. duebeni duebeni* in Brittany, Western France (Krebs et al., 2010), but at least 2000 km apart. Moreover, we find Microsporidia that have previously been associated with Lake-Baikal-endemic amphipod hosts: *Brandtia latissima latior* (FJ756059) and *Acanthogammarus victorii* (FJ756171) (Qiu et al., unpublished) that are 99.15% and 98.09% identical to the British M. sp-711 sequence, which extends the range of the MOTU H for ca. 5700 km eastward. Finally, our study illustrates that species range might also be underestimated for several of the formally described species. This is the case for *Cucumispora roeselii*, a species initially detected in *G. roeselii* in Poland and in Western Europe (Bojko et al., 2017; Quiles et al., 2019). Now it is known to exist in *G. balcanicus* across the whole Balkan region (Fig. 3G).

The problem with many rare parasites is that their biology remains understudied, preventing in-depth interpretations. They have only been detected using molecular tools (like in the present study), *C. roeselii* being the only one complemented by an anatomical description (Bojko et al., 2017). Therefore, it is not known whether they are pathogens, or if their primary transmission pathway is vertical, horizontal, or both. Yet, Microsporidia are of seminal importance for the biology of their gammarid hosts. Some of them are known to increase the proportion of females in gammarid populations by feminization, as observed in some *Dictyocoela* spp. or *Nosema granulosis* (Terry et al., 2004; Haine et al., 2004). However, because this sex ratio distortion allows the parasites to strongly increase in frequency locally, it is improbable that rare MOTUs, always found in low numbers locally, are sex ratio distorters. These rare MOTUs may be more likely to be pathogenic. Many Microsporidia significantly affect their hosts survival rate or behaviour. For example, *C. dikerogammari* alters the survival rate of its typical host, the invasive *D. villosus*, and impact its predatory efficiency (Bacela-Spychalska et al., 2012, 2014). Other Microsporidia have been showed to modulate co-existence between competitors or predators, by changing the ability of gammarids to avoid predation (Mac Neil et al., 2003).

For two rare *G. balcanicus* Microsporidia, *Cucumispora dikerogammari*

and *C. ornata*, our survey did not significantly expand the geographic range, but extended the known host range. The biology of these two species is better known. They are pathogenic to their two typical gammarid hosts (*D. villosus* and *D. haemobaphes*, respectively) and use mainly horizontal transmission (Ovcharenko et al., 2010; Bojko et al., 2015, 2017). Since *D. villosus* and *D. haemobaphes* are invasive throughout Europe (Rewicz et al., 2015; Jazdzewska et al., 2020) and carry pathogens during the invasion process (Wattier et al., 2007; Bojko et al., 2019), their Microsporidian parasites have the potential to shift hosts and threaten local gammarid species (Bacela-Spychalska et al., 2012; Bojko et al., 2019). The scattered infection of *C. dikerogammari* in *G. balcanicus* suggests that these infections could have been acquired by interspecific horizontal transfers at sympatric sites after contact with *D. villosus*; however, no sites are known where *G. balcanicus* and *D. villosus* currently live in sympatry, and given their ecological requirements such a situation is highly improbable. We therefore have to speculate that *C. dikerogammari* spores could have been transported from other sites or have infected paratenic hosts (unknown and never detected until now). With our discovery of *C. ornata* in *G. balcanicus*, this pathogen is now known to infect seven gammarid species across Europe (Fig. 3G).

The last rare Microsporidium infecting *G. balcanicus* is puzzling. *Enterocytopora artemiae* b01 haplogroup's closest relative is a parasite infecting *G. roeselii* in Greece (Quiles et al., 2019), but also (and most remarkably) known, showing 98.97% of identity, from the brine shrimp, *Artemia franciscana*, present in the USA, France, and Israel (Rode et al., 2013). Within this taxon we also find the Baikalian sequence recorded from the locally endemic gammarid *Micruropus wahlili* (FJ756186). In our study, this Microsporidium was found in *G. balcanicus* from a Romanian site, which is more than 500 km away from the seashore. It is intriguing that these parasites mostly recorded from salt-water hosts can be found in freshwater animals hundreds of kilometres from the sea. Quiles et al. (2019) hypothesised a long-distance transport of microsporidian spores by, e.g., migratory shorebirds to explain infection of the freshwater *G. roeselii* by a salt-water parasite. This new gammarid host and site determined in this study might help to explain the same process, but here the distance from the seashore is much larger. It has previously been described that some microsporidian species may persist outside their host and may still be horizontally transmitted after spore desiccation (Vizoso and Ebert, 2005). We hypothesize that it should be the case for *E. artemiae*. This hypothesis is strengthened by the life cycle of the *Artemia* host. *Artemia* produce resistant thick-shelled eggs (cysts) in case of drying environmental conditions. These eggs can remain in a dormant state and dry for several years. This resistance to extreme desiccation could have been selected for in the parasite *E. artemiae* following coevolution with their hosts, explaining the possibility for long-lasting travel. This would nevertheless imply that these parasites are not specific to *Artemia*, which remains to be demonstrated experimentally. The last possibility is that *E. artemiae* could be a parasite transiently acquired from infected sympatric aquatic invertebrate, as pointed out by Grabner (2017). Indeed, even if Microsporidia are frequently found in the freshwater environment (Stentiford et al., 2013), it may be predicted that thousands of microsporidian taxa remain undescribed from aquatic hosts, because they are able to infect a vast range of hosts, and because of the relative lack of pathogen census of aquatic organisms.

Our study suggests that the Microsporidia noted in this study are ancient groups capable of infecting European gammarids. For some, we may propose that their common ancestors infected the hosts common ancestors, and then diversified following the hosts' diversification via co-evolution. Different haplogroups of each parasite MOTUs often infect different host species (Fig. 2), at wide geographic ranges. This hypothesis is strengthened by the evidence of new haplogroups for parasite MOTUs infecting *G. balcanicus*, which is a species complex with high local lineage endemism (Mamos et al., 2016) living in relative isolation from other European gammarid species (Copilas-Ciocianu et al., 2014).

Such a co-differentiation pattern has been suggested for *Dictyocoela* spp. from amphipods, both at large and local scales (Park et al., 2020; Quiles et al., 2020). Exploring such a pattern further may benefit from the application of additional highly variable genetic markers in parasites, such as RPB1 (RNA polymerase II subunit 1), as already exemplified by Pretto et al. (2018), at least on microsporidian MOTUs where several haplogroups have now been detected in different gammarid species (e.g. MOTUs A and B).

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ijppaw.2021.01.004>.

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