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Molecular phylogeny provides new insights on the taxonomy and composition of *Lyperosomum* Looss, 1899 (Digenea, Dicrocoeliidae) and related genera

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ABSTRACT

Lyperosomum Looss, 1899 is one of the largest genera of the Dicrocoeliidae and is one of the best examples of the systematic complexity and taxonomic instability within this family. We present the molecular analyses based on novel sequences of nuclear and mitochondrial genes obtained from 56 isolates of adult flukes and larval stages of dicrocoeliids belonging to *Lyperosomum, Skrjabinus*, *Zonorchis* as well as previously available sequence data. According to obtained results we propose to return *Zonorchis clathratus* and *Z. petiolatus* into *Lyperosomum*, and to recognize *L. alagesi* as a synonym of *L. petiolatum*. Our study shows that *L. petiolatum* commonly occurs in Europe in corvids as well as in several species of migratory songbirds, e.g. *Sylvia atricapilla*. At the same time, the Turdidae appear to host a distinct species of *Lyperosomum*. The phylogenetic analysis has clearly demonstrated the paraphylepic nature of *Lyperosomum* and indicated the need of its thorough revision preferably using specimens from type hosts and type territories of nominal species. In addition, inclusion of numerous not yet sequenced dicrocoeliid genera into future phylogenetic studies is necessary to clarify the interrelationships of taxa within the family and stabilize its system.

1. Introduction

Lyperosomum Looss, 1899 is one of the more speciose genera of the digenean family Dicrocoeliidae. The genus is characterized by the worldwide distribution and currently contains 33 species, the majority of them are found in biliary ducts of birds (Panin, 1984; Pojmańska, 2008). Two species, i.e. *L. soricis* (Diesing, 1858) and *L. transcarpathicus* (Bychovskaja-Pavlovskaja, Vysotzkaja and Kulakova, 1970), were described from shrews (Eulipotyphla), and one species, *L. intermedium* Denton and Kinsella, 1972, was described from the rice rat *Oryzomys palustris* (Denton and Kinsella, 1972); the latter species was moved to *Dicrocoelium* Dujardin, 1845 by Panin (1984). The taxonomy of the Dicrocoeliidae has been unstable and many species were transferred between various genera, mostly due to the high morphological similarity between dicrocoeliid taxa as well as the lack of understanding of the taxonomic value of traditionally used morphological characters (Yamaguti, 1958, 1971; Panin, 1984; Pojmańska, 2008). The genus *Lyperosomum* is one of the best examples of the systematic complexity and taxonomic instability within this group of digeneans.

Initially, Looss (1899) included 4 species in *Lyperosomum* with *L. longicauda* (Rudolphi, 1809) selected as a type species; at the time the genus was placed into the subfamily Dicrocoelinae along with *Dicrocoelium*. Over the time, a number of additional species have been described or transferred into *Lyperosomum* by different authors (Skrjabin and Evranova, 1952). In turn, some existing members of *Lyperosomum* have been transferred into other genera, e.g., *Oswaldoia* Travassos, 1919 and *Lyperosomoides* Yamaguti, 1971. Shtrom (1940) proposed a new classification of the Dicrocoelinae, dramatically changed the content of some existing genera and emphasized that *Lyperosomum* likely contained a number of species that were not closely related to

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each other. He transferred most species of *Lyperosomum* known at the time into either *Brachylecithum* Shtrom, 1940 or *Corrigia* Shtrom, 1940, while *Lyperosomum* retained only the type species and received 11 species transferred from other genera. Almost simultaneously, Travassos (1944) published his monographic revision of the Dicrocoeliidae. He also transferred a number of *Lyperosomum* species into other genera, e. g., *Olssoniella* Travassos, 1944 or *Luztrema* Travassos, 1941. According to Travassos (1944) *Lyperosomum* was characterized by a short forebody and conspicuous ventral sucker which made it similar to *Zonorchis* Travassos, 1944. However, the two genera differed by the relative position of the testes (oblique in *Lyperosomum* and symmetrical in *Zonorchis*). These revisions also proposed numerous other changes within the Dicrocoeliidae with *Zonorchis* and *Platynosomum* being particularly affected.

According to the most recent classification of the Dicrocoeliidae by Pojmańska (2008) *Lyperosomum* is characterized by the testes positioned closely to the ventral sucker, the ovary at substantial distance from the posterior testis, the genital pore usually located anterior to the intestinal bifurcation, the vitellarium forming two relatively short lateral bands of follicles and extending anteriorly past the level of the ovary, and the ventral sucker larger than the oral sucker. However, some of the species currently included in *Lyperosomum* do not fit one or several of the aforementioned criteria.

Denton and Krissinger (1975) concluded that *Lyperosomum* contains "a confusing assemblage of species of diverse morphology and uncertain relationships". One of the best examples of such confusion is the tumultuous taxonomic history of *Dicrocoelium petiolatum* Railliet, 1900. Shtrom (1940) and Travassos (1944) independently suggested its transfer to *Lyperosomum*; then it was moved to *Zonorchis* by Denton and Byrd (1951) and *Dicrocoelioides* by Dollfus (1954); thereafter, Panin (1984) placed it back in *Zonorchis* and most recently, the whole genus *Zonorchis* was synonymized with *Skrjabinus* Bhalerao, 1936 by Pojmańska (2008).

DNA sequence data provide an important additional, complementary set of characters for phylogenetic inference. Although the history of the molecular systematic studies of the Dicrocoeliidae is relatively short, these studies have already provided important insights into the interrelationships among some key dicrocoeliid taxa and their systematic position (Tkach et al., 2001, 2018; Ribas et al., 2012; Hildebrand et al., 2015, 2016; Aldhoun et al., 2018; Pinto et al., 2018; Hildebrand and Tkach, 2019). Among other contributions, the molecular phylogenetic analyses strongly suggested that the subfamily-based structure as proposed in previous systems of the family, including the most recent revision by Pojmańska (2008) is not supported by the phylogeny and needs to be abandoned (Tkach et al., 2018). Nevertheless, the progress in revealing the interrelationships among dicrocoeliid taxa has been overall slow, mostly due to the insufficient representation of the dicrocoeliid diversity (comprising 47 genera and more than 400 species) in the molecular phylogenetic studies.

We obtained novel DNA sequences for adult and larval stages of several species of dicrocoeliids, mainly from the *Lyperosomum petiolatum/collurionis* group. We present the results of molecular phylogenetic analyses using nuclear and mitochondrial markers and discuss the obtained data in relation to the systematic position, morphological variability, host specificity and life cycles of these taxa.

2. Material and methods

2.1. Sample collection

Specimens of adult flukes from the family Dicrocoeliidae Looss, 1899 were collected by the authors mostly as a part of long-term helminthological studies carried out in the Czech Republic (Moravia) by Jiljí Sitko and in different regions of the Americas and Africa by Vasyl Tkach. Some additional specimens of adult digeneans, were obtained from birds and small mammals found dead from various causes in different regions of Poland (mainly Lower Silesia and Mazovia districts). Rudimentary-tailed dicrocoeliid xiphidocercariae enclosed in daughter-sporocysts were collected from slugs in different regions of Poland. In the same localities, snails and terrestrial isopods were checked for the presence of dicrocoeliid larval stages (Table 1). Both adult and larval stages were collected live, rinsed in saline, heat-killed with hot water, preserved in 70% ethanol and stored at −20 °C. Measurements and images were taken with an Olympus BX50 microscope equipped with Olympus DP25 digital camera.

2.2. Molecular data

2.2.1. PCR amplification

DNA was extracted from single individuals of adult digeneans and metacercaria or from a single sporocyst containing cercariae using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR reactions were performed with the use of KAPA2G Robust HotStart ReadyMix (Sigma-Aldrich) or OneTaq Quick-load Mastermix (New England Biolabs). Approximately 1200 nucleotide (nt) long fragment of partial sequence of the nuclear large ribosomal subunit gene (LSU) including variable domains D1–D3 was amplified by PCR with the forward primers LSU5, digl2 and the reverse primer 1500R (Tkach et al., 2003). The thermocycling profile was as follows: 3 min denaturation at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C; and 5 min extension at 72 °C. PCR primers and additional dicrocoeliid-specific internal forward primer di890f and reverse primers di390r and di970r (Tkach et al., 2018) were used in sequencing reactions. Extracted DNA was also used for the amplification of the partial mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene and the partial mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) gene. The 400–420 nt long *cox*1 fragment was amplified using the forward primers COIDF1 and the reverse primers COITR1 or COIDR1 (Hildebrand et al., 2016) as well as the forward primer ACOX650F (reverse ACOX650R) (Kudlai et al., 2015), with the following thermocycling profile: 3 min denaturation at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C; and 5 min extension at 72 °C. An approximately 520bp long fragment of *nad*1 gene was amplified using the forward primer NDJ11 and the reverse primer NDJ2a (Kostadinova et al., 2003). The thermocycling profile was as follows: denaturation at 95 °C for 3 min, followed by 35 cycles with 30 s denaturation at 95 °C, 20 s primer annealing at 47 °C, and 45 s at 72 °C for primer extension, with a final extension step of 7 min at 72 °C. Sequences of all primers used in this study are provided in Table 2. Obtained PCR products were purified with Exo-BAP Kit (EURx, Poland), Exo-Sap IT PCR Product Clean-up kit (Affymetrix, USA) or QIAquick Gel Extraction Kit (Qiagen, Germany). Purified products were sequenced directly using ABI BigDye™ chemistry (Applied Biosystems, USA) on an ABI Prism 373xl or an ABI Prism 3100™ automated sequencers.

2.2.2. Phylogenetic analysis

Phylogenetic analysis was performed based on partial sequences of the nuclear large ribosomal subunit rRNA (28S) gene using the newly generated sequences and matching sequences of the representatives of all dicrocoeliid genera available in GenBank (Table 1) including all sequences of the genera formerly included in the Dicrocoeliinae. Forward and reverse sequences were assembled using ContigExpress (Vector NTI Advance 11, Invitrogen) and Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor) software. Contiguous sequences were submitted to GenBank (Table 1). 28S sequences were aligned using Clustal W implemented in MEGA v7 (Kumar et al., 2016). The alignment was trimmed to the length of the shortest sequence. Phylogenetic trees were constructed using Bayesian inference (BI) as implemented in the MrBayes version 3.2.6 software (Ronquist and Huelsenbeck, 2003). The general time reversible model with estimates of invariant sites and gamma distributed among-site variation (GTR + I + G) was chosen as the best-fitting nucleotide substitution model for dataset using

Table 1
The list of dicrocoeliids used in the molecular analyses. GenBank numbers of new sequences obtained in this study are in bold. Abbreviations: A – Aves, C – Crustacea, G – Gastropoda, M – Mammals.

Digenean taxa current/previous names	Host species and geographic origin	Hosts	GenBank Nos		
			28S	*cox*1	*nad*1
Lyperosomum petiolatum/L. alagesi	*Pica pica* Czech Republic	A	**MK478480 MK478473 MK626683**	**MK445302 MK445292**	**MK391419 MK391420**
L. petiolatum/L. alaudae	*Alauda arvensis* Czech Republic	A	**MK478485**	**MK445315**	–
L. petiolatum/L. collurionis	*Sylvia atricapilla* Czech Republic	A	**KU212193 MK478474**	**KU212192 MK445303 MK621923**	**MK391422 MK391421 MK621918 MK621919 MK621920**
***Lyperosomum* sp. 1**	*Acrocephalus arundinaceus* Czech Republic	A	**MK496656**	**MK445285**	–
***Lyperosomum* sp. 2**	*Delichon urbica* Czech Republic	A	**MK496657 MK626682**	–	**MK391431**
L. petiolatum	*Emberiza schoeniclus* Czech Republic	A	**MK478475**	**MK445304**	**MK391423**
L. petiolatum	*Cyanistes caerulaeus* Czech Republic	A	**MK478476**	**MK445305**	**MK391424**
L. petiolatum/L. platynosomoides	*Parus major* Czech Republic	A	**MK478483**	**MK445299**	–
L. petiolatum	*Motacilla alba* Czech Republic	A	**MK478477 MK626684**	**MK445306**	**MK391425 MK621921**
L. petiolatum/L. dujardini	*Prunella modularis* Czech Republic	A	**MK478484 MK478478 MK626685**	**MK445307 MK445300**	**MK391426 MK621922**
Lyperosomum* cf. *turdia/Zonorchis petiolatum	*Turdus philomelos* Czech Republic	A	**MK478496**	**MK445292**	**MK391427**
Lyperosomum* cf. *turdia/Z. petiolatum	*Turdus merula* Czech Republic	A	**MK478497**	**MK445293 MK445294 MK445295**	**MK391428 MK391429 MK507897**
Lyperosomum* cf. *turdia/Z. petiolatum	*Turdus merula* Poland	A	**MK478486**	**MK445291**	–
L. petiolatum/Z. petiolatum	*Corvus frugilegus* Czech Republic	A	**MK478479**	–	–
L. petiolatum/Z. petiolatum	*Corvus frugilegus* Poland	A	**MK478472 MK621181**	**MK445301 MK621924**	**MK391418**
L. petiolatum/Z. petiolatum	*Garrulus glandarius* Poland	A	**MK478481**	**MK445296**	–
***L. petiolatum/Zonorchis* sp.**	*Erinaceus roumanicus* Poland	M	**MK478482**	**MK445297**	–
***L. petiolatum* sporocysts 1**	*Limax maximus* Poland	G	**MK478487 MK618573 MK618574 MK618575**	**MK445308 MK621925 MK621926 MK621927**	–
***L. petiolatum* sporocysts 1**	*Deroceras reticulatum* Poland	G	**MK478488 MK618576**	**MK445309 MK621928**	–
***L. petiolatum* sporocysts 1**	*Cepaea nemoralis* Poland	G	**MK478489**	**MK445310**	–
***L. petiolatum* sporocysts 2**	*Limax maximus* Poland	G	**MK478491**	**MK445312**	–
***L. petiolatum* metacercariae 1**	*Porcellio scaber* Poland	C	**MK478492 MK618577 MK618578 MK618579**	**MK445313 MK621929 MK621930 MK621931**	–
***L. petiolatum* metacercariae 1**	*Ligia* sp. Poland	C	**MK618580**	**MK621932**	–
***L. petiolatum* metacercariae 2**	*Porcellio scaber* Poland	C	**MK478481**	**MK445296**	–
***Lyperosomum* sp. 3**	*Pogoniulus scolopaceus* Uganda	A	**MK480326**	–	–
Lyperosomum clathratum/Zonorchis clathratus	*Apus apus* Czech Republic	A	**MK478493 MK478494**	**MK445289 MK445290 MK445287 MK445288**	**MK391430**
Skrjabinus kalmikensis	*Delichon urbica* Czech Republic	A	**MK478495**	**MK445286**	**MK391432**
Zonorchis alveyi	*Zonotrichia albicollis* USA	A	**MK480327**	–	–
Zonorchis delectans	*Caryothraustes poliogaster* Costa Rica	A	**MK480329**	–	–
***Zonorchis* sp.**	*Phaenostictus mcleannani* Costa Rica	A	**MK480328**	–	–
Stromitrema koshewnikowi	*Hirundo rustica* Czech Republic	A	**MK474483**	**MK445284**	–
Anenterotrema auritum	*Glossophaga soricina* Ecuador	M	MH158566	–	–
Brachydistomum ventricosum	*Erithacus rubecula* Czech Republic	A	KU563713	–	–
Brachylecithum lobatum	*Corvus corone* Czech Republic	A	KU212200	–	–
Dicrocoelium dendriticum	*Marmota bobak* Ukraine	M	AF151939	–	–
Dicrocoelium dendriticum	–		–	NC025280	NC025280
Dicrocoeliidae sp. cercariae	*Discus pauper* Japan	G	MG845913	–	–
Eurytrema pancreaticum	Sheep China	M	KY490000	–	–
Eurytrema pancreaticum	Sheep China	M	KY490004	–	–
Lutziella microacetabulare	*Hipposideros armiger* Vietnam	M	MH158562	–	–
Lutztrema attenuatum	*Turdus merula* Poland	A	KT387687	–	–
Lyperosomum collurionis	*Sylvia atricapilla* Czech Republic	A	AY222259	–	–
Lyperosomum sarothrurae	*Sarothrura pulchra* Democratic Republic of the Congo	A	KP765767	–	–
Lyperosomum transcarpathicus	*Sorex minutus* Ukraine	M	AF151943	–	–
Lyperosomum intermedium	*Oryzomys palustris* USA	M	MH158563	–	–
Lyperosomum sp.	*Turdus merula* Czech Republic	A	MG560864	–	–
Metadelphis lenti	*Lonchophylla robusta* Panama	M	MH158568	–	–
Platynosomum illiciens	*Callithrix penicillata* Brazil	M	MH156571	–	–
Platynosomum illiciens	*Mus musculus* Brazil	M	MH156570	–	–
Pojmanskatrema balcanica	*Neomys fodiens* Bulgaria	M	MK426285	–	–

Table 2
List of primers used in present study.

LOCUS	PRIMER	SEQUENCE (5′–3′)	REFERENCE
28*S*	LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	Tkach et al. (2003)
	digl2	AAGCATATCACTAAGCGG	Tkach et al. (2003)
	1500R	GCTATCCTGAGGGAAACTTCG	Tkach et al. (2003)
	di890f	GAGGGCCAATAGTCTGTGGTG	Tkach et al. (2018)
	di390r	GCTTGCAGTTCAACTCCACC	Tkach et al. (2018)
	di970r	ACTGCGCCTGTGGGTTTCG	Tkach et al. (2018)
***COX*1**	ACOX650F	CAGCATATGTTTTGGTTTTTTGG	Kudlai et al. (2015)
	COIDF1	TATTGTTTCAGCATATGTTTTG	Hildebrand et al. (2016)
	COIDR1	CAACAAACCAAGTATCATGCAAC	Hildebrand et al. (2016)
	COITR1	CAACAACAAACCAAGTATCATG	Laskowski and Rocka (2014)
***NAD*1**	NDJ11 (JB11)	AGATTCGTAAGGGGCCTAATA	Morgan et al., 1998
	NDJ2a	CTTCAGCCTCAGCATAAT	Kostadinova et al. (2003)

JModelTest version 2.1.4 software (Guindon and Gascuel, 2003; Darriba et al., 2012). Sequence of *Encyclometra colubrimurorum* (Rudolphi, 1819), GenBank accession number AF184254, was used as the outgroup based on the previously published phylogenies (Tkach et al., 2001, 2018). BI analysis was performed as follows: Markov chain Monte Carlo (MCMC) chains was run for 2,000,000 generations, log-likelihood scores were plotted and the final 75% of trees were used to produce the consensus tree.

In addition, partial sequences of the mitochondrial *cox*1 and *nad*1 genes obtained from adults and larvae of dicrocoeliids were aligned as above, trimmed to the length of the shortest sequence in each case and concatenated for the combined phylogenetic analysis. The *cox*1 dataset included a greater number of sequences and thus was also utilized for pairwise nucleotide comparisons carried out using the BioEdit 7.0.9 program (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA, 2007). The phylogenetic analyses were carried out using the BI in the MrBayes program. The parameters for each of the concatenated datasets were estimated separately, using the Akaike information criterion (AIC) calculated by the JModelTest 2.1.4 software. The HKY + G model of nucleotide substitution was identified as the best fitting model for the *cox*1 and *nad*1 datasets independently. The sequence of *Dicrocoelium dendriticum* (NC 025280) was used as outgroup in this analysis based on the results of our 28S rDNA analysis. BI analysis of mitochondrial sequences was performed with the same parameters as the 28S rDNA analysis except for the different nucleotide substitution models. Phylogenetic trees were visualized using the TreeView software.

3. Results

We obtained sequence data from a total of 56 isolates of dicrocoeliids, including adult stages from birds (20 species) and mammals (1) and larval stages from slugs (2), snails (1) and isopods (2). Fifty-one partial 28S rDNA sequences and sixty-four mitochondrial (partial *cox*1 and *nad*1 genes) sequences were obtained from adult trematodes belonging to the genera *Lyperosomum*, *Skrjabinus*, *Zonorchis* and *Stromitrema* (Table 1).

Upon trimming to the length of the shortest sequence the 28S alignment was 1159 nt long; only two ambiguously aligned nucleotide positions were excluded from the analysis. The Bayesian phylogenetic analysis of the 28S rDNA alignment generated a phylogenetic tree containing a number of strongly supported clades as well as several unresolved polytomies (Fig. 1). Rather unexpectedly, sequences of dicrocoeliids obtained from a variety of passeriform birds and morphologically identified as *Z. petiolatus* (specimens from *Corvus frugilegus* and *Garrulus glandarius*), *L. alagesi* (specimens from *Pica pica*), *L. collurionis* (specimens from *Sylvia atricapilla*), *L. dujardini* (specimens from *Prunella modularis*), *L. platynosomoides* (specimen from *Parus major*) and as *Lyperosomum* sp. (specimens from *Motacilla alba* and *Emberiza schoeniclus*), proved to be identical to each other and to the sequences previously deposited in GenBank as *L. collurionis* from *Sylvia atricapilla* (AY222259, KU212193, MG560854, MG560860), *G. glandarius* (MG560861) and *P. modularis* (MG560863). Moreover, sequences from the juvenile dicrocoeliids found in hedgehog *Erinaceus roumanicus* were also identical to the sequences listed above.

Sporocysts and cercariae obtained from slugs and snails (*Limax maximus*, *Deroceras reticulatum*, *Cepaea nemoralis*), and metacercariae isolated from isopods (*Porcellio scaber*, *Ligia* sp.) in different regions of Poland (Lower Silesia, Mazovia, Pomerania) corresponded morphologically to the description of *L. petiolatum* (syn. *Dicrocoelioides petiolatum* and *Z. petiolatus*) larvae provided by Timon-David (1960). All our isolates of sporocysts and cercariae from molluscs as well as metacercariae from isopods showed 100% similarity in 28S rRNA with the sequences from birds mentioned above.

All members of *Lyperosomum* appeared in the tree within a major, 100% supported clade that also included representatives of *Skrjabinus*, *Zonorchis*, *Platynosomum*, *Eurytrema* and newly described genus *Pojmanskatrema* Hildebrand and Tkach, 2019 (Hildebrand and Tkach, 2019). Interestingly, this major group of the dicrocoeliids did not include any of the species from mammals present in the analysis (Fig. 1). This “avian” clade included two subclades. One comprised L. *petiolatum* (syn. *Z. petiolatus*), *L. collurionis*, *Lyperosomum* sp. from *Turdus merula* and *T. philomelos*, and *L. clatrathum* (syn. Z. *clathratus*) from *Apus apus* (Fig. 1, Table 1). The second clade consisted of a well-supported (100%) cluster of *Zonorchis* from the Americas (the region where the type species of *Zonorchis* was described) and *L. sarothrurae* from Africa. It also included dicrocoeliids likely acquired by migratory birds on their wintering grounds, such as *Skrjabinus kalmikensis* and *Lyperosomum* sp. from *Delichon urbica* and *Acrocephalus arundinaceus* (Fig. 1, Table 1). These latter specimens morphologically resembled *L. oswaldoi*, but did not fully correspond to its description.

Elsewhere in the tree, *Stromitrema koshewnikowi* clustered with *Lutziella microacetabulare* albeit with somewhat low posterior probability support (89%). Since both taxa form extremely long branches, we consider this topology with some caution.

The *cox*1 alignment was 363 nt long and did not require any gaps whereas the *nad*1 alignment was 462 nt long and also did not require any gaps. Analysis of *cox*1 mtDNA sequences showed 100% identity of isolates from all corvid birds as well as from *Erinaceus roumanicus* and the majority of the isolates from larvae. Sequences of adult trematodes from *S. atricapila*, *E. schoeniclus*, *C. caerulaeus* and sporocysts found in *Limax maximus* (Lower Silesia, Poland), were identical to each other and differed by only 3 nucleotides from specimens from the Corvidae. The sequences of dicroceliids from *M. alba* and *P. modularis* were identical to each other, but slightly different from sequences of specimens obtained from other passerine hosts. At the same time, we observed higher levels of sequence divergence among isolates from different specimens of *Turdus* spp. and *Apus apus*. The pairwise *cox*1 sequence divergence presented as percentage of similarity and the absolute numbers of variable sites is shown in Table 3.

The concatenated alignment of the two mitochondrial genes was

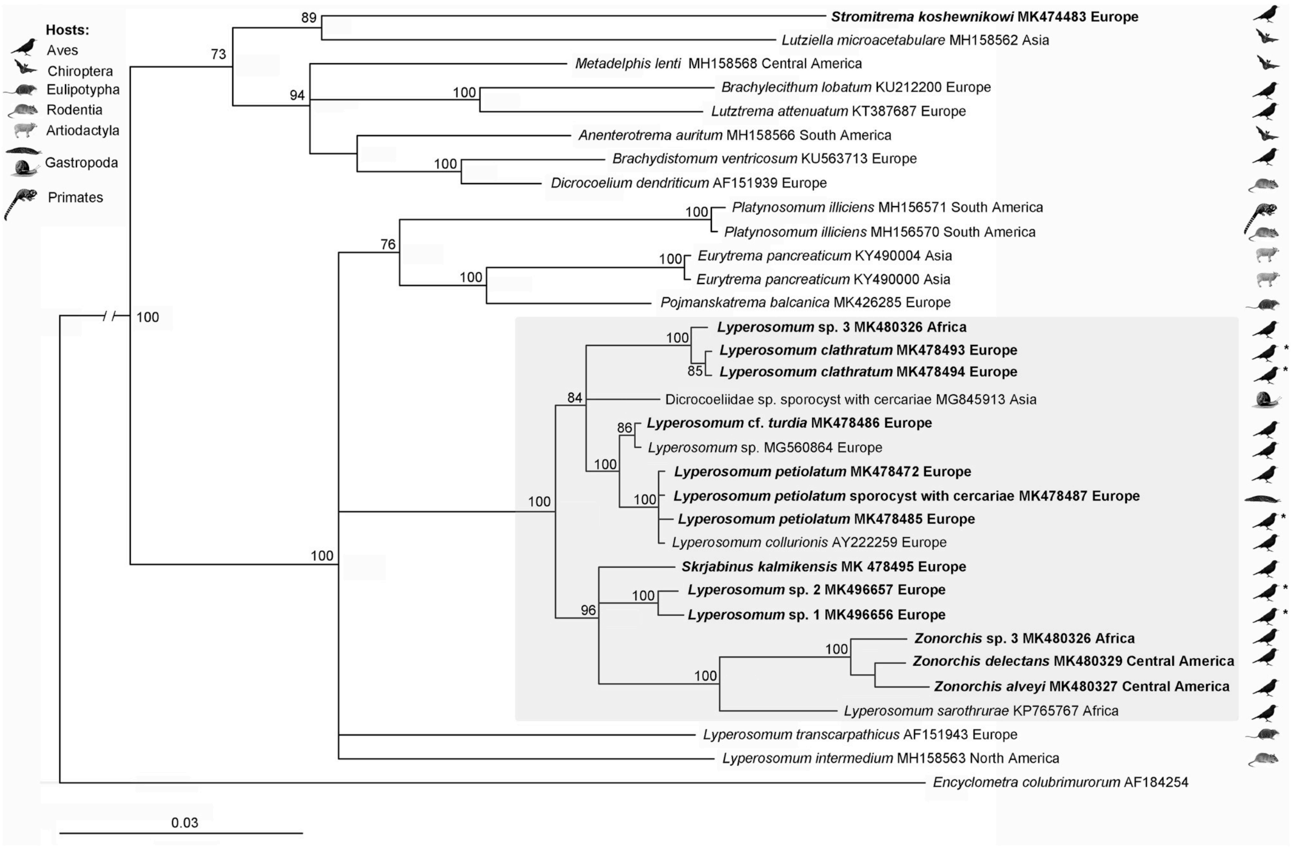

Fig. 1. Phylogenetic interrelationships of Dicrocoeliidae based on Bayesian analysis of partial sequences of the 28 S rDNA gene. Numbers above internodes indicate posterior probabilities greater than 70%. The scale bar indicates the number of substitutions per site. New sequences obtained in this study are in bold. The symbol (*) marks migrating bird. Additional data regarding the sequences are presented in Table 1.

825 nt long. The BI phylogenetic analysis of concatenated mitochondrial sequences (*cox*1 + *nad*1) from closely related dicrocoeliids parasitic in birds produced a tree topology (Fig. 2) generally similar to that seen in the 28S tree, with specimens from *Turdus* spp. forming a distinct, 98% supported clade (Fig. 2).

4. Discussion

Our molecular analysis based on the partial sequences of the 28S rRNA gene shows a significant discord between the molecular data and the current classification of the Dicrocoeliidae based on morphological characters alone which further corroborates the conclusions made by Tkach et al. (2018). Our study included a substantial diversity of flukes that currently are, or historically were, classified as *Lyperosomum* (Fig. 1, Table 1). Similar to the results of Tkach et al. (2018) our data further demonstrate non-monophyly of the sub-families within the Dicrocoeliidae recognized by Pojmańska (2008). In part, species of *Lyperosomum* showed closer relationships with the representatives of the subfamily Leipertrematinae instead of the Dicrocoeliinae where they were placed by Pojmańska (2008). Our results also allow to re-evaluate the relative value of some of the morphological characters traditionally used in the dicrocoeliid systematics. For instance, the location of the testes is one of the features often utilized for the differentiation among dicrocoeliid taxa. While it remains a useful character for distinguishing among species, in our analysis the genera *Lutziella*, *Stromitrema* and some species of *Metadelphis* with symmetrical testes showed close affinities with representatives of *Brachylecithum* or *Lutztrema* which have tandem testes (Fig. 1). Another interesting result in this respect is the high level of support for the clade containing *Zonorchis* from the Americas (flukes with elliptical, fusiform body and symmetrical testes located close to the ventral sucker and to each other, with short vitellaria) and *Lyperosomum sarothrurae* (Baer, 1959) from Africa (a highly elongated fluke with tandem testes and long vitelline fields) (Fig. 1). This suggests the need for a re-evaluation of the morphological criteria used in dicrocoeliid systematics.

The 28S tree contains a well-supported clade formed by *Lyperosomum* parasitic in birds as well as the genera *Skrjabinus* and *Zonorchis*, but this clade does not include all representatives of *Lyperosomum* (Fig. 1). Although some of the previous phylogenetic studies as well as our results strongly suggest the need for separation of some species currently placed in *Lyperosomum* (e.g., *L. intermedium* and *L. trascarpathicus*, both from mammals), we refrain from introducing systematic/nomenclatural changes at this time. Denser sampling of dicrocoeliid taxa for molecular analyses as well as additional morphological studies are necessary for confident delineation among taxa separated by our phylogenetic results. Unfortunately, the cursory original diagnosis of genus *Lyperosomum* by Looss (1899) does not provide a sufficient ground for its clear differentiation from other dicrocoeliid genera. Looss (1899) separated species of *Dicrocoelium* with elongated body into a new genus *Lyperosomum* with *L. longicauda* (Rudolphi, 1809) as a type-species. Unfortunately, we did not have in our material specimens fully corresponding to the description of *L. longicauda*. Some of our specimens from the Corvidae with elongated body had diagonally positioned testes characteristic of *L. longicauda*, but their eggs were larger than in previous descriptions of the species (Dolfus, 1957; Macko and Macková, 1995).

Our molecular study based on 28S rDNA demonstrated that specimens from *C. frugilegus*, *G. glandarius*, *P. pica* and *S. atricapilla* were identical (Fig. 1, Table 1). Traditionally, the flukes from these birds were identified based on their host specificity, as *Z. petiolatus* (from *C. frugilegus*, *G. glandarius*), *L. alagesi* (*P. pica*) and *L. collurionis* (*S. atricapilla*). In addition, the 28S sequences of larval forms, i.e. sporocysts and

Table 3

Pairwise comparisons of 363 nt long fragment of mitochondrial *cox*1 gene of *Lyperosomum* specimens from different avian hosts collected in this study. Numbers of variable nucleotide positions are above diagonal; percentages of pairwise nucleotide similarity are below diagonal. The alignment did not contain indels.

Lyperosomum isolates from different hosts	*Corvus frugilegus*	*Pica pica*	*Alauda arvensis*	*Sylvia atricapilla*	*Motacilla alba*	*Prunella modularis*	*Turdus merula* 1	*Turdus merula* 2	*Turdus philomelos*	*Apus* 1	*Apus* 2	*Apus* 3	*Delichon urbica*
Corvus frugilegus MK445301	–	0	3	3	3	2	25	29	30	50	51	49	59
Pica pica MK445292	100	–	3	3	3	2	25	29	30	50	51	49	59
Alauda arvensis MK445315	99.2	99.2	–	0	4	5	26	30	31	51	52	50	61
Sylvia atricapilla MK445303	99.2	99.2	100	–	4	5	26	30	31	51	52	50	61
Motacilla alba MK445306	99.2	99.2	98.9	98.9	–	5	26	30	31	52	53	51	58
Prunella modularis MK445307	99.4	99.4	98.6	98.6	98.6	–	23	27	28	52	53	51	59
Turdus merula 1 MK445293	93.3	93.3	93.0	93.0	93.0	93.8	–	5	6	47	47	45	56
Turdus merula 2 MK445295	92.2	92.2	92.0	92.0	92.0	92.8	98.6	–	1	50	52	50	58
Turdus philomelos MK445292	92.0	92.0	91.7	91.7	91.7	92.5	98.4	99.7	–	51	53	51	59
Apus apus 1 MK445287	86.6	86.6	86.4	86.4	86.1	86.1	87.4	86.6	86.4	–	2	2	52
Apus apus 2 MK445288	86.4	86.4	86.1	86.1	85.8	85.8	87.4	86.1	85.8	99.4	–	2	54
Apus apus 3 MK445290	86.9	86.9	86.6	86.6	86.4	86.4	88.0	86.6	86.4	99.4	99.4	–	52
Delichon urbica MK445315	84.2	84.2	83.7	83.7	84.5	84.2	85.0	84.5	84.2	86.1	85.6	86.1	–

metacercariae from slugs, snails and woodlice, showed 100% identity to the adult digeneans from these 4 avian hosts. Combination of molecular results and morphological analysis allowed us to identify all these adult flukes and larval stages as *L. petiolatum*. It should be noted that sequences of mitochondrial DNA of *L. petiolatum* isolates obtained from different hosts showed some intraspecific variation (Table 3).

So far, the life-cycles of only 15 dicrocoeliids are known (Skrjabin and Evranova, 1952; Krissinger, 1984; Panin, 1984; Manga-González et al., 2005; Pinto et al., 2014; Hildebrand et al., 2016). One type of the life-cycles (observed in members of *Brachylecithum*, *Brachydistomum*, *Lutztrema*) is characterized by the presence of long-tailed xiphidocercariae, which leave the first intermediate host (a land snail) in mucoid balls that are eaten by the second intermediate host (an arthropod). Dicrocoeliids with the second type of life-cycles (*Conspicuum*, *Eurytrema*, *Platynosomum*) possess xiphidocercariae with a short or rudimentary tail, which leave the snail enclosed in daughter-sporocysts. The life cycle of *L. petiolatum* (syns. *D. petiolatum* and *Z. petiolatus*) belongs to the second type, and was revealed experimentally by Timon-David (1960), with the snail *Helicella* (*Helicopsis*) *arenosa* and terrestrial isopods (*Armadillo officinalis, Armadillidium vulgare*) used as intermediate hosts (Timon-David, 1960). Thus, in the present study, the full life cycle of *L. petiolatum* was confirmed for the first time from the nature. Unfortunately, a full comparison of our data on the morphology of the life cycle stages with those reported by Timon-David (1960) is difficult because the latter author did not provide ranges for the majority of morphometric characters and did not mention some essential characters. Nevertheless, the morphology of the life cycle stages in our material fits well the description by Timon-David. Based on our data from natural infection and the data from experimental infection by Timon-David (1960) *L. petiolatum* apparently has a relatively low specificity to the intermediate hosts.

Lyperosomum petiolatum is one of the most commonly reported *Lyperosomum* species in the Central Europe. As previously noted by several authors (Denton and Bird, 1951; Mettrick, 1963; Macko, 1968; Macko and Macková, 1995) this species exhibits significant morphological variability which is confirmed by our morphological study based on freshly collected material. Genetically identical samples from different avian hosts examined by us included forms with oblique and symmetrical testes as well as forms with the varying length of vitelline fields. We have also observed the change in proportion of the acetabulum to body size ratio in the process of fluke maturation (Fig. 3). Both the literature and our data demonstrate that *L. petiolatum* does not show a narrow definitive host specificity. The inconsistency in the relative position of testes, namely the presence of a morphotype with symmetrical testes was the reason for the transfer of *L. petiolatum* into *Zonorchis* by Travassos (1944) and Denton and Byrd (1951). At the same time, several morphologically similar species, e.g., *L. alagesi* (Skrjabin and Udinzev, 1930), *L. alaudae* (Shtrom and Sondak, 1935) and *L. collurionis* (Skrjabin and Isaitschikoff, 1927) remained in *Lyperosomum*.

Dicrocoeliids occurring in the Turdidae were traditionally identified as *Z. petiolatus* due to their morphological similarity to flukes parasitizing corvids (Denton and Bird, 1951; Panin, 1984; Mettrick, 1963). Our 28S sequences from specimens originated from *Turdus merula* and *T. philomelos* were identical to the sequences of *Lyperosomum* sp. from *Turdus* sp. (MG560864, MG560865) recently published by Aldhoun et al. (2018). At the same time, sequences from *Turdus* differed by 5 nt (99.6% similarity) from sequences from the specimens obtained from corvids (*C. frugilegus*, *G. glandarius* and *P. pica*; Fig. 1). The results from 28S rDNA were further corroborated by the mtDNA sequences which showed 6.7–8.0% difference between specimens from *Turdus* spp. and corvids, *C. frugilegus* and *P. pica* (Table 3; Fig. 2). This strongly suggests the status of the form parasitizing Turdidae as a separate species. Morphological comparison of our material with published descriptions did not allow for an unambiguous species identification of *Lyperosomum* from *Turdus* spp., although they appear to be somewhat similar to *L.*

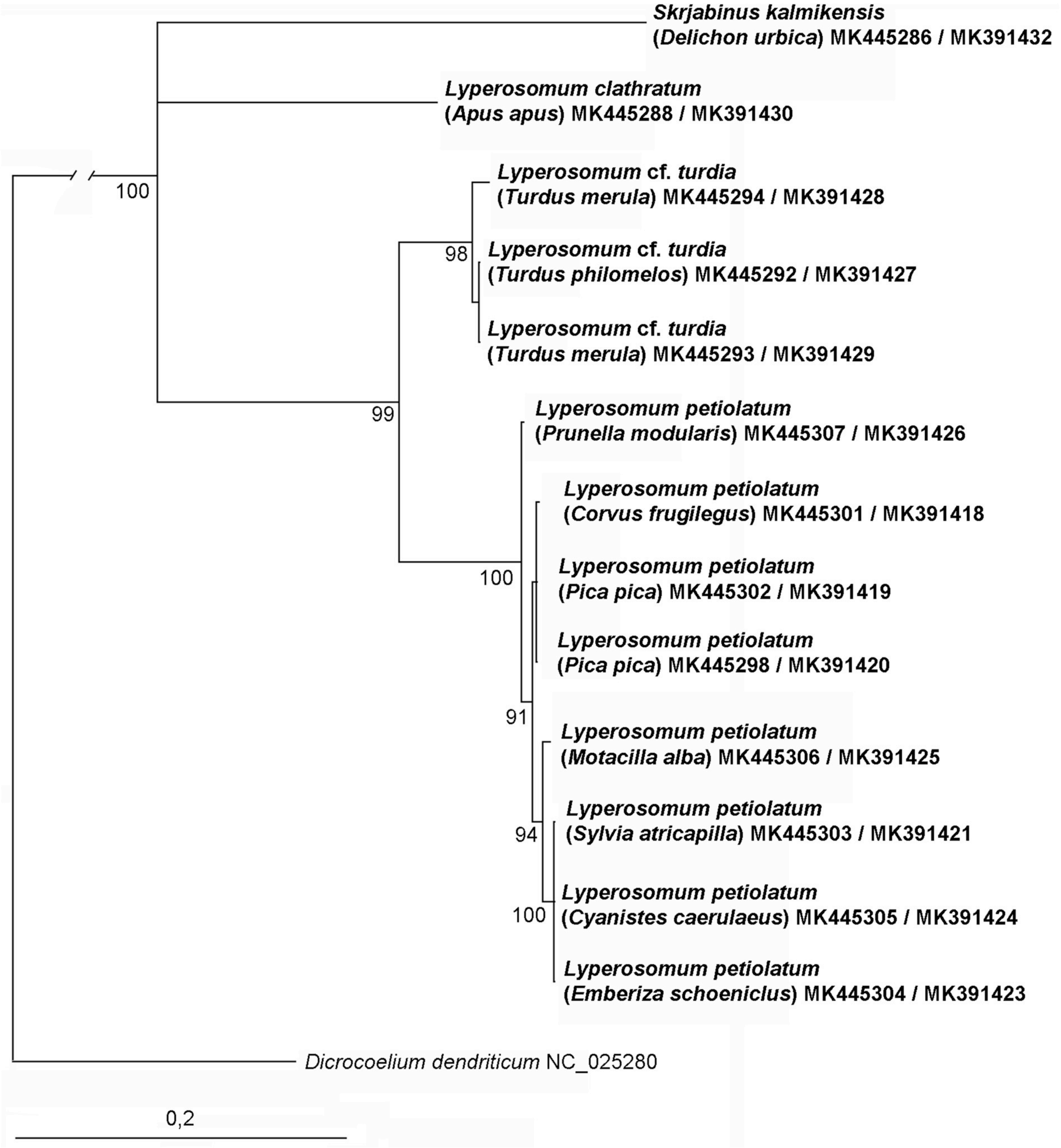

Fig. 2. Bayesian analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) gene combined with nicotinamide adenine dinucleotide dehydrogenase subunit 1 gene (*nad*1) of *Lyperosomum* spp. constructed using MrBayes. Numbers below internodes indicate posterior probabilities greater than 70%. All sequences, except for *D. dendriticum*, have been obtained in this study. Additional data regarding the sequences are presented in Table 1.

turdia (Ku, 1938) described from *T. merula* and *T. cardis* in China and Japan. However, due to the great geographic distance between our collecting sites and the type territory of *L. turdia* we are hesitant to assign this name to the European specimens in the absence of sequence data from the Far East.

Lyperosomum clathratum is characterized by symmetrical testes which was previously a reason for the transfer of this species into *Zonorchis* by Odening (1964). However, it appeared on the phylogenetic trees within *Lyperosomum* as a sister taxon to the clade of *L. petiolatum* + *Lyperosomum* from *Turdus* spp. (Figs. 1 and 2). The genus *Zonorchis* was originally established for digeneans founds in the Americas (the type species is *Z. microrchis* (Travassos, 1916) from Brazil) and is represented in our tree by three species characterized by the typical *Zonorchis* morphotype, all collected from birds in North and Central America (Fig. 1). These species form a 100% supported clade not closely related to *L. clathratum* which suggests that the relative position of testes alone is not a suitable character for distinguishing among genera in this group of digeneans. *Lyperosomum clathratum* does not seem to be a typical representative of the European fauna and is likely acquired by *A. apus* on wintering grounds in Africa, based on the fact that we only found this species in spring in birds returning after overwintering in Africa. This hypothesis is partly supported by the close relationship between this species and *Lyperosomum* sp. from the speckled tinkerbird *Pogoniulus scolopaceus* in Uganda.

One of the main problems in the systematics of *Lyperosomum* and the Dicrocoeliidae as a whole is the insufficient knowledge of the extent of the intraspecific morphological variability. This variability is associated in part with the crowding effect which can be quite pronounced due to

Fig. 3. Representatives of *Lyperosomum petiolatum* from different hosts: a – *Pica pica*; b – *Garrulus glandarius*; c – *Corvus frugilegus*; d – *Corvus frugilegus*, subadult specimen; e, f – *Sylvia atricapilla*. *Lyperosomum* sp., from *Turdus merula*: g – specimen fixed after death; h – specimen fixed under pressure. Scale bars – 1 mm.

the parasitism in a limited space such as the gall bladder and bile ducts (e.g., Tkach and Bray, 1995; Pinto et al., 2015, 2017). In addition, these flukes grow continuously throughout their lives, and their anatomical features undergo partial changes including the size and proportions of the gonads (Macko, 1968; Sitko, 1994, 1995). Thus, in longer living birds such as some corvids, the same species may have a somewhat different appearance than in a bird with a shorter life span. Considering that many dicrocoeliid species were described based on a single specimen or very few specimens (e.g., *L. collurionis*, *L. alagesi*, *L. alaudae*, *S. kalmikensis*, *S. skrjabini*) the role of morphological variability was likely underestimated in dicrocoeliid taxonomy. When it comes to the higher level systematics, recently published molecular data as well as the results of the present study have demonstrated that the relative value of some of the morphological criteria broadly used in dicrocoeliid systematics (e.g., relative position of testes, body size and proportions, presence/absence of digestive system) needs to be re-assessed using a combination of morphological examination of properly fixed specimens and evidence provided by DNA sequences.

5. Conclusions

The obtained molecular data and the morphological examination of newly collected material combined with literature data, allow us to make several systematic conclusions and nomenclature changes.

1. Based on our results we propose the following taxonomic changes:
   a) we return *Zonorchis clathratus* and *Z. petiolatus* into *Lyperosomum* as *L. clathratum* and *L. petiolatum*.
   b) we recognize *L. alagesi* as a synonym of *L. petiolatum*
   c) Turdidae host a distinct species of *Lyperosomum* morphologically similar to *L. petiolatum*.
2. The dicrocoeliids parasitic in *Sylvia atricapilla* in Central Europe belong to *L. petiolatum* and not to *L. collurionis.* We disagree with Aldhoun et al. (2018) who proposed to place *L. collurionis* among synonyms of *L. petiolatum*; the lack of specimens matching the original description of *L. collurionis* from the type host *Lanius collurio* prevents a quality re-description of the species at this time.
3. *Lyperosomum petiolatum* occurs commonly in Europe in corvids as well as in some migratory songbirds besides *S. atricapilla*, e. g., *Emberiza schoeniclus Motacilla alb*a and *Prunella modularis.*
4. Some morphological characters previously considered as critically important for separation between major lineages of the Dicrocoeliidae, proved to be useful only at species level or even demonstrate intraspecific variability. Subfamilies based on traditional characters turned out to be non-monophyletic. Therefore, we agree with the recent decision by Tkach et al. (2018) to abandon the subfamily-based classification of the Dirocoeliidae.
5. Phylogenetic relationships of species previously included in *Lyperosomum* demonstrated the paraphyletic nature of the genus and indicate the need of its thorough revision based on both morphological and molecular data.
6. The systematic status and interrelationships among members of *Zonorchis* occurring in the New World and the Old World need further clarification. However, a well-grounded decision cannot be made without inclusion of several other morphologically close genera, e.g., *Conspicuum* and *Lubens*, into the future phylogenetic studies.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.03.020.

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