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Description of *Myxobolus balatonicus* n. sp. (Myxozoa: Myxobolidae) from the common carp *Cyprinus carpio* L. in Lake Balaton

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Abstract *Myxobolus balatonicus* n. sp. was detected in the gill filaments of the common carp *Cyprinus carpio* L. collected in Lake Balaton, Hungary. Its oval plasmodia measuring 600–800 × 300–400 µm were located intravasally in the filamental arteries. The spores measured 11.2 ± 0.92 × 9.5 ± 0.41 × 7.4 ± 0.33 µm and had two equal polar capsules with six filamental turns. Both morphology and DNA sequence analysis revealed that *M. balatonicus* n. sp. is distinct from the ten species of *Myxobolus* Bütschli, 1882 described from the European common carp and the 21 species described from the Asian common carp subspecies. Phylogenetic analysis placed *M. balatonicus* n. sp. in a clade of gill-infecting myxobolids.

Introduction

The common carp *Cyprinus carpio* L. is the most important species of freshwater fish culture. Its parasite fauna is well studied all over the world. The works of Akhmerov (1960) and Donec & Shulman (1984) contain abundant data on myxozoan parasites of the European common carp and their Asian relatives in River Amur and the synopsis by Chen & Ma (1998) provides detailed data on the parasites of carp cultured in China. To date, 21 species of the genus *Myxobolus* Bütschli, 1882 from Asia and ten species from other parts of the world have been described (Eiras et al., 2005, 2014). Unfortunately, the descriptions of most of these species are poor and restricted to data on spore shape and size. Also, line drawings are not sufficiently illustrative to allow identification of the species level. Most descriptions lack information on the plasmodium stages, and other cyprinids and fishes of genetically distant genera are recorded as hosts in addition to common carp. Correct data including histology, pathology and molecular results are available for six European species: (*M. basilamellaris* Lom & Molnár, 1983, *M. cyprini* Doflein, 1898, *M. cyprinicola* Reuss, 1908, *M. dispar* Thélohan, 1895, *M. encephalicus* Mulsow, 1911 and *M. intrachondrealis* Molnár, 2000) (see Lom & Molnár, 1983; Molnár & Kovács-Gayer, 1985; Dyková et al., 1986; Antychowicz & Reichert 1987; Dyková & Lom 1988; Molnár 2000, 2002a, 2009; Cirkovic et al., 2010) and for three Asian species (*M. artus* Achmerov, 1960, *M. musseliusae* Yakovchuk, 1979 and *M. tsangwuensis* Chen & Ma, 1998) (see Ogawa et al., 1992; Liu et al., 2013; Huang et al., 2014).

In a long-term parasitological survey conducted on fishes from Lake Balaton in Hungary, a new species of the genus *Myxobolus* was found. This paper provides a description of *Myxobolus balatonicus* n. sp. in the gills of the common carp and differentiates the new form

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from the known *Myxobolus* spp. based on its morphology, site selection and 18S rDNA sequences.

Materials and methods

Samples

Lake Balaton is the largest lake in Central Europe. This shallow lake is a favourite recreational area in Hungary, its utilisation for fisheries being only secondary. Due to the control and building up of the lakeshore, there is minimal natural propagation of the common carp, and therefore >2-year-old common carp specimens from fish ponds are being introduced into the lake every year to facilitate recreational fishing activities. The permanent stocking of common carp from fish ponds to the lake is the main reason why during the regular surveys performed earlier relatively little attention was paid to parasites of the common carp. Despite this fact, during our survey of fresh fish catches comprised of several fish species at the Keszthely fishery on 31 March 2009, we examined the gills of 14 common carp specimens belonging to the 30–45 cm size range, and selected a 40 cm long specimen showing signs of myxosporean infection visible to unaided eye (gill plasmodia). This infected carp specimen was transferred to the laboratory alive in an oxygenated plastic bag and kept in a concrete tank. On the day after its collection the fish was sedated with clove oil and killed with a cervical cut. No signs of myxosporean infection were found on the remaining 13 specimens. All applicable institutional and national guidelines for the care and use of animals were followed.

In the framework of a complete parasitological examination, the hemibranchia of the gills and the fins were cut and examined under a dissecting microscope for the presence of *Myxobolus* spp. plasmodia. Myxozoan spores from the isolated and opened cysts were first studied in wet mounts, and then some of the spores were placed in glycerine jelly under a cover slip and preserved as a reference slide. Another subsample of the spores collected from a single matured plasmodium were placed into 1.5 ml tubes and stored at −20°C for subsequent molecular study. The vitality of the spores was checked by placing them into a 0.4% solution of urea. Spores of a given plasmodium were regarded as mature when at least 90% of the spores had extruded polar filaments in that solution. Unfixed spores were examined using an Olympus BH2 microscope fitted with Nomarski differential interference contrast optics. Fresh spores were photographed with an Olympus DP20 digital camera and measurements were taken from fresh spores and from digitised photos. All measurements are given in micrometres and are given as the range followed by the mean ± standard deviation and the number of measurements in parentheses.

Tissue samples from infected organs containing developing and mature plasmodia were fixed in Bouin's solution, embedded in paraffin wax, cut into 4–5 µm sections, and stained with haematoxylin and eosin.

Molecular data

For DNA extractions, samples preserved in 80% ethanol were centrifuged at 8,000*g* for 10 min to pellet the myxospores and the ethanol removed. DNA was extracted using a Qiagen DNeasyTM tissue kit (animal tissue protocol, Qiagen, Hilden, Germany) and eluted in 75 µl of AE buffer. The 18S rDNA was amplified using the primers ERIB1 and ERIB10 (Table 1) in a 25-µl reaction mixture, which comprised 2 µl extracted genomic DNA, 5 µl 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentas, Burlington, Ontario, Canada), 0.325 µM of each primer, 2.5 µl 10× Taq buffer (MBI Fermentas), 1.25 µl 25 mM $MgCl_2$, 0.1 µl Taq polymerase (1 U) (MBI Fermentas) and 13.5 µl distilled water. The PCR cycle consisted of an initial denaturation step at 95°C for 3 min, followed by 35 cycles at 94°C for 50 s, 56°C for 50 s, 72°C for 80 s, plus a terminal extension at 72°C for 7 min. This was followed by a second round of PCR with the MYX1F-SphR primer pair (Table 1). The total volume of the nested PCR reactions was 50 µl, which contained 1 µl amplified DNA, 10 µl 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentas), 0.325 µM of each primer 5 µl 10× Taq buffer (MBI Fermentas), 2.5 µl 25 mM $MgCl_2$, 0.2 µl Taq polymerase (2 U) (MBI Fermentas) and 30.3 µl water. Amplification conditions in the second round were: 94°C for 50 s, 56°C for 50 s, 72°C for 90 s for 35 cycles, and the cycle was terminated with an extension at 72°C for 10 min. Both PCR cycles were performed in a PTC-200 thermocycler (MJ Research, St. Bruno, Quebec, Canada). The PCR products were electrophoresed in 1.0% agarose gels in Tris-Acetate-EDTA (TAE) buffer gel stained with 1% ethidium

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Table 1 Primers used for PCR or sequencing

Primer	Sequence	Source
ERIB1	5′-ACCTGGTTGATCCTGCCAG-3′	Barta et al. (1997)
ERIB10	5′-CTTCCGCAGGTTCACCTACGG-3′	Barta et al. (1997)
Myx1F	5′-GTGAGACTGCGGACGGCTCAG-3′	Hallett & Diamant (2001)
SphR	5′-GTTACCATTGTAGCGCGCGT-3′	Eszterbauer & Székely (2004)
ACT1fr	5′-TTGGGTAATTTGCGCGCCTGCTGCC-3′	Hallett & Diamant (2001)
MC5	5′-CCTGAGAAACGGCTACCACATCCA-3′	Molnár et al. (2002)
MC3	5′-GATTAGCCTGACAGATCACTCCACA-3′	Molnár et al. (2002)
MB5r	5′-ACCGCTCCTGTTAATCATCACC-3′	Eszterbauer (2004)
MB5f	5′-GATGATTAACAGGAGCGGTTGG-3′	Eszterbauer (2004)

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156 bromide and then purified with the EZ-10 Spin column
157 PCR Purification Kit (Bio Basic Inc., Markham,
158 Ontario, Canada). Purified PCR products were se-
159 quenced using the primers listed in Table 1 and ABI
160 BigDye Terminator v3.1 Cycle Sequencing Kit with
161 an ABI 3100 Genetic Analyser.

162 The phylogenetic analyses were executed with
163 MEGA 6.06 (Tamura et al., 2013). The various forward
164 and reverse sequence segments were assembled in the
165 alignment editor. Published myxozoan sequences were
166 downloaded from the GenBank based on the Blast
167 matches; *Myxobolus cerebralis*, Hofer, 1903 was
168 chosen as an outgroup. Nucleotide sequences were
169 aligned with CLUSTAL W (Thompson et al., 1994)
170 and the alignment was corrected manually using the
171 alignment editor. DNA pairwise distances were calcu-
172 lated using p-distance model. Maximum likelihood
173 (ML) analysis was performed to determine the phylo-
174 genetic position of the analysed sample. The data set
175 was tested for the nucleotide substitution model of best
176 fit; the model selected using the Akaike Information
177 Criterion (AIC) was GTR+G+I. Bootstrap values
178 based on 1,000 resampled datasets were generated.

179 ***Myxobolus balatonicus*** **n. sp.**

180 *Type-host*: *Cyprinus carpio* L.
181 Type-locality: Western basin of Lake Balaton, near
182 the town of Keszthely (46°45′12.4″N 17°14′55.6″E),
183 Hungary.
184 *Site of tissue development*: Efferent arteries of the gill
185 filaments.
186 *Prevalence*: 7 % (1/14 of the 30 to 45 cm long fish;
187 based on gross observation of macroscopic plasmodia).
Intensity: 3 to 8 plasmodia per hemibranch. 188
Type-material: Voucher spores of *M. balatonicus* n. 189
sp. in glycerine-gelatine, phototypes deposited in the 190
parasitological collection of the Zoological Depart- 191
ment, Hungarian Natural History Museum, Budapest 192
(Coll. No. HNHM-18212). 193
Representative sequence: 18S rDNA sequence for *M.* 194
balatonicus n. sp. is deposited in GenBank under 195
accession number KP205545. 196
Etymology: The species is named after the type- 197
locality, Lake Balaton in Hungary. 198

Description (Figs. 1–3) 199

Vegetative stages 200

Ellipsoidal plasmodia (600–800 × 300–400) filled 201
with spores were found in the gill filaments of a 202
3-year-old common carp (*Cyprinus carpio* L.). 203

Spores 204

Spores ellipsoidal in frontal view (Figs. 1A, 2, 3) 205
and lemon shaped in sutural view (Fig. 1B, Fig. 3A). 206
Length of spores 10.1–12.1 (11.2 ± 0.92; n = 50), 207
width 8.8–10.1 (9.5 ± 0.41; n = 50), thickness 208
7.1–7.9 (7.4 ± 0.33; n = 11). Polar capsules drop- 209
like, equal in size, slightly converging anteriorly, 210
4.9–6 (5.5 ± 0.24; n = 50) long, 3–3.7 (3.3 ± 0.21; 211
n = 50) wide. Six filament coils arranged perpen- 212
dicular to capsule length wound up densely in polar 213
capsule. Intercapsular appendix large, cuneiform. 214
Sutural protrusion forms circular rim around spore 215
emerging about 0.6 to 1.1 over spore surface; suture 216
rim emerges from spore surface 0.7 to 0.9 at 217
posterior end of spore. Sutural edge markings 7, 218
distinct, one at posterior end large. Wall at posterior 219

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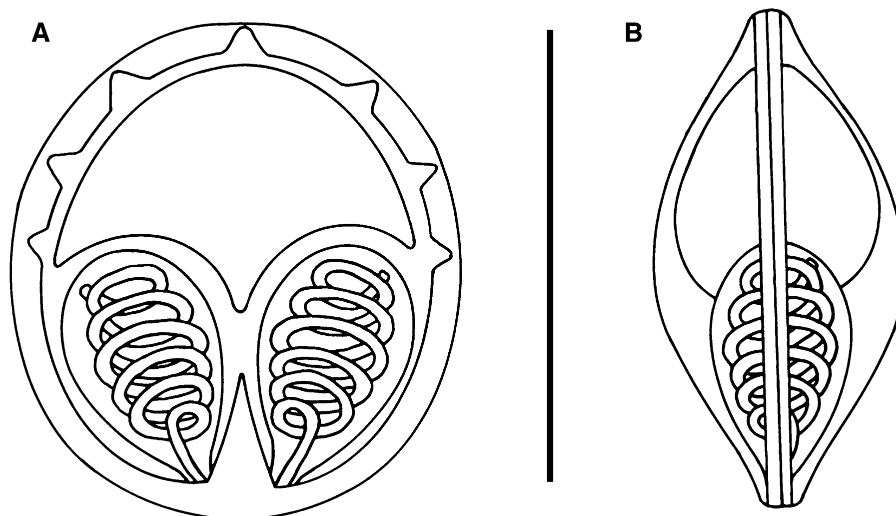

Fig. 1 Schematic drawings of *Myxobolus balatonicus* n. sp. A, Spore in frontal view; B, Spore in sutural view. *Scale-bar*: 10 µm

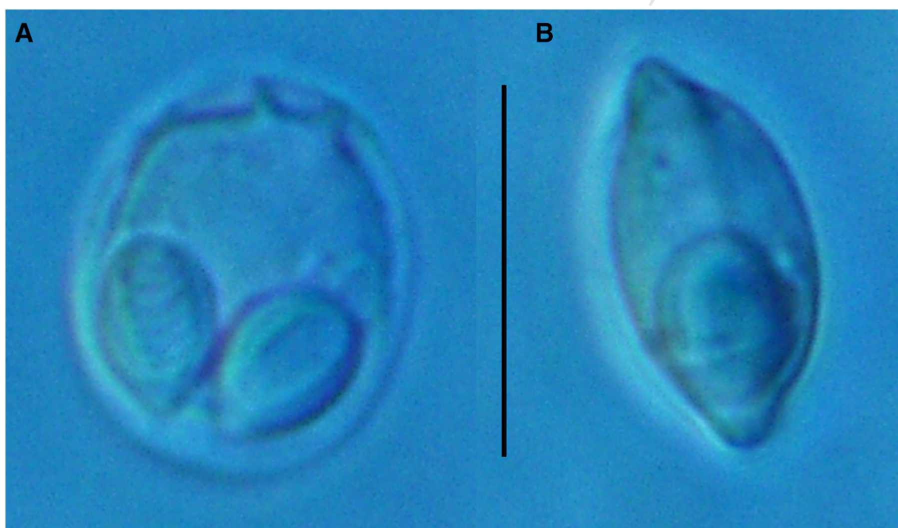

Fig. 2 Fresh spores of *Myxobolus balatonicus* n. sp. A, Spore in frontal view; B, Spore in sutural view (note the well-developed edge markings, especially the posterior one). *Scale-bar*: 10 µm

220 end thickened. Sporoplasm single, binucleated, with
221 iodinophilous vacuole in spore. Mucous envelope not
222 found.

223 Histology

224 In histological sections, plasmodia were found in the
225 efferent artery at about middle part of the filaments
226 (Fig. 4). In longitudinal sections of the filaments it was
227 well visible that the oval plasmodia are located inside
228 the lumen and both the anterior and posterior ends of
229 the plasmodium dilatations of the artery were seen.
230 The secondary lamellae in the infected sections were
231 shorter than in the uninfected filaments and in the
232 uninfected part of the damaged filaments. Although
233 the capillary network of the neighbouring lamellae
234 was less filled with blood, these capillaries still
235 received blood from the artery through a narrow gap
236 between the plasmodium and the endothelium of the
237 artery where the blood could flow through (Fig. 5).

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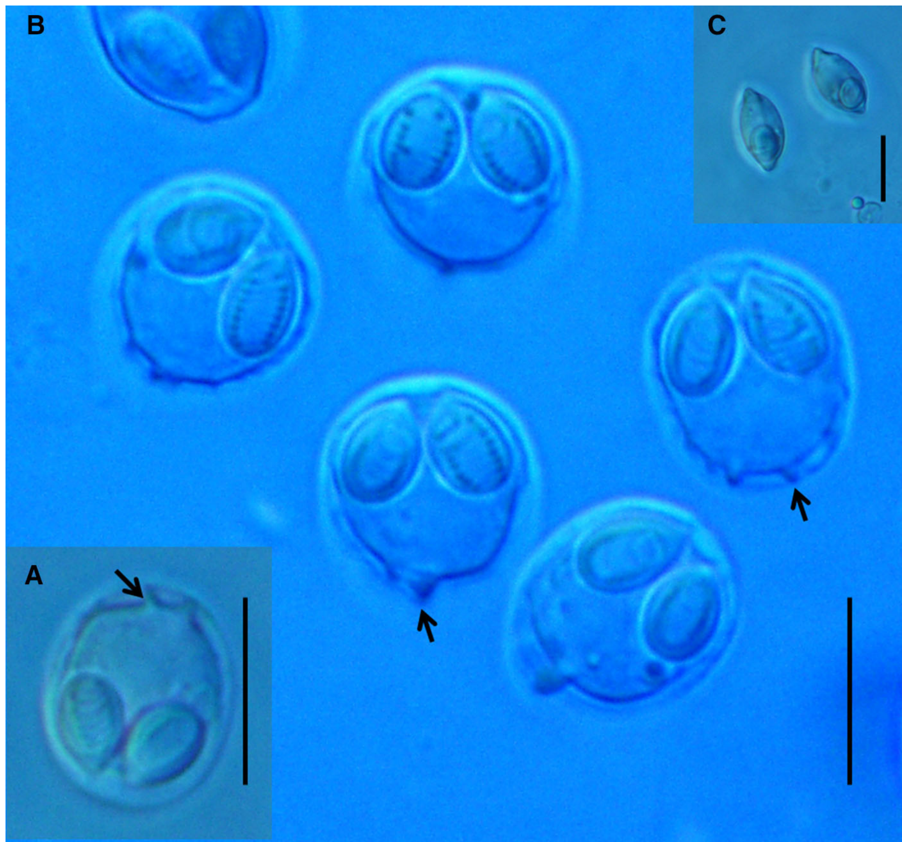

Fig. 3 Spores of *M. balatonicus* n. sp. A, B, Spores in frontal view; note that most show a slight thickening at posterior end (*arrows*), C, Spores in sutural view. *Scale-bars*: 10 µm

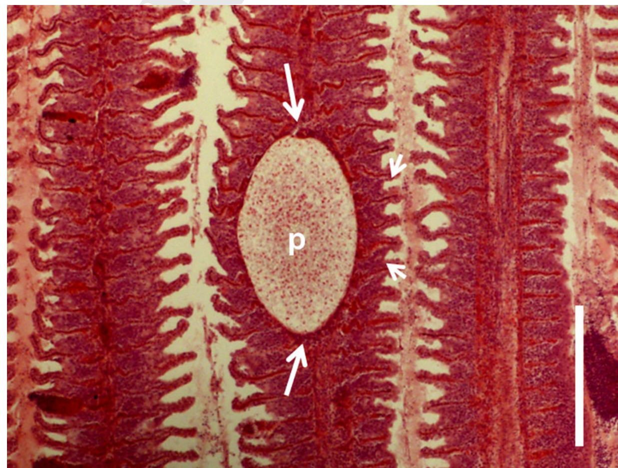

Fig. 4 Oval-shaped plasmodium (p) of *M. balatonicus* n. sp. in one of the gill filaments of a common carp, located in the lumen of the efferent artery of the gill filament. Lamellae (*short arrows*) around the plasmodium are shorter than in other filaments and in the non-infected part of the affected filament. The gill artery is enlarged at the anterior and posterior ends of the plasmodium and filled by blood cells (*long arrows*). Histological section, haematoxylin and eosin staining. *Scale-bar*: 300 µm

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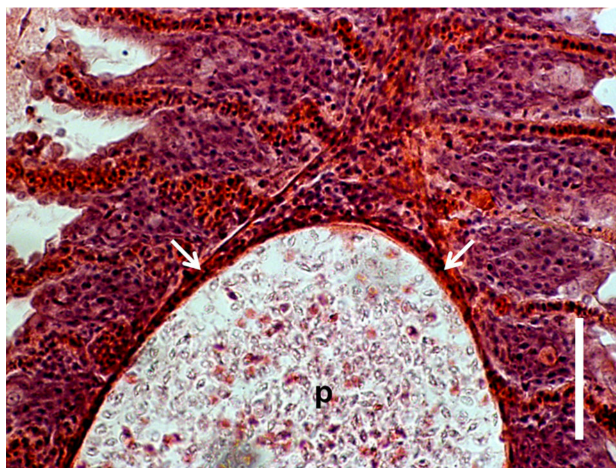

Fig. 5 The plasmodium (p) leaves a narrow gap at the periphery to the blood flow (*arrows*), through which the blood can run across the lamellae. Histological section, haematoxylin and eosin staining. *Scale-bar*: 100 µm

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238 Molecular data

239 The 18S rDNA sequence of *M. balatonicus* n. sp.
240 differed from all known sequences of *Myxobolus* spp.
241 known from the common carp and also from those of
242 other myxozoan species. The phylogenetic position of
243 *M. balatonicus* n. sp. was sister to *M. dispar*, another
244 gill-infecting parasite of carp, with high bootstrap
245 support (Fig. 6). *Myxobolus cyprinicola* was sister to
246 this pair, but this species develops in carp intestines.
247 The pairwise distances showed remarkable differences
248 between *M. balatonicus* n. sp. on the one hand and *M.*
249 *dispar* and *M. cyprinicola*, on the other, with sequence
250 identities reaching only 94.0% and 89.6%, respectively.

251 Differential diagnosis

252 The number of myxozoan species described from the
253 common carp is high. Twenty-one species have been
254 recorded by Molnár (2009) from the Asian subspecies
255 of the common carp and ten species from the European
256 variant. Most of the species from the Asian carp have
257 poor descriptions based only on the shape and size of
258 the spores (e.g. species described by Akhmerov, 1960
259 and Chen & Ma, 1998). In most cases, descriptions
260 lack data on the plasmodium stages and several
261 genetically distant hosts have been also recorded; we
262 suppose that some of these species are synonymous.
263 Of the *Myxobolus* spp. described from the gills of the
264 Asian common carp, *M. hanchuanensis* Chen & Ma,
265 1998, *M. obovoides* Li & Ni, 1973 and *M. oviformis*
266 Thélohan, 1892 have larger spores than *M. balatonicus*
267 n. sp. The spores of *M. artus* and *M. liaoningensis*
268 Chen & Ma, 1998 have larger dimensions in width
269 than in length. The spores of *M. acinosus* Nie & Li,
270 1973 have a posteriorly tapering pyriform shape, and
271 *M. amurensis* Akhmerov, 1960 has a small tip at the
272 anterior end. *Myxobolus haematopterus* Yukhimenko,
273 1986 and *M. liaoningensis* Chen & Ma, 1998 have
274 similar roundish spores, but their intercapsular ap-
275 pendix is small (Chen & Ma, 1998; Eiras et al., 2005;
276 Molnár, 2009; Eiras et al., 2014). The location of the
277 plasmodia of *M. balatonicus* n. sp. in the gill filaments
278 of the common carp resembles that of *M. dispar* and
279 *M. musseliusae*. However, the latter species form
280 large, elongated spores with two different polar
281 capsules. Seemingly the latter three species belong
282 to the vascular type of myxosporidia (Molnár, 2002b),
283 but a recent study (Liu et al., 2013) has revealed that
284 *M. musseliusae* does not develop in the vascular lumen
285 but forms plasmodia attaching to the artery from the
286 outside in the connective tissue of the filament. In
287 addition to spore morphology, *M. balatonicus* n. sp.
288 differs from the species discussed above and in its 18S
289 rDNA sequences and site selection in the gills.

Discussion 290

291 To date, the occurrence of six *Myxobolus* species (*M.*
292 *basilamellaris*, *M. cyprini*, *M. cyprinicola*, *M. dispar*,
293 *M. encephalicus* and *M. intrachondrealis*), all specific

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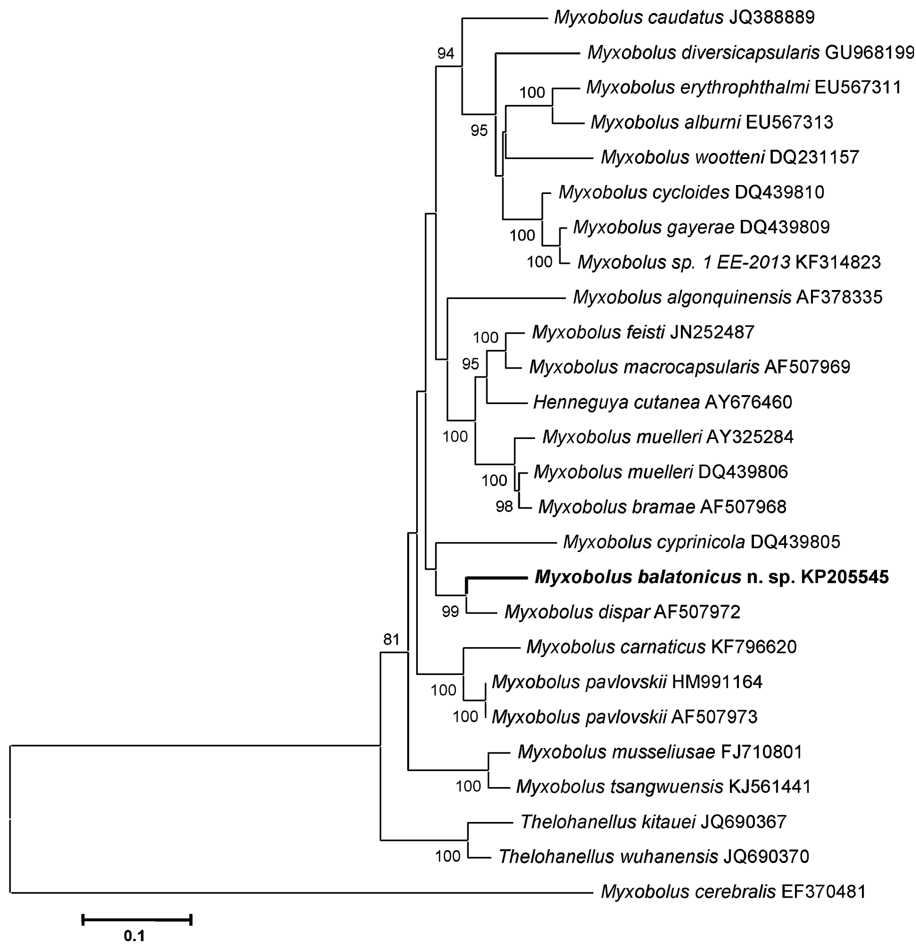

Fig. 6 Phylogenetic position of *Myxobolus balatonicus* n. sp. from common carp. The tree was constructed on the basis of the 18S rDNA sequences using maximum likelihood analysis. *Myxobolus cerebralis* was used as the outgroup. Bootstrap values <70 were omitted. The scale-bar indicates the expected number of substitutions per site

to the common carp, are known from Hungary (Lom & Molnár, 1983; Molnár & Kovács-Gayer, 1985; Molnár et al., 1999; Molnár, 2000, 2002a; data on the common occurrence of *M. encephalicus* were provided by personal communication of Dr. György Csaba). Of these, *M. cyprini* and *M. cyprinicola* were reported also from Lake Balaton (Molnár & Székely 1995; Molnár 2002a). Székely & Molnár (1997) also observed *M. cyprini* along with *M. dispar* in the kis-Balaton water reservoir. Another species, *M. dogieli* Bykhovskaya-Pavlovskaya & Bykhovski, 1940, originally described from the common carp, has also been described from the common bream *Abramis brama* L. in Lake Balaton (Molnár et al., 2008). All of these species have a well-defined organ and tissue specificity. Three of them (*M. basilamellaris, M. dispar* and *M. intrachondrealis*) were known to infect the gills of carp. However, *M. basilamellaris* forms plasmodia only at the base of the gill filaments, the plasmodia of *M. intrachondrealis* develop inside the cartilage of the gill arch, and only the plasmodia of *M. dispar* develop inside the arteries of the gill filaments. Despite the common location, the two species developing on the gill filaments (*M. dispar* and *M. balatonicus* n. sp.) can be easily distinguished from each other by the shape of their plasmodia. The plasmodia of *M. dispar* are large and have an elongated shape, in contrast with the oval-shaped plasmodia of *M. balatonicus* n. sp. The two species differ from each other also in spore morphology. The polar capsules of *M. dispar* have different sizes whereas those of *M. balatonicus* n. sp. are equal in

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size. Moreover, the 18S rDNA sequence of the new species described here is clearly distinguishable from those of all other known species. Due to the relatively low prevalence of *M. balatonicus* n. sp. its pathological significance cannot be established, although it seems to be obvious that plasmodia filling the lumen of filament arteries obstruct the blood flow. The observation that the passage of the blood was ensured through a narrow gap between the plasmodium and the wall of the arteries suggests that blocking of arteries is not complete. Although the common carp is a fish species with worldwide distribution, Froufe et al. (2002) and Molnár (2009) suppose that it is a fish of Asian origin, and until the intensive fish transfers its parasite fauna had been restricted to a few specific parasites. After the introduction of the Amur wild common carp to the European part of Russia and the regular imports of the coloured carp from Asia, the parasite fauna of the common carp is gradually expanding and new species hitherto known only in China, Japan and the Amur Basin might also appear in the common carp in Europe.

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