First Central European record of the fungus Prolixandromyces triandrus Santam. (Ascomycota: Laboulbeniales), a parasite of veliid bugs (Heteroptera: Veliidae), with notes on its biology and DNA barcoding

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First Central European record of the fungus *Prolixandromyces triandrus* Santam. (Ascomycota: Laboulbeniales), a parasite of veliid bugs (Heteroptera: Veliidae), with notes on its biology and DNA barcoding

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**ABSTRACT**

A new distribution record for *Prolixandromyces triandrus* Santam. (Ascomycota: Laboulbeniales) is presented from the Bük Mountains in northeastern Hungary, from the host species *Velia* (*Plesiovelia*) *saulii* Tamanini, 1947 (Heteroptera: Veliidae). Hitherto, this fungal parasite had only been observed in the western Mediterranean region and the Macaronesia Archipelago. *Prolixandromyces. triandrus* seems to be abundant in the reported Hungarian host population. Additionally, ribosomal DNA barcodes for this fungal species are also presented. Incidence of the parasite and potential of the molecular investigations of host-parasite relationships of this ectoparasitic fungus is discussed. A brief review is given of known hosts of *P. triandrus* and of Laboulbeniales from aquatic/semiaquatic insect hosts in Hungary.

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**KEYWORDS**

Laboulbeniales; Heteroptera; Veliidae; ribosomal DNA; Hungary

**Introduction**

The Laboulbeniales (Ascomycota: Laboulbeniomycetes) are an order of specialised fungi with an ectoparasitic lifestyle on arthropods — mainly Coleoptera, but also other orders and classes across three subphyla (Haelewaters 2012; Haelewaters et al. 2015a). In the most recent comprehensive work on the microscopic fungi of Hungary, which listed previously known and also unpublished species based on herbarium collections (Bánhegyi, Tóth, Ubrizsy, and Vörös 1985), altogether 88 species of the Laboulbeniales were recorded. Since then, data on this obscure fungal group have been scarce in Hungary (e.g., Huldén 1985; Majewski 2008; Pfliegler 2014; Báthori, Pfliegler, and Tartally 2014, 2015).

Broad-shouldered water striders (Heteroptera: Veliidae) are known as hosts for Laboulbeniales (Benjamin 1970; Weir 2008). In Hungary, thus far no Laboulbeniales have been observed on Veliidae. In this study, we screened specimens of this host family for the presence of Laboulbeniales in the country. So far in Hungary, the only reported
Laboulbeniales fungi infecting Heteroptera are Coreomyces elongatus Speg. and C. italicus Speg. on members of Corixidae, another aquatic heteropteran family (Bánhegyi et al. 1985). Considering aquatic/semiaquatic host insects, a much higher number of Laboulbeniales species has been recorded on aquatic beetles (families Dryopidae, Dytiscidae, Gyrinidae, Haliplidae, and Hydrophilidae) from the country (summarised in Table 1).

The genus Prolixandromyces R.K. Benj. was established by Benjamin (1970) for two species parasitic on species of Veliidae from the New World. The description of Prolixandromyces was based on two distinguishing characteristics: the superposition of the basal and suprabasal cells of the receptacle and the very long antheridial tubes formed distally on a three-celled appendage (four-celled in Prolixandromyces triandrus) (Benjamin 1970; Weir 2008). Weir (2008) placed Prolixandromyces in the Stigmatomyceteae tribe of the Laboulbeniaceae family, characterised by a simple three-celled receptacle with the basal cell forming the foot, the suprabasal cell giving rise typically to a single, stalked perithecium and the terminal cell subtending a few- to many-celled antheridial appendage. After the description of P. corniculatus R.K. Benj. and P. veliae R.K. Benj., the number of known species rose to five (Benjamin 1981), all recorded from Velia spp. from Central America. The first and only European species, P. triandrus, was described from Spain on the host Velia (Plesiovelia) caprai Tamanini, 1947 (Santamaría 1988). Since then, it has been collected on the same host in Portugal (Santamaría 1992), France (Santamaría, Balazuc, and Tavares 1991), and Morocco (Santamaría and Rossi 1999); on Velia (Plesiovelia) lindbergi Tamanini, 1954 in the Canary Islands (Santamaría et al. 1991); on Velia (Plesiovelia) sarda Tamanini, 1947 in Sardinia; and on Velia (Plesiovelia) saullii Tamanini, 1947 in mainland Italy (Santamaría and Rossi 1999). Two further Old World species of the genus have also been described recently from Africa and East Asia on other Veliids (Weir 2008).

Table 1. Review of Laboulbeniales on aquatic/semiaquatic insect (Coleoptera and Heteroptera) hosts recorded from Hungary (only hosts recorded in the corresponding references are listed).

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Host species (in Hungary)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoicomyces aquatilis (F. Picard)</td>
<td>Hydrochus spp. (Hydrophilidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Autoicomyces crassus Speg.</td>
<td>Berosus spp. (Hydrophilidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Autoicomyces melanocerus Speg.</td>
<td>Dryopus spp. (Dytiscidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Cantharomyces italicus Speg.</td>
<td>Bidessus spp., Hygrotus spp. (Dytiscidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Chitonomyces bidessarius Thaxt.</td>
<td>Bidessus spp., Hygrotus spp. (Dytiscidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Chitonomyces hydropori Thaxt.</td>
<td>Hydroporus spp., Hygrotus spp. (Dytiscidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Chitonomyces melanurus Peyr.</td>
<td>Laccophillus spp. (Dytiscidae)</td>
<td>Balazuc (1974), Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Chitonomyces paradoxxus (Peyr.) Thaxt.</td>
<td>Laccophillus spp. (Dytiscidae)</td>
<td>Balazuc (1974), Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Chitonomyces truncatus Speg.</td>
<td>Laccophillus spp. (Dytiscidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Helodiomyces elegans F. Picard</td>
<td>Dryopus spp. (Dytiscidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Hydraeomyces halipli (Thaxt.) Thaxt.</td>
<td>Haliplus spp. (Haliplidae)</td>
<td>Bánhegyi (1950), Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Laboulbenia gynincola Speg.</td>
<td>Gymirus spp. (Gyrinidae)</td>
<td>Bánhegyi, (1940), Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Misgomyces coneglanensis (Speg.) Thaxt.</td>
<td>Laccobius spp. (Hydrophilidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Prolixandromyces triandrus Santam.</td>
<td>Velia saullii Tamanini, 1947 (Veliidae)</td>
<td>This study, Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Rhyynchophoromyces anacaea (Fabricius, 1792)</td>
<td>Anacaena limbata (Fabricius, 1792) (Hydrophilidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
<tr>
<td>Scheloske</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhyynchophoromyces rostratus (Thaxt.)</td>
<td>Enochus spp., Helochares spp. (Hydrophilidae)</td>
<td>Bánhegyi et al. (1985)</td>
</tr>
</tbody>
</table>
Material and methods

Morphology

Host specimens were collected with an entomological net, killed, stored in 80% ethanol, and then examined under a stereomicroscope. For determination, Weir’s work (2008) was used. The sex of the host and the presence/absence of fungal thalli were recorded. An infected specimen was photographed with a DSLR camera with macro set. Several thalli were prepared in Heinz PVA mounting medium for microscopic observations. Light microscopy images were taken with an Olympus BD40 microscope equipped with an Olympus 40× lens and a digital microscope camera, with the Olympus DP Controller software. Images were stacked using ZereneStacker software and enhanced in Photoshop (Adobe). Infected specimens have been put in storage in 80% ethanol at the Department of Evolutionary Zoology and Human Biology, University of Debrecen (Debrecen, Hungary). One microscopic slide has been deposited in the Botanical Collection of the Hungarian Natural History Museum (Budapest, Hungary).

Morphometrics

Differences in thallus dimensions and shapes were measured following De Kesel and van den Neucker (2005) and De Kesel and Haelewaters (2014) with some modifications. We measured eight variables from 20 mature intact thalli randomly chosen from altogether five host specimens using the microscope set-up described above. These parameters were used (abbreviations and explanations are given between parentheses): total thallus length (TL, from foot to perithecial apex); length of the receptacle (RL); perithecium length (PL, without cell VI); perithecium width (PW); height of cell I (cell I); height of cell II (cell II); height of cell III (cell III); and length of the appendages (App). Some ratios were also calculated: PL/PW; TL/PL; TL/App; TL/RL. In general, the morphology of the specimens was compared to the specimens from V. (P.) caprai from Spain described in detail by Weir (2008).

DNA isolation and barcoding

DNA was isolated from five thalli according to the microwave extraction protocol of Haelewaters et al. (2015b): thalli were placed in 0.5-mL PCR tubes, and microwave-treated (750 W for 5 min). Subsequently 50 μL ddH$_2$O was added to the tube, and the thalli were manually crushed with a sterile pipette tip while viewed under a dissecting microscope. The PCR tubes were then incubated at $-20^\circ$C for 10 min. For each PCR reaction, 10 μL of the extracted material was used. PCR was carried out using the primer pairs NS1–NS4 (White, Bruns, Lee, and Taylor 1990), LR0R–LR5 (Vilgalys and Hester 1990), ITS1f–ITS4_kyo1 (Gardes and Bruns 1993; Toju, Tanabe, Yamamoto, and Sato 2012) for ribosomal small (SSU) and large (LSU) subunits, and ITS1-5.8S-ITS2 (ITS) regions, respectively, as described in Haelewaters et al. (2015b). PCR protocols were the following: 95°C for 5 min, 30×(94°C 50 s, Tm 50 s, 72°C 50 s), 72°C 5 min. Tm was set to 50°C for primers NS1–NS4, and 55°C for primers LR0R–LR5 and ITS1f–ITS4_kyo1. We used an Applied Biosystems 2720 thermal cycler and final volume of 50 μL. PCR products were loaded onto 1.4% agarose gels for electrophoresis at 100 V for 15 min and UV
transillumination was used to check the product size. With our PCR conditions, it was possible to obtain single, specific bands of products. PCR products were cleaned with a Geneaid DF100 PCR cleaning kit and sequenced in both directions. The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems) was used for sequencing. Cycle sequencing PCR was performed according to the manufacturer’s protocol. Products of the sequencing reactions were purified using post-reaction clean-up columns (Sigma-Aldrich). Capillary sequencing was performed on an ABI Prism 3100-Avant Genetic Analyser instrument (Applied BioSystems). Sequences were trimmed and manually edited at ambiguous sites. Edited sequences were deposited in ENA (European Nucleotide Archive).

Results

Prolixandromyces triandrus Santam. (Figures 1A, B)

Material examined
65 thalli, Hungary, Heves County, Nagyvisnyó (48°09′02.4″N 20°26′54.6″E), small brook, on a female Velia (Plesiovelia) saullii specimen (on the legs and antennae), 15.VII.2015, leg. J.F. 207 thalli, Hungary, Heves County, Nagyvisnyó (48°09′02.4″N 20°26′54.6″E), small brook, on altogether 11 male and 12 female V. (P.) saullii specimens (on the legs and antennae in both sexes), 9.IX.2015, leg. J.F.

Remarks
Several thalli from the host specimen collected on 15 September 2015 were mounted on a microscope slide, which have been deposited at BP (slide number: 107914). DNA barcode sequences from the same material were deposited in ENA with the following accession numbers: SSU (LT158294), LSU (LT158295), ITS (LT158296).

Figure 1. (Colour online) Prolixandromyces triandrus Santam. on Velia (Plesiovelia) saullii Tamanini, 1947 host (a) and single thalli (b) with transmitted light microscopy (scale: 200 μm).
Prevalence of infection

In this study, we collected and examined altogether 50 V. saulii specimens and almost half of these proved to be infected by P. triandrus (11 males and 13 females, while 15 males and 11 females were uninfected). The level of infection varied across host specimens, ranging from few to dozens of thalli. Thalli were found only on the legs and antennae of the hosts, not on their bodies. Figure 2 shows the recorded numbers of thalli on host specimens.

Morphological description and comparison of the Bükk population

Characters of the P. triandrus specimens collected in the Bükk Mountains matched the morphological data presented by Weir (2008) for Spanish specimens: thalli are erect, nearly straight, orange brown (Figure 1), with a total length ranging from 218 to 358 \( \mu \text{m} \), with only four thalli smaller than the range of 295–420 \( \mu \text{m} \) measured in the reference above. The receptacle is relatively short, on average 1/5.6 of total length (compared to \(~1/6\) in the reference). Its average length of 52.9 \( \mu \text{m} \) falls between the range of 42 and 70 \( \mu \text{m} \) given for the Spanish specimens. The average length of cell I was smaller (33.4 \( \mu \text{m} \)) than the range in the reference (45–55 \( \mu \text{m} \)), while cells II and III fall between Weir’s (2008) ranges. The arrangement and shape of cells I, II, III, and VI correspond to the reference. Appendage: the length of the appendage was more variable (44–132 \( \mu \text{m} \); 89.8 \( \mu \text{m} \) on average) than the range measured for the Spanish specimens (98–105 \( \mu \text{m} \)), but its arrangement and shape match the referenced description: basal cell triangular, superposed above by a parallel-sided cell (both sterile), the uppermost subtending two long and thin cells that separate the three antheridia. Perithecium: its shape matches the description in the reference, with more variable length (170–309 \( \mu \text{m} \), on average 238 \( \mu \text{m} \)) than in the reference (258–275 \( \mu \text{m} \)). It is broadest near the middle and 41–55 \( \mu \text{m} \) wide (average 58.7 \( \mu \text{m} \); this range falls between 38 and 60 \( \mu \text{m} \) in the reference). Details of the measurements of the Bükk specimens are given in Table 2.

Figure 2. Numbers of Prolixandromyces triandrus Santam. thalli on female (×) and male (○) host specimens of Velia (Plesiovelia) saulii Tamanini, 1947.
Discussion

The occurrence of *P. triandrus* in Hungary represents the first record of this fungus from Central Europe and the second host record from *V. (P.) saulii*. The morphology of the thalli collected in this study matches the description of Spanish specimens from *V. (P.) caprai* described by Weir (2008) in general, with the Hungarian specimens being in the smaller lower part of the range given in the reference, have a shorter cell I, and a perithecium and appendage more variable in size than the Spanish specimens. The newly generated DNA sequences of *P. triandrus* will prove useful in Laboulbeniales molecular research, since for the genus *Prolixandromyces*, which is suggested to belong in the Stigmatomycetae (Weir 2008), no sequences were available to date. As the currently known range of the species extends from the Canary Islands (where it is associated with the endemic *V. lindbergi* host) to Hungary and several hosts with often non-overlapping distributions are known, the comparison of possible sequence and morphological divergence between different genetic lineages of *P. triandrus* represents a direction for further study. The existence of host-specific and/or geographically restricted distinct lineages (or even cryptic species with low morphological divergence) may be possible and biogeographically interesting, as recently reported in the genus *Hesperomyces* infecting different ladybird (Coccinellidae) species (Haelewaters and van Wielink 2016).

Our data of infected hosts may enable some speculations on the biology of *P. triandrus*. The number of thalli per host was variable both in female and male bugs and about half of the specimens were infected in both sexes (42% of males and 54% of females). Highly infected hosts (with more than 20 thalli) were rare, but recorded in both sexes. Position specificity driven by the mode of the copulation of the host has been described in some aquatic Laboulbeniales parasitizing beetles (Scheloske 1976a, 1976b; Goldmann and Weir 2012), but we did not find support for a marked position effect. Semiaquatic bugs of the superfamily Gerroidea mate with the male on top of the female, while the male’s legs may come into contact with the female’s legs and antennae (e.g., Arquist 1988; Kovac and Krocke 2013). However, all infected specimens in our collection had the thalli of *P. triandrus* attached to one or more of the legs and/or antennae in both sexes and the fungus never occupied the host’s abdominal surface, which theoretically would be a favourable place for transmission during copulation.

### Table 2. Morphometrics of *Prolixandromyces triandrus* Santam. from Bükk Mountains. Parameters are described in the section ‘Material and methods’.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean (μm)</th>
<th>Std. dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>294.15</td>
<td>32.12</td>
</tr>
<tr>
<td>RL</td>
<td>52.91</td>
<td>4.51</td>
</tr>
<tr>
<td>PL</td>
<td>238.01</td>
<td>33.73</td>
</tr>
<tr>
<td>PW</td>
<td>58.74</td>
<td>6.55</td>
</tr>
<tr>
<td>Cell I</td>
<td>33.39</td>
<td>2.56</td>
</tr>
<tr>
<td>Cell II</td>
<td>28.78</td>
<td>2.63</td>
</tr>
<tr>
<td>Cell III</td>
<td>28.10</td>
<td>3.41</td>
</tr>
<tr>
<td>App</td>
<td>89.84</td>
<td>15.87</td>
</tr>
<tr>
<td>PL/PW</td>
<td>4.05</td>
<td>0.29</td>
</tr>
<tr>
<td>TL/PL</td>
<td>1.24</td>
<td>0.06</td>
</tr>
<tr>
<td>TL/App</td>
<td>3.39</td>
<td>0.87</td>
</tr>
<tr>
<td>TL/RL</td>
<td>5.60</td>
<td>0.82</td>
</tr>
</tbody>
</table>

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Disclosure statement

No potential conflict of interest was reported by the authors.

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