1 **Molecular and morphological characterization of the cercariae of *Lecithodendrium***
2 ***linstowi* (Dollfus, 1931), a trematode of bats, and incrimination of the first intermediate**
3 **snail host, *Radix balthica***
4

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14 Running title: Cercariae of *Lecithodendrium linstowi*
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SUMMARY

Lecithodendrium linstowi is one of the most prevalent and abundant trematodes of bats, but the larval stages and intermediate hosts have not been identified. We present the first molecular and morphological characterization of the cercariae of *Lecithodendrium linstowi* based on phylogenetic analysis of partial fragments of LSU and ITS2 rDNA. The first intermediate host was incriminated as *Radix balthica* by DNA barcoding using cox1 and ITS2 sequences, although the snail morphologically resembled *R. peregra,* emphasising the requirement for molecular identification of lymnaeids as important intermediate hosts of medical and veterinary impact. The application of molecular data in this study has enabled linkage of life cycle stages and accurate incrimination of the first intermediate host.

Key words: *Lecithodendrium linstowi*, xiphidiocercariae, *Radix balthica*, bats, LSU, ITS2, cox1

39 KEY FINDINGS

40 First molecular and morphological identification of the cercariae of *Lecithodendrium linstowi*

41 Description of the cercariae of *L. linstowi* using light and scanning electron microscopy

42 First molecular incrimination of *Radix balthica* as intermediate host of *L. linstowi*

43 Recommendation to screen intermediate hosts for digeneans and their endosymbiont

44 *Neorickettsia*

45 INTRODUCTION

46 Trematode life cycles are complex, usually employing multiple hosts, and often with low
47 specificity in the definitive vertebrate host. Resolution of their life cycles is therefore
48 challenging, requiring direct linkage of morphologically distinct larval stages such as cercariae
49 with adults (Brant *et al*. 2006). Furthermore, life cycle elucidation in the laboratory can be
50 technically and ethically problematic. DNA sequencing and the development of databases with
51 species-specific reference DNA sequence data have enabled larval and adult trematodes to be
52 matched and hosts accurately incriminated, thus informing taxonomy, biodiversity and
53 epidemiology (Brant *et al*. 2006).
54
55 The Lecithodendriidae (Digenea: Plagiorchiida) are a prime example of taxonomic uncertainty
56 due to missing links between larval and adult stages. These parasites infect insectivorous
57 vertebrates and typically use prosobranch molluscs as first intermediate hosts. The emergent
58 cercariae encyst as metacercariae in aquatic insect larvae, which are later ingested as adult
59 insects by foraging definitive hosts. More detailed life cycle elucidation exists for only a few
60 species (reviewed in Kudlai *et al*. 2015) making it difficult to assess the diversity of these
61 parasites and their contribution to trematode communities in host populations. In addition,
62 identification to species level is important as lecithodendriids are common hosts of intracellular
63 endosymbiotic *Neorickettsia* bacteria (Rickettsiales, Anaplasmataceae), which can cause
64 debilitating and sometimes fatal diseases in vertebrates, including humans (Greiman *et al*. 2017).
65
66 Published reports on Lecithodendriidae in the UK are limited to early morphological studies (e.g.
67 Nicoll, 1923; Brown, 1933) and a detailed study of gastrointestinal *Lecithodendrium* spp. in

68 pipistrelle bats by Lord *et al.* (2012) who used molecular analysis to revise phylogenetic
69 relationships between lecithodendriid species. Otherwise, there are morphological reports of
70 xiphidiocercariae under provisional names such as *Cercaria helvetica* XII Dubois, 1928 (Nasir
71 and Erasmus, 1964), now known to be phylogenetically close to, but not identical to, *L. linstowi*
72 (Kudlai *et al*. 2015), illustrating the importance of molecular confirmation. Here, we report the
73 first identification of the cercariae of *L. linstowi* using molecular and morphological approaches
74 and molecular incrimination of the snail intermediate host. The cercariae were collected during
75 the UK Digenean Diversity Project, a molecular study of digeneans infecting freshwater snails in
76 the UK.
77
78 MATERIALS AND METHODS
79 *Collection and screening of snails*
80 Eighty-three *Radix* sp. (Lymnaeidae) snails were collected by hand net from the Queen's River,
81 Bushy Park, Surrey, England (51°24'42"N; 0°20'27"W) in September 2013. This artificial river
82 was created in the 17th century to bring water from the Colne River to Hampton Court Palace
83 (Bushy Park Management Plan, The Royal Parks, 2014, unpublished). Snails were individually
84 placed in 50 ml glass beakers containing filtered, dechlorinated water and were screened for
85 emergent cercariae by microscopy in the laboratory. Only one snail, identified as *R. peregra*
86 based on shell morphology, was observed to shed xiphidiocercariae.
87
88 *Morphological description of cercariae*
89 Cercariae were fixed in 4% formaldehyde solution and stored in 70% ethanol prior to processing.
90 Cercariae examined by light microscopy were stained with acetocarmine, dehydrated in a graded

91 ethanol series, cleared in HistoChoice (Sigma-Aldrich, UK) and mounted in Canada balsam.
92 Image capture and measurements of cercariae were made using a Nikon Eclipse NiE microscope
93 and NIS-Elements BR (Nikon Instruments, UK) software. Cercariae examined by scanning
94 electron microscopy were dehydrated in a graded ethanol series, dried in hexamethyldisilazane,
95 attached to stubs using double sided tape, sputter coated with gold palladium and examined
96 under a Zeiss EVO 50 scanning electron microscope. Upon confirmation of species, parasite
97 reference material was deposited at the Natural History Museum, London, UK (accession
98 numbers NHMUK 2017.6.15.1-2).
99
100 *Molecular analysis*
101 Total genomic DNA was isolated from a pool of ten 96% ethanol-fixed morphologically
102 identical cercariae using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc.) following the
103 manufacturer's protocol. PCR was performed to amplify partial fragments of the large ribosomal
104 subunit (LSU) using primers LSU28S (forward; TAGGTCGACCCGCTGAAYTTAAGCA) and
105 1500R (reverse; GCTATCCTGAGGGAAACTTCG) as described by Olson *et al.* (2003). The
106 internal transcribed spacer region (ITS) was amplified using primers; p1 (forward;
107 GTCGTAACAAGGTTTCCGTAGGTG) and p2 (reverse;
108 TATGCTTAAATTCAGCGGGTAATC) according to Wang *et al.* (2009).
109
110 In order to accurately identify the *Radix* species acting as an intermediate host, DNA was also
111 extracted from a tissue snip from the foot of the infected snail using the same methods described
112 above, but with an extended 24 h initial digest. A partial fragment of the mitochondrial
113 cytochrome c oxidase 1 gene (cox1) was amplified with PCR using primers LCO1490

114 (GGTCAACAAATCATAAAGATATTGG) and HCO2198

115 (TAAACTTCAGGGTGACCAAAAAATCA) using protocols described by Folmer *et al.* (1994)

116 and the ITS2 region was amplified using primers NEWS (TGTGTCGATGAAGAACGCAG)

117 and RIXO (TTCTATGCTTAAATTCAGGGG) (Almeyda-Artigas *et al.* 2000).

118

119 PCR amplicons generated from both the cercariae and the snail were visualized in 1% agarose

120 gels stained with gel red (Bioline™) prior to sequencing using the same PCR primers with

121 Fluorescent Dye Terminator Sequencing Kits (Applied Biosystems™) run on an Applied

122 Biosystems™ 3730XL automated sequencer. Resultant sequences were assembled in

123 BioEdit (Hall, 1999) and corrected manually to resolve ambiguous base calls. BLASTn searches

124 were performed at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to provide initial

125 identification and to ensure no contamination and sequences were submitted to GenBank

126 (accession numbers: MF498820- MF498823).

127

128 *Phylogenetic analysis*

129 The MUSCLE algorithm (http://www.ebi.ac.uk) was used to align the generated sequences with

130 retrieved GenBank sequences: (i) for lecithodendriid spp., *Maritrema* spp., *Microphallus* spp.

131 and *Collyriclum faba* were used as out-groups; (ii) for *Radix* spp., *Lymnaea stagnalis* was used

132 as the outgroup. Since most of the available lecithodendriid sequences on GenBank were ITS2,

133 the complete ITS sequence from this study and other retrieved sequences were trimmed to the

134 ITS2 fragment prior to analysis.

135

136 Neighbour joining (NJ) and maximum likelihood (ML) methods were employed to perform
137 phylogenetic reconstruction for the parasite and the snail species using MEGA v6 (Tamura *et al*.
138 2013). For the xiphidiocercariae, NJ trees based on ITS2 and LSU were constructed under the
139 conditions of the Kimura 2 parameter model (K2P). Based on the lowest Bayesian information
140 criterion, MEGA6 identified that the K2P model with a gamma distribution best fit the ITS2 and
141 LSU data thus both ML analyses were performed under the conditions of this model. For *Radix*
142 spp., the NJ analysis for both the ITS2 and cox1 were performed under the conditions of the K2P
143 model, but the ML analysis was performed using the Tamura 3 parameter with gamma
144 distribution for ITS 2 and the Hasegawa-Kishino-Yano with gamma distribution for cox1. In all
145 analyses nodal support values were estimated using 1000 bootstrap replicates.
146
147 RESULTS
148 Morphological description of *Lecithodendrium linstowi* cercariae
149 The body was oval-elongate and very contractile, usually longer than the tail (Table 1, Figs 1A-
150 C). The oral sucker was sub-terminal, round-oval with a small central stylet (Fig. 1B, D). The
151 ventral sucker was round-oval, located posterior to the mid-body (Figs 1C,E). Fine spines and
152 type 1 sensory papillae with tegumental collars covered the body tegument (Figs 1D-F). The tail
153 was simple with indented margins, without a finfold, spines or sensory papillae (Fig. 1G). Three
154 pairs of penetration gland cells filled with granules were located anterior to the ventral sucker
155 with ducts opening anteriorly either side of stylet. The pharynx was small and the intestinal tract
156 was indistinct. The v-shaped excretory vesicle was thin-walled ending in a sub-terminal
157 excretory pore. Numerous cystogenous cells and refractile granules obscured structures in the
158 body (Fig 1B).

159 *Molecular and phylogenetic analysis*

160 The xiphidiocercariae sequences were 930 base pairs (bp) long for the complete ITS (365 bp
161 after trimming to the ITS2 fragment) and the partial fragments of the LSU were 1110 bp.
162 BLAST searches on GenBank and pairwise *p*-distance comparisons of ITS2 and LSU sequences
163 demonstrated that the cercariae were an exact match to *L. linstowi*. Phylogenetic analyses based
164 on the ITS2 and LSU alignments for NJ and ML showed that the novel sequences clustered with
165 *L. linstowi* adult sequences from bats and formed a clade with *Lecithodendrium* sp. cercariae
166 (syn. *Cercaria helvetica* XII Dubois, 1928) (Fig. 2A). Comparison of uncorrected pairwise
167 genetic distance (*p*-distance) between both species using MEGA v6 revealed greater genetic
168 divergence in ITS2 (0.014, 1.4%) than LSU (0.006, 0.6%).
169
170 The generated ITS2 and cox1 sequences for the snail were 440 and 570 bp respectively.
171 Phylogenetic analysis based on both molecular markers and NJ/ML methods (Fig. 2B) produced
172 congruent hypotheses regarding the placement of the novel sequences from this study. Three
173 main sub-clades of *Radix* spp. were observed: *R. ampla* + *R. lagotis*; *R. labiata* + *R. auricularia*
174 and *R. balthica* in the ITS2 tree and *R. ampla* + *R. labiata; R. auricularia* and *R. balthica* in the
175 cox1 tree. In both trees the sequence from this study clustered within the *R. balthica* clade (Fig.
176 2B).
177
178 DISCUSSION

179 We report the first molecular and morphological identification of the cercariae of *L. linstowi* and
180 incrimination of *R. balthica* as the molluscan first intermediate host. The rDNA LSU and ITS2
181 data confirm that the xiphidiocercariae in this study were *L. linstowi* based on 100% sequence

182 similarity to adults from *Nyctalus noctula* (common noctule) in the Ukraine (Tkach *et al*., 2000)
183 and *Pipistrellus pipistrellus* in the UK (Lord *et al*. 2012). Phylogenetic analysis of ITS2 and
184 cox1 identified the snail host of *L. linstowi* as *R. balthica*, although it morphologically resembled
185 *R. peregra,* and therefore further supports synonymy of *R. balthica* with *R. peregra* as proposed
186 by Bargues *et al*. (2001) and Lawton *et al*. (2015). The data emphasises the need for molecular
187 identification of lymnaeid snails to determine their role as intermediate hosts in the life cycles of
188 digeneans, particularly those of medical and veterinary importance.
189
190 *Lecithodendrium linstowi* is a generalist trematode species that is one of the most prevalent and
191 abundant helminths of Eurasian bats (Esteban *et al*. 2001; Lord *et al*. 2012) and also infects the
192 Hungarian harvest mouse (*Micromys minutus pratensis*) (Matskási, 1971). Its prevalence can be
193 partly explained by the ubiquity of *R. balthica*. Adults of *L. linstowi* were first reported in the
194 UK by Lord *et al*. (2012) from the duodenum and upper jejunum of pipistrelle bats (*P.*
195 *pipistrellus* and *P. pygmaeus*). Bushy Park is an important bat habitat with nine bat species
196 recorded since 2004 (Bushy Park Management Plan, The Royal Parks, 2014 unpublished) so
197 further lecithodendriid species are likely to exist at this location, particularly since *L. linstowi* is
198 commonly associated with *L. spathulatum* which probably shares aquatic insect larvae hosts
199 (Lord *et al*. 2012). There is no evidence available for negative health impacts of lecithodendriid
200 species on bat hosts.
201
202 Phylogenetic reconstruction illustrates a well-supported relationship between cercariae and
203 adults of *L. linstowi* (Tkach *et al*. 2000; Lord *et al*. 2012) and confirms the separate
204 *Lecithodendrium* clade proposed by Lord *et al*. (2012). Analysis of p-distance estimates of

205 divergence verify that *L. linstowi* and *Lecithodendrium* sp. (syn. *Cercaria helvetica* XII Dubois,
206 1928) from *Bithynia tentaculata* (Kudlai *et al.* 2015) are closely related separate species. The
207 observed differences were within levels usually recorded among closely related congeneric
208 species such as *Echinostoma caproni* and *E. paraensei* (Vilas *et al*. 2005). Both species have
209 non-virgulate, morphologically similar xiphidiocercariae, although *L. linstowi* is smaller (Table
210 1). The lack of a virgula organ in *L. linstowi* demonstrates that this trait is not an absolute
211 synapomorphy for lecithodendriids (Lotz and Font, 2008) and cannot be used as a broad
212 phylogenetic characteristic.
213
214 The application of molecular approaches in the current study has enabled taxonomic linkage of
215 cercariae of *L. linstowi* to adult stages without attempting life cycle elucidation, and accurate
216 incrimination of the snail host, thus emphasising the essential role of DNA sequencing in
217 understanding digenean life cycles. Future molecular studies will be required to identify the
218 second intermediate hosts of *L. linstowi* to achieve resolution of its life cycle. The intermediate
219 host species for many bat parasites are unknown and the lack of reference material and DNA
220 sequence data hinders an understanding of parasite biodiversity in bats. As highlighted by Lord
221 and Brookes (2014), protected species status in the UK means that bats, unless dead or
222 euthanized due to injury, cannot be directly examined. Molecular based surveys of first and
223 second intermediate hosts are therefore important for long term monitoring of parasitic infections
224 in endangered bat populations and other vertebrates and the identification of emerging zoonoses.
225 Lecithodendriidae in bats have been identified as hosts of *Neoricketssia* in Egypt, the
226 Philippines, Thailand, North and South America. *Neoricketssia* are vertically transmitted
227 through the parasite life cycle, but can be horizontally transmitted to vertebrate hosts and cause

228 disease (Greiman *et al*. 2017). *Lecithodendrium* sp. harbours *Neoricketssia risticii*, which causes
229 the debilitating and sometimes fatal disease equine monocytic ehrlichiosis (Potomac horse fever)
230 in the Americas. Horses are probably infected through inadvertently consuming metacercariae in
231 insect hosts, while grazing or drinking (reviewed in Vaughan *et al*. 2012). It is therefore
232 important to screen accessible intermediate hosts for both digeneans and their endosymbiont
233 bacteria to provide new insights into neorickettsial-digenean epidemiology.
234

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239

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243

244

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315 **Figure legends**

316

317 Fig. 1 Cercariae of *Lecithodendrium linstowi* from *Radix balthica*. (A)-(C) Entire cercaria. (A)
318 line drawing, scale bar = 25 µm. (B) Photomicrograph, stylet (black arrow), penetration glands
319 (stippled arrows), ventral sucker (white arrow), scale bar = 25 µm. (C) Scanning electron
320 micrograph, scale bar = 20 µm. (D)-(G) Scanning electron micrographs showing characteristic
321 features, including spinose body tegument. (D) Subterminal oral sucker, stylet detached during
322 processing, scale bar = 2 µm. (E) Ventral sucker, sensory papillae (arrows), scale bar = 2 µm. (F)
323 Junction of body with tail, sensory papillae (arrows), scale bar = 5 µm. (G) Simple tail, scale bar
324 = 10 µm.

325

326 Fig. 2 Phylogenetic identification of *Lecithodendrium linstowi* and *Radix balthica*. (A)
327 Phylogenetic reconstructions based on (i) ITS2 and (ii) LSU sequences of Lecithodendriidae
328 used for the identification of xiphidiocercariae infecting *Radix balthica* from Bushy Park, Surrey,
329 UK. (B) Phylogenetic reconstructions based on (i) ITS2 and (ii) cox1 sequences used for
330 identification of *Radix balthica* from Bushy Park, Surrey, UK. Trees were constructed using the
331 Maximum Likelihood method. The scale shows the number of nucleotide substitutions per site
332 between sequences. The nodal support is given in NJ and ML bootstraps respectively and shows
333 values >50%. Sequences from this study are indicated in bold.

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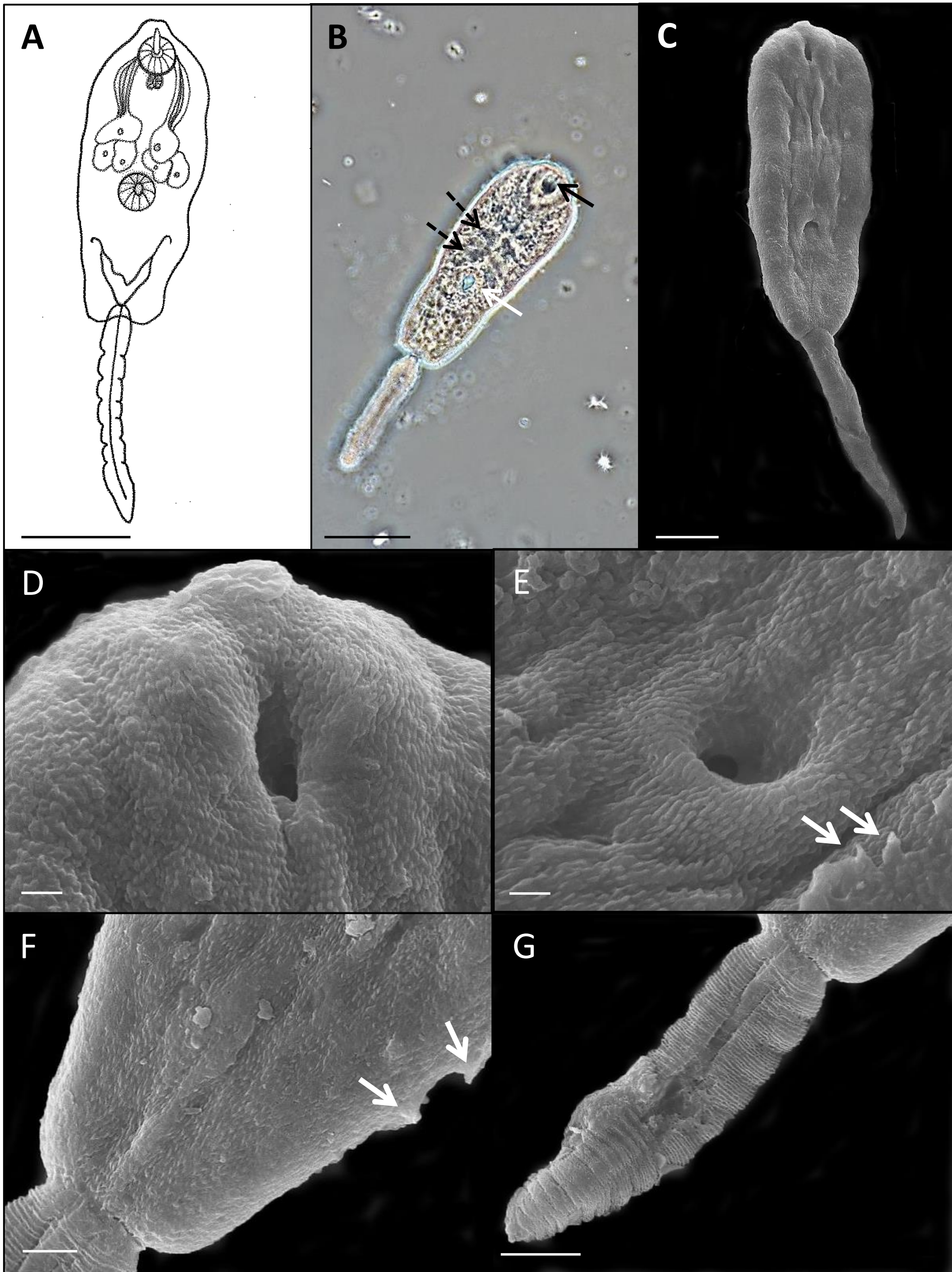
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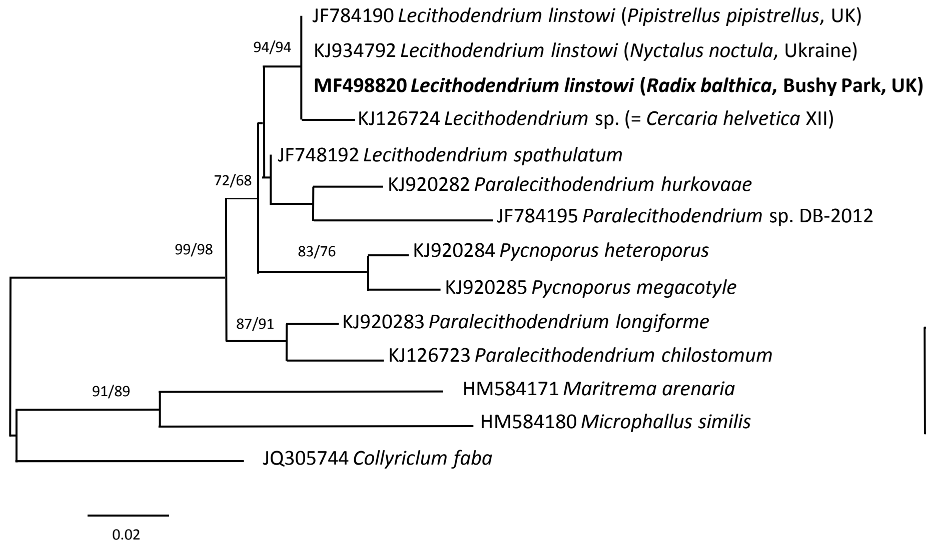
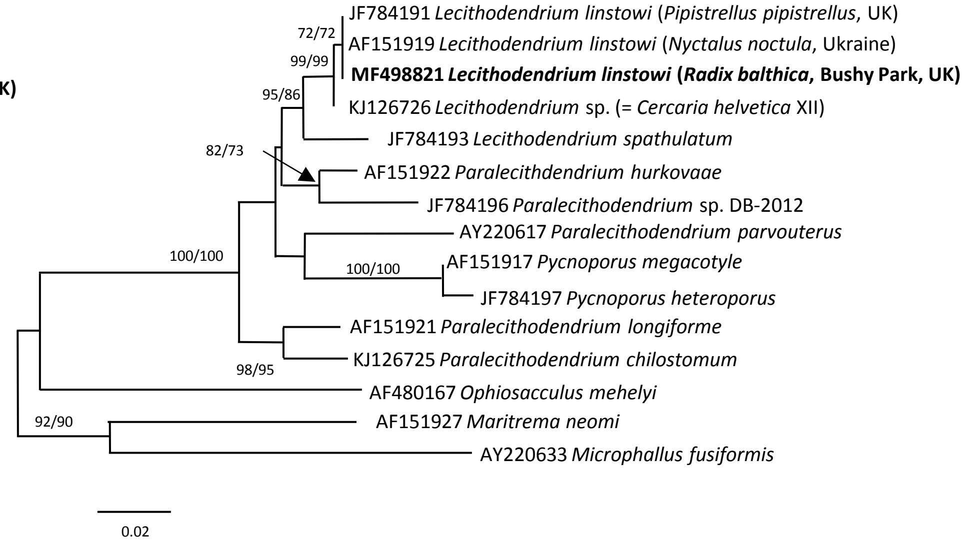
A) Phylogenetic identification of *Lecithodendrium linstowi*

i) Reconstruction based on ITS2

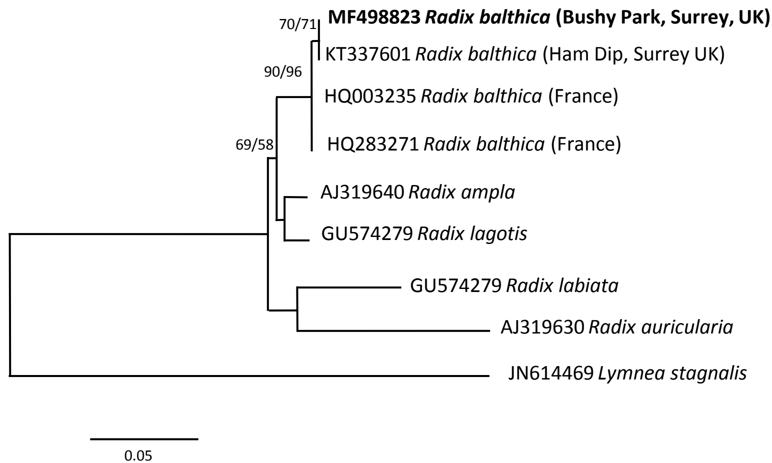


ii) Reconstruction based on LSU



B) Phylogenetic identification of *Radix balthica*

i) Reconstruction based on ITS2



ii) Reconstruction based on cox1