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Position specificity in the genus *Coreomyces* (*Laboulbeniomycetes*, *Ascomycota*)

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Abstract: To study position specificity in the insect-parasitic fungal genus *Coreomyces* (*Laboulbeniaceae*, *Laboulbeniales*), we sampled corixid hosts (Corixidae, Heteroptera) in southern Scandinavia. We detected *Coreomyces* thalli in five different positions on the hosts. Thalli from the various positions grouped in four distinct clusters in the resulting gene trees, distinctly so in the ITS and LSU of the nuclear ribosomal DNA, less so in the SSU of the nuclear ribosomal DNA and the mitochondrial ribosomal DNA. Thalli from the left side of abdomen grouped in a single cluster, and so did thalli from the ventral right side. Thalli in the mid-ventral position turned out to be a mix of three clades, while thalli growing dorsally grouped with thalli from the left and right abdominal clades. The mid-ventral and dorsal positions were found in male hosts only. The position on the left hemelytron was shared by members from two sister clades. Statistical analyses demonstrate a significant positive correlation between clade and position on the host, but also a weak correlation between host sex and clade membership. These results indicate that sex-of-host specificity may be a non-existent extreme in a continuum, where instead weak preferences for one host sex may turn out to be frequent.

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INTRODUCTION

Members of the *Laboulbeniales* are minute ascomycete fungi that average 0.2 mm in length and seldom exceed 1 mm in length, although some species have been reported to grow beyond 2 mm (Giard 1892, Thaxter 1896, Santamaría 1998). They are obligate ectoparasites on arthropods tied to their host throughout the entire life cycle, and many species appear to be specific to a single or a few closely related host species (Scheloske 1969, Huldén 1983, Majewski 1994). The order comprises four families with around 140 genera and in excess of 2000 described species (Kirk *et al.* 2008), but some estimates suggest that the true number of species worldwide is in the interval 15 000–75 000 (Weir & Hammond 1997).

Ascospores are assumed to be transmitted between hosts mainly through direct contact during various behavioural interactions, e.g., mating (Richards & Smith 1954, 1955, Whisler 1968, De Kesel 1993, 1995, 1996) or, to a more limited extent, through spore-contaminated host habitat (Arwidsson 1946, Lindroth 1948, Scheloske 1969, De Kesel 1995). Infection experiments in *Herpomyces* have shown that species infecting cockroaches are highly specific to one or a few species in the same genus (Richards & Smith 1954). Only occasionally did weak infections occur in host species distantly related to the main host. De Kesel (1996), on the other hand, demonstrated that *Laboulbenia slackensis*, seemingly strictly host specific in nature, was able to infect a broad range of carabid hosts under artificial conditions. This indicates that the physiological properties of the host do not explain host specificity in *L. slackensis*. The closely related *L. littoralis* is found in the same habitat (coastal

marshland) but on a different host belonging to another coleopteran suborder, indicating that the species is restricted by habitat rather than host and that host shifts within the same habitat may result in speciation (De Kesel & Haelewaters 2014). Another example of habitat specificity is *Rickia wasmannii*, which infects several unrelated arthropod hosts sharing the same environment in *Myrmica* ant nests (Pfliegl *et al.* 2016). On the other hand, relatively few nominal species are known to exhibit a broad host range in nature, and some of these may turn out to consist of complexes of distinct species upon closer examination, e.g. with molecular tools (Weir & Blackwell 2005, Haelewaters & De Kesel 2017).

The *Laboulbeniales* are unique among the fungi in displaying “position specificity” (Peyritsch 1875, Thaxter 1896, Benjamin & Shanor 1952, Whisler 1968), a term coined to describe the phenomenon that a nominal parasite species only inhabits a specific, restricted part of its host species’ anatomy. Position specificity is also known in a variety of parasites in the animal kingdom, for instance in the flatworm class Monogenea (Littlewood 1997) and among the water mites (Martin 2004). In many cases among the *Laboulbeniales*, however, there is no simple one-to-one relationship between the parasite and its position on the host. It has been observed, for example, that positions may become less unique as time after infection passes and that this could be explained by secondary infections mediated by the behaviour of the host (Whisler 1968). In some cases, the same nominal species has been observed to inhabit different positions in males and females that come into contact during mating (Peyritsch 1875). Thaxter (1896) countered that positions were not as strictly upheld as suggested by Peyritsch

and that mating alone could not fully explain this pattern. An even more extreme hypothesis, “sex-of-host specificity”, was advanced by Benjamin & Shanor (1952) and Benjamin (1971), who suggested that each host sex is inhabited by one member of a pair of closely related parasite species, each member often with unique morphological traits and in a unique position on the host. Scheloske (1976) rejected sex-of-host specificity and argued that the different nominal species found on males and females are merely morphotypes of the same species (or exceptionally, a few species). Several examples of di- and polymorphic species have later been described where the authors follow Scheloske (e.g. Rossi & Kotrba 2004, Santamaria & Faille 2009). However, the parasite infects male and female hosts in the same position, which is difficult to explain by mating behaviour only (Majewski 1994, Rossi & Kotrba 2004, Santamaria & Faille 2009). Recent molecular investigations in the genera *Chitinomyces* and *Hesperomyces* suggest that nominal position-specific species more or less correspond to species as independent evolutionary units, that positions on the host may be different between the sexes, and that there may be intraspecific morphological differences correlated with either host sex and/or the position on the host (Goldmann & Weir 2012, Goldman et al. 2013).

This study is focused on the genus *Coreomyces* (Fig. 1), in which all members have been claimed to exhibit position specificity (identical between host sexes) but not sex-of-host specificity (Thaxter 1931, Majewski 1994). The genus is known from all continents except Australia (Tavares 1985) and includes 21 nominal and accepted species (Mycobank 2018), all of which parasitize members of the two closely related hemipteran families Corixidae and Micronectidae (Thaxter 1931, Nieser 2002). The host ranges of the *Coreomyces* species

are poorly understood, because claims of host and position specificity are often based on few observations. The most thorough morphological investigations of the genus, focus on the eastern European species (Majewski 1973, 1994, 2003, 2008). Distribution ranges are poorly known and many nominal species are known only from a few sites. Only a handful species have been reported from more than one country and a single one is considered more or less cosmopolitan (Thaxter 1931, Sugiyama & Hayama 1981, Majewski 1988, Santamaria et al. 1991, Majewski 1994, Shen et al. 2006).

The aims of this study were to test (1) to what extent thalli growing in different positions on their corixid hosts correspond to species as independent evolutionary units in the sense of de Queiroz (2007), and (2) the extent to which species (understood as independent evolutionary units) display position specificity, host specificity, or sex-of-host specificity. Our sampling included corixid populations in southern Sweden and Denmark, and we obtained DNA sequence data from several markers and numerous *Coreomyces* individuals from different host positions, sexes and species. This approach was made possible by recent advances in acquiring DNA sequence data from *Laboulbeniales* (Haelewaters et al. 2015, Sundberg et al. 2018).

MATERIALS AND METHODS

Sampling

During 2014–2015 we sampled corixids along a route from the province of Uppland in central Sweden to the province of Skåne in the southernmost part of the country, as well as in the Copenhagen area in Denmark. The geographical sampling range spans roughly 300 km in the longitudinal direction and 550 km in the latitudinal. Potential localities, i.e., small ponds, were identified from satellite images (see Table 1 for coordinates). The corixids were captured by sweeping a reinforced colander along the bottom of the pond. Captured animals were killed and preserved in 99.7 % ethanol, which was replaced after a few hours for the best preservation of the fungal DNA. Infected animals with the most developed thalli were sorted out under a dissecting microscope (Olympus SZ1145 TR), placed in 99.7 % ethanol, and then stored at -20 °C.

Thalli were detached from 76 corixid individuals and crushed according to the protocol by Sundberg et al. (2018). We used from one (or when poorly developed) up to six thalli from each position. The positions of the thalli were documented with a microscope camera (Moticam 5) connected to the dissecting microscope. After the study, host specimens were deposited at the Museum of Evolution (UPSZ).

Molecular procedures

For DNA amplification we used the Terra PCR Direct Polymerase Mix (Clontech) and KAPA3G Plant PCR Kit (Kapa Biosystems). The PCR settings and primers followed the protocol described by Sundberg et al. (2018). PCR products showing clear single bands on an agarose gel were enzymatically cleaned with FastAP Thermosensitive Alkaline Phosphatase combined with Exonuclease I (Thermo Fisher Scientific Inc.). The concentrations and quality of the PCR products were checked on a 2100 Bioanalyzer (Agilent Technologies). We used four molecular markers: the small subunit (nrSSU), internal transcribed spacer

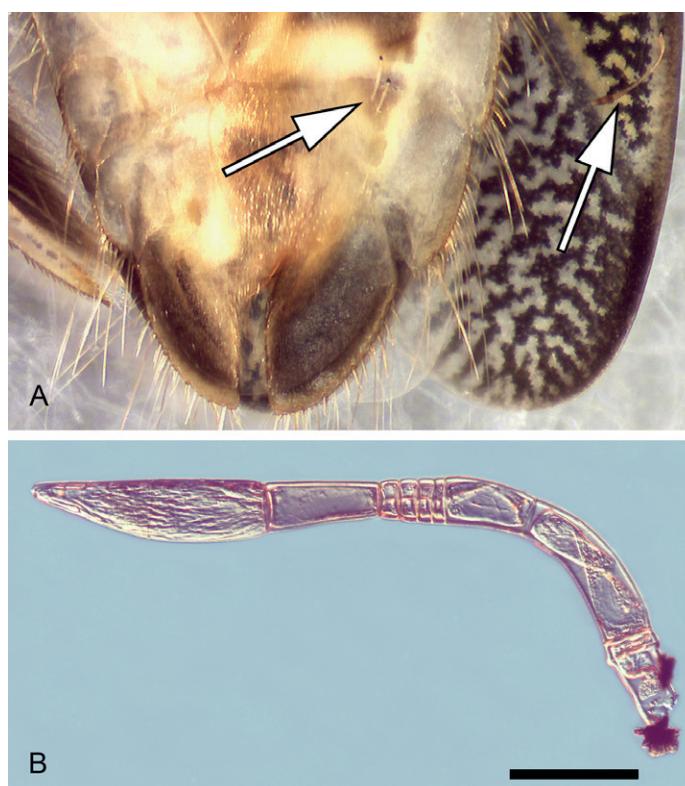


Fig. 1. Examples of *Coreomyces* sp. **A.** Thalli at the ventral side of the abdomen and at the inferior side of the left hemelytral margin of the corixid host. **B.** Detached thallus from the inferior margin of the left hemelytron. Scale bar = 100 µm.

Table 1. *Coreomyces* sp.: position on host, clade affiliation, isolate ID, host species and sex, site of collection with coordinates and GenBank accession numbers for the different molecular markers. CV = midventrally on the abdomen, in male hosts only. LV = inferior side of the exterior margin of the left hemelytron, LV = ventrally on the left side of the abdomen, RV = ventrally on the right side of the abdomen, RD= dorsally on the right side of the abdomen near the margin, in male hosts only. GenBank accession numbers beginning with KY were first published in Sundberg *et al.* (2017).

Position	Clade	Isolate	Host	Host sex	Collection site	WGS84 decimal (lat, lon)	ITS	nrLSU	nrSSU	mtSSU
LV	Green	H_1-1	<i>Sigara lateralis</i>	female	Sweden, Halland	57.136752, 12.271385	KY293257	KY350525	KY523236	KY523212
LW	Orange	H_2-1	<i>Sigara striata</i>	male	Sweden, Halland	57.131477, 12.265763	KY293277	KY350551	—	—
LW	Orange	Og_1-1	<i>Callicorixa praeusta</i>	male	Sweden, Östergötland	58.393054, 15.579268	—	—	—	MG602616
LV	?	Og_1-2	<i>Callicorixa praeusta</i>	male	Sweden, Östergötland	58.393054, 15.579268	—	—	—	MG602617
CV	Red	Sjae_1-1	<i>Sigara iactans</i>	male	Denmark, Sjælland	55.706659, 12.508814	MG602605	MG602627	—	MG602615
LW	Blue	Sjae_1-2	<i>Sigara iactans</i>	male	Denmark, Sjælland	55.706659, 12.508814	KY293265	KY350540	—	—
LW	Orange	Sjae_2-1	<i>Sigara striata</i>	male	Denmark, Sjælland	55.679423, 12.418692	KY293263	KY350533	KY523242	KY523217
LW	Orange	Sjae_3-1	<i>Sigara iactans</i>	male	Denmark, Sjælland	55.754983, 12.548575	KY293275	KY350549	—	—
LW	?	Sk_1-1	?	?	Sweden, Skåne	55.726704, 13.185147	—	—	—	MG602618
CV	Orange	Sk_1-10	<i>Sigara lateralis</i>	male	Sweden, Skåne	55.726704, 13.185147	KY293283	KY350557	—	—
LW	Orange	Sk_1-2	<i>Sigara iactans</i>	male	Sweden, Skåne	55.726704, 13.185147	KY293262	KY350532	KY523241	KY523216
LW	Orange	Sk_1-3	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.726704, 13.185147	—	KY350534	KY523243	KY523218
LV	Green	Sk_1-4	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.726704, 13.185147	KY293264	KY350535	—	—
LW	Blue	Sk_1-5	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.726704, 13.185147	KY293267	KY350542	—	—
LW	Blue	Sk_1-6	<i>Callicorixa praeusta</i>	male	Sweden, Skåne	55.726704, 13.185147	KY293268	KY350543	—	—
LW	Orange	Sk_1-7	<i>Sigara striata</i>	male	Sweden, Skåne	55.726704, 13.185147	KY293269	KY350544	—	—
LW	Blue	Sk_1-8	<i>Sigara distincta</i>	male	Sweden, Skåne	55.726704, 13.185147	KY293270	KY350545	—	—
LW	Blue	Sk_1-9	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.726704, 13.185147	KY293271	KY350546	—	—
LW	Blue	Sk_2-1	<i>Sigara striata x dorsalis?</i>	female	Sweden, Skåne	55.580009, 13.163021	KY293228	KY350499	—	—
LV	Green	Sk_2-2	<i>Sigara iactans</i>	female	Sweden, Skåne	55.580009, 13.163021	KY293229	KY350500	—	—
LW	Orange	Sk_2-3	<i>Sigara iactans</i>	female	Sweden, Skåne	55.580009, 13.163021	KY293230	—	—	—
LV	Green	Sk_2-4	<i>Sigara striata</i>	male	Sweden, Skåne	55.580009, 13.163021	—	—	KY523224	—
RV	Red	Sk_2-5	<i>Sigara iactans</i>	male	Sweden, Skåne	55.580009, 13.163021	KY293231	—	—	—
LW	Orange	Sk_2-6	<i>Sigara striata x dorsalis?</i>	male	Sweden, Skåne	55.580009, 13.163021	—	KY350501	KY523225	—
LV	Green	Sk_2-7	<i>Sigara striata x dorsalis?</i>	male	Sweden, Skåne	55.580009, 13.163021	KY293233	—	—	—
LW	Blue	Sk_3-1	<i>Sigara striata</i>	male	Sweden, Skåne	55.677399, 13.061255	KY293234	KY350503	KY523227	KY523205
LV	Green	Sk_3-2	<i>Sigara striata</i>	male	Sweden, Skåne	55.677399, 13.061255	—	KY350504	—	—

Table 1. (Continued).

Position	Clade	Isolate	Host	Host sex	Collection site	WG584 decimal (lat, lon)	ITS	nrlSU	nrSSU	mtSSU
LV	Green	Sk_3-3	<i>Sigara striata</i> × <i>dorsalis</i> ?	female	Sweden, Skåne	55.677399, 13.061255	KY293235	KY350505	KY523228	—
LV	Green	Sk_4-1	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.557086, 14.337586	KY293238	KY350506	—	—
LV	Green	Sk_4-2	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.557086, 14.337586	KY293239	KY350507	—	—
LV	Green	Sk_4-3	<i>Sigara lateralis</i>	male	Sweden, Skåne	55.557086, 14.337586	KY293240	KY350508	—	—
RD	Green	Sk_4-4	<i>Sigara lateralis</i>	male	Sweden, Skåne	55.557086, 14.337586	KY293241	KY350509	KY523229	—
LV	Green	Sk_4-5	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.557086, 14.337586	KY293242	KY350510	—	KY523206
RV	Red	Sk_4-6	<i>Sigara striata</i> × <i>dorsalis</i> ?	male	Sweden, Skåne	55.557086, 14.337586	KY293244	KY350511	—	—
LV	Green	Sk_4-7	<i>Sigara iactans</i>	female	Sweden, Skåne	55.557086, 14.337586	KY293243	KY350512	—	KY523207
RD	Green	Sk_4-8	<i>Sigara iactans</i>	male	Sweden, Skåne	55.557086, 14.337586	KY293245	KY350513	—	KY523208
RD	Red	Sk_4-9	<i>Sigara iactans</i>	male	Sweden, Skåne	55.557086, 14.337586	KY293246	KY350514	—	—
LV	Green	Sk_5-1	<i>Hesperocorixa sahlbergi</i>	female	Sweden, Skåne	55.585991, 14.157691	—	KY350517	—	—
LW	Blue	Sk_5-2	<i>Sigara distincta</i>	male	Sweden, Skåne	55.585991, 14.157691	KY293250	KY350518	KY523232	—
LV	Green	Sk_5-3	<i>Sigara distincta</i>	male	Sweden, Skåne	55.585991, 14.157691	KY293251	KY350519	KY523233	KY523209
LW	Blue	Sk_5-4	<i>Callicorixa praeusta</i>	female	Sweden, Skåne	55.585991, 14.157691	KY293252	KY350520	KY523234	KY523210
LV	Green	Sk_5-5	<i>Callicorixa praeusta</i>	female	Sweden, Skåne	55.585991, 14.157691	KY293253	KY350521	—	—
LW	Blue	Sk_5-6	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.585991, 14.157691	KY293254	KY350522	—	—
LW	Blue	Sk_5-7	<i>Callicorixa praeusta</i>	male	Sweden, Skåne	55.585991, 14.157691	KY293255	KY350523	KY523235	KY523211
LV	Green	Sk_5-8	<i>Callicorixa praeusta</i>	male	Sweden, Skåne	55.585991, 14.157691	KY293256	KY350524	—	—
LV	Green	Sk_6-1	<i>Sigara lateralis</i>	female	Sweden, Skåne	55.573821, 14.273054	KY293258	KY350527	KY523237	KY523213
CV	Blue	Sk_6-2	<i>Sigara lateralis</i>	male	Sweden, Skåne	55.573821, 14.273054	KY293272	KY350547	—	—
CV	Blue	Sk_6-3	<i>Sigara lateralis</i>	male	Sweden, Skåne	55.573821, 14.273054	KY293273	KY350548	—	—
RV	Red	Sk_7-1	<i>Sigara fallenii</i>	male	Sweden, Skåne	55.57512, 13.208926	KY293259	KY350528	KY523238	KY523214
LW	Blue	Sk_7-2	<i>Sigara fallenii</i>	male	Sweden, Skåne	55.57512, 13.208926	KY293276	KY350550	—	—
LW	Blue	Sk_7-3	<i>Sigara fallenii</i>	female	Sweden, Skåne	55.57512, 13.208926	KY293278	KY350552	—	—
LW	Blue	Sk_7-4	<i>Sigara fallenii</i>	male	Sweden, Skåne	55.57512, 13.208926	KY293282	KY350556	—	—
LW	Blue	Sk_8-1	<i>Sigara striata</i> × <i>dorsalis</i> ?	male	Sweden, Skåne	55.771642, 12.955582	KY293266	KY350541	—	—
RV	Red	Sk_8-2	<i>Sigara striata</i>	male	Sweden, Skåne	55.771642, 12.955582	KY293274	—	—	—
LW	Blue	Sk_8-3	<i>Sigara striata</i>	male	Sweden, Skåne	55.771642, 12.955582	KY293281	KY350555	—	—

Table 1. (Continued).

Position	Clade	Isolate	Host	Host sex	Collection site	WG584 decimal (lat, lon)	ITS	nrlSU	nrSSU	mtSSU
RV	Red	Sm_1-1	<i>Sigara fassarum</i>	male	Sweden, Småland	57.802609, 14.272442	KY293247	KY350515	—	—
LW	Blue	Sm_1-2	<i>Sigara fassarum</i>	female	Sweden, Småland	57.802609, 14.272442	—	—	KY523231	—
RV	Red	Sm_1-3	<i>Sigara falleni</i>	male	Sweden, Småland	57.802609, 14.272442	KY293249	KY350516	—	—
RV	Red	Sm_2-1	<i>Callicorixa praewusta</i>	female	Sweden, Småland	57.776057, 14.151442	—	KY350526	—	—
LW	Blue	Sm_3-1	<i>Callicorixa praewusta</i>	male	Sweden, Småland	57.778705, 14.228196	KY293261	KY350530	KY523240	—
LV	Green	Sm_3-2	<i>Callicorixa praewusta</i>	male	Sweden, Småland	57.778705, 14.228196	—	KY350531	—	—
LW	Orange	UpL_1-1	?	?	Sweden, Uppland	59.84386, 17.735076	—	—	—	MG602606
LV	Green	UpL_1-2	<i>Sigara dorsalis</i>	male	Sweden, Uppland	59.84386, 17.735076	MG602597	MG602619	MG640370	MG602607
LW	Orange	UpL_1-3	<i>Paracorixa concinna</i>	male	Sweden, Uppland	59.84386, 17.735076	MG602604	MG602626	MG640374	—
LW	Blue	UpL_1-4	<i>Sigara distincta</i>	male	Sweden, Uppland	59.84386, 17.735076	KY293279	KY350553	—	—
LW	Blue	UpL_1-5	<i>Sigara distincta</i>	female	Sweden, Uppland	59.84386, 17.735076	KY293280	KY350554	—	—
LW	Blue	UpL_2-1	<i>Callicorixa praewusta</i>	male	Sweden, Uppland	59.867169, 17.71259	MG602598	MG602620	MG640371	MG602608
LW	Blue	UpL_2-10	<i>Callicorixa praewusta</i>	male	Sweden, Uppland	59.867169, 17.71259	KY293237	KY350502	—	KY523204
LW	Blue	UpL_2-2	?	?	Sweden, Uppland	59.867169, 17.71259	MG602599	—	—	MG602609
LW	Blue	UpL_2-3	<i>Callicorixa praewusta</i>	male	Sweden, Uppland	59.867169, 17.71259	MG602600	MG602621	MG640372	MG602610
LV	Green	UpL_2-4	<i>Callicorixa praewusta</i>	male	Sweden, Uppland	59.867169, 17.71259	—	—	—	MG602611
LW	Blue	UpL_2-5	<i>Callicorixa praewusta</i>	male	Sweden, Uppland	59.867169, 17.71259	MG602601	MG602622	—	MG602612
LW	Blue	UpL_2-6	<i>Callicorixa praewusta</i>	female	Sweden, Uppland	59.867169, 17.71259	—	MG602623	MG640373	MG602613
LW	Blue	UpL_2-7	?	?	Sweden, Uppland	59.867169, 17.71259	MG602602	MG602624	—	MG602614
CV	Red	UpL_2-8	<i>Sigara iactans</i>	male	Sweden, Uppland	59.867169, 17.71259	MG602603	MG602625	—	—
LW	Blue	UpL_2-9	<i>Sigara distincta</i>	female	Sweden, Uppland	59.867169, 17.71259	KY293236	—	KY523226	—

(ITS) region (including ITS1, 5.8S, and ITS2), the large subunit (nrLSU) of the nuclear ribosomal RNA gene, and the small subunit of the mitochondrial ribosomal RNA gene (mrSSU). Sequencing was carried out by Wyzer Biosciences Inc., Cambridge (MA), and Macrogen Inc., Amsterdam, with the primers ITS4, ITS5, 5.8Shs2, 5.8Shs4, ctb6, LRhs1, LRhs3, LR7 NS4, NShs1, NShs4, NShs2, NShs3, mrSSU1, and mrSSU3R (Vilgalys & Hester 1990, White *et al.* 1990, Zoller *et al.* 1999, Sundberg *et al.* 2018).

Data analysis

Newly produced sequences were edited using Sequencher v. 5.4 and Geneious v. 7.1.9 and aligned together with previously published sequences (Sundberg *et al.* 2018) using MAFFT v. 7.312 (Katoh & Standley 2013). We used the E-INS-i algorithm with the PAM1 matrix for the nrSSU and mrSSU alignments, and the PAM20 matrix for the ITS and nrLSU alignments. The entire alignments were used in downstream analyses, i.e. no data were excluded.

Likelihood model selection as well as maximum likelihood analyses were performed using IQ-TREE v. 1.6 beta 4 (Nguyen *et al.* 2015, Kalyaanamoorthy *et al.* 2017). The best-fitting GTR family model was selected using the Bayesian Information Criterion (BIC) among candidates with one, two, or six substitution rates, with and without gamma-distributed rate heterogeneity among sites and a proportion of invariable sites. For the ITS data, we additionally selected the best-fitting time-irreversible RY Lie Markov model (Sumner *et al.* 2012, Fernández-Sánchez *et al.* 2015, Woodhams *et al.* 2015). Full likelihood optimization was carried out under each candidate model. For each marker, we performed searches for the best trees as well as non-parametric bootstrap analyses with 1 000 replicates under the selected model. The purpose of estimating a tree under a time-irreversible Lie Markov model was to infer the placement of the root. This is not a trivial task in Laboulbeniomycetes, because available DNA sequences are few and sequence divergence within the class is extreme compared to the amount of variation present in our data. The only marker displaying substantial variation in our data, the ITS, is essentially unalignable with any other available sequences. Therefore, we decided to instead use the information contained inside our data for rooting.

Bayesian inference was carried out using MrBayes v. 3.2.6 (Ronquist & Huelsenbeck 2003, Ronquist *et al.* 2012). We used the same best-fitting models as in the maximum likelihood analyses. Priors included a uniform distribution on topology, a uniform (0, 1) distribution on the proportion of invariable sites, a (1, 1) beta distribution on the transition/transversion rate ratio (when applicable), and a (1, 1, 1, 1) Dirichlet on state frequencies (when applicable). We assumed a compound Dirichlet prior on branch lengths (Rannala *et al.* 2012, Zhang *et al.* 2012). The gamma distribution component of this prior was set to $\alpha = 1$ and $\beta = \alpha / (\text{ML tree length})$, whereas the Dirichlet component was set to the default (1, 1). Three parallel Markov chain Monte Carlo (MCMC) runs were performed, each with four parallel chains and the temperature increment parameter set to 0.15 (Altekar *et al.* 2004). The appropriate degree of heating was determined by observing swap rates between the cold and hot chains in preliminary runs. Every 1000th tree was sampled. Runs were diagnosed every 10^6 generations, removing the first 50 % of the tree sample as burnin, and were set to halt automatically when converged before a maximum of 100×10^6 generations. Convergence was defined as an average standard deviation of splits (with frequency ≥ 0.1) across runs ≤ 0.01 .

We used mPTP v. 0.2.3 in maximum likelihood mode to delineate species by locating the transition points between inter- and intraspecific processes in the accumulation of substitutions on the individual gene trees (Kapli *et al.* 2017). The original Poisson tree process (PTP) implementation assumes a single rate distribution across the tree for the coalescent process (Zhang *et al.* 2013), whereas the multi-rate Poisson tree process (mPTP) model allows multiple rate distributions, one for each inferred species. We applied both single-rate and multi-rate models to the already rooted Lie Markov model ITS tree and to a rooted version of the nrLSU tree. The nrSSU tree was not included in the analysis, because one potential species was represented by a singleton, whereas mrSSU data were excluded owing to lack of resolution. Likelihood ratio tests were performed to assess whether the multiple-rates model had better fit to the data than a single-rate model.

We performed multinomial logistic regression using the *multinom()* function of R package nnet v. 7.3.12 (Venables & Ripley 2002). Clade membership (four categories based on ITS, nrLSU, and nrSSU phylogenies) was treated as the response variable and position on host (five categories), host sex (male, female) and host genus (four categories) as predictor variables. We set green clade membership, host genus *Sigara*, host sex female, and left ventral position on the host as baseline categories. The *Anova()* function of R package car v. 2.1.6 (Fox & Weisberg 2011) was subsequently used to test the null hypotheses of no effect of each predictor term on the response using type-II chi-square likelihood ratio tests. We also carried out log-linear analysis on the same data using the *loglm()* function of R package MASS v. 7.3.47 (Venables & Ripley 2002). Starting from the saturated model, we carried out stepwise backward regression to find the best model according to the Akaike Information Criterion (AIC). All analyses, including 71 observations without missing data, were carried out with R v. 3.4.3 (R Core Team 2017).

RESULTS

Sampling outcome

Members of the genus *Coreomyces* were found on 10 species of corixids belonging to four genera: *Callicorixa*, *Hesperocorixa*, *Paracorixa*, and *Sigara* (Table 1). Three new host species for *Coreomyces* were noted, *Sigara dorsalis*, *S. fossarum*, and *S. iactans*. Six individuals of *Sigara* showed intermediary traits and were determined as potential hybrids. Among the hosts, 48 were males and 24 females (four individuals were lost during handling and not included in downstream statistical analyses). *Coreomyces* thalli were encountered in five different positions on the corixids (Fig. 2): inferior side of the exterior margin of the hemelytron (LW), ventrally on the left side of the abdomen (LV), ventrally on the right side of the abdomen (RV), dorsally on the right side of the abdomen near the margin (RD), and mid-ventrally on abdomen (CV).

Molecular data

All sequences produced are listed in Table 1 with GenBank accession numbers. Those beginning with KY were published by Sundberg *et al.* (2018). Alignments and resulting gene trees are available from TreeBASE (<https://treebase.org>) under study number S21919.

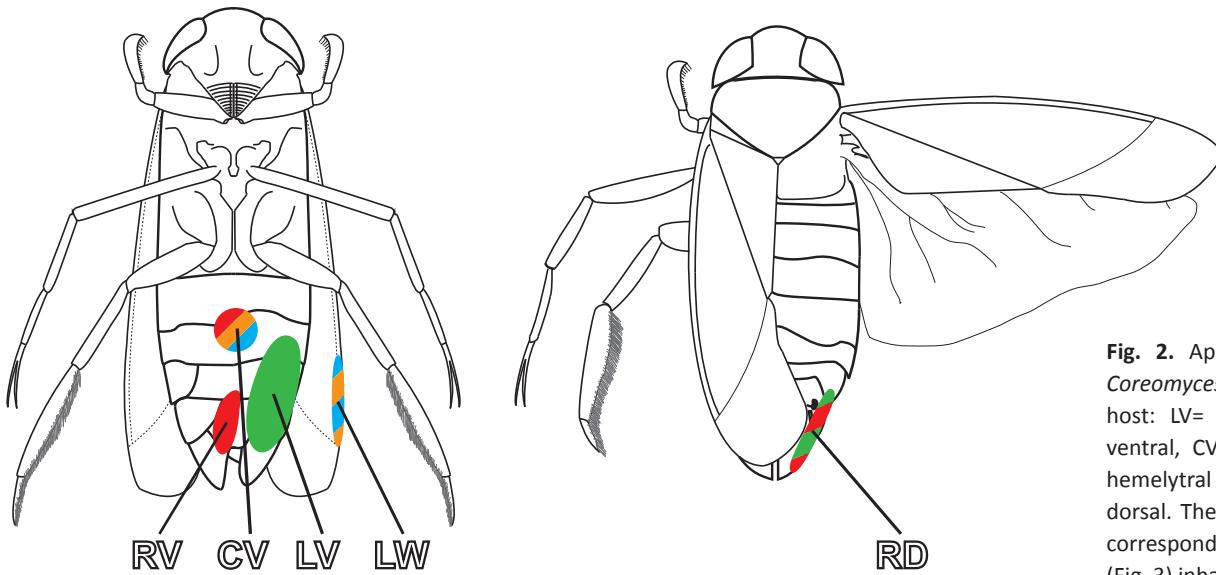


Fig. 2. Approximate positions of *Coreomyces* individuals on the host: LV= left ventral, RV= right ventral, CV= midventral, LW= left hemelytral margin, and RD= right dorsal. The colour of the positions corresponds to the colour of clades (Fig. 3) inhabiting these positions.

The ITS data is comprised of 62 sequences and 1 241 aligned positions, the nrLSU data 63 sequences and 1293 aligned positions, the nrSSU data 24 sequences and 1 059 aligned positions, and the mrSSU 27 sequences and 680 aligned positions. The ITS, nrLSU, nrSSU, and mrSSU data include 248, 21, 5, and 1 variable positions, respectively. The best among the 1-, 2-, and 6-rate GTR family models selected by the BIC were HKY+I for the ITS and nrLSU, and JC+I for the nrSSU. The best time-irreversible RY Lie Markov model selected for the ITS was RY6.7b. The ln likelihood scores of the best trees found by IQ-TREE under a GTR family model were -3363.666, -1953.347, and -1514.566 for the ITS, nrLSU, and nrSSU respectively, whereas the score was -3357.614 for the best ITS tree found under the RY6.7b model. The Bayesian inferences, depending on the marker, stopped after $2-4 \times 10^6$ generations under the convergence criterion in use. The ln harmonic mean estimations of marginal likelihoods in the Bayesian inference were -3496.416, -2009.554, and -1531.517 for the ITS, nrLSU, and nrSSU, respectively. The individual gene trees, with their corresponding bootstrap branch support and posterior probabilities, are summarized in Fig. 3A–D. Four major clades (green, red, orange, blue) are displayed in colour. The time-irreversible ITS tree in Fig. 3A indicates that the root of the tree is situated on the branch separating the green clade from the rest. This tree, contrary to all other, is rooted and has separate support for the two daughter branches attached to the root node. The relatively low bootstrap support values for these daughter branches compared to the corresponding branch in the unrooted and time-reversible ITS tree in Fig. 3B indicate that there is some uncertainty about the placement of the root. The four clades are monophyletic in the ITS and nrLSU trees (Fig. 3A–C). In the ITS tree, all four groups have strong support ($\geq 80\%$ bootstrap proportions and ≥ 0.95 posterior probabilities), whereas in the nrLSU tree, three branches are strongly supported and the fourth slightly less so (71%, 0.71). The nrSSU tree (Fig. 3D) provides strong support for two of the branches but weak support for the green clade (note that the red clade has no support as it is represented by a single sequence). Finally, the mrSSU data, consisting of only two haplotypes differing in a single mutation, is represented by a haplotype network in Fig. 3E. This network shows that the orange clade is resolved as a unique haplotype relative to the rest.

Poisson tree process modelling on the ITS and nrLSU trees indicates that the four major clades represent independent species. A model with multiple rates turned out to be worse than a model with a single rate for the ITS and a model with multiple rates was not significantly better than a model with a single rate for the nrLSU (likelihood ratio test, $p=1.00$). Therefore, we report here the results from the single-rate model.

Multinomial logistic regression rejects the null hypothesis that position on the host has no effect on clade membership ($p < 2 \times 10^{-16}$), whereas there is no indication that host genus or host sex affect clade membership ($p = 0.87$ and 0.34, respectively). Relative risk ratios (effect sizes), however, indicate that even among the non-significant predictors, some switches among host genera and host sex may confer an increased probability of clade membership: Relative risk ratios for *Sigara*→*Callicorixa* and *Sigara*→*Paracorixa* were 547 and 5×10^{13} for switching to the red and orange clade, respectively, whereas male→female was associated with relative risk ratios 1 and 8×10^8 for switching to the blue and orange clades, respectively. A model including all three predictors will, using the maximum predicted probability for each observation, classify 83 % of the observations correctly. Removing the only significant predictor, position on the host, results in 44 % correctly classified observations. For comparison, a random classification has a probability of 2×10^{-24} of being 44 % correct (four categories and 71 observations, 31 of which are correctly classified). The log-linear analysis with stepwise backward regression stopped at a model including the four variables plus the interactions between (1) clade and position and (2) host sex and position (chi-square likelihood ratio, $p = 1$). Taken together, the statistical analyses suggest what is obvious from looking at the phylogenetic trees in Fig. 3: There is a strong correlation between clade membership and the position of the fungus on the corixid host: Thalli in position LW came out as two sister groups (blue and orange), meaning that there seem to be two species occupying the same position (Fig. 2). All samples in position LV group in one clade (green). Position RV thalli group into the red clade. The thalli in position RD came out inside two different clades in the tree (the green and red). Finally, thalli from position CV belong to the red, orange and blue clades.

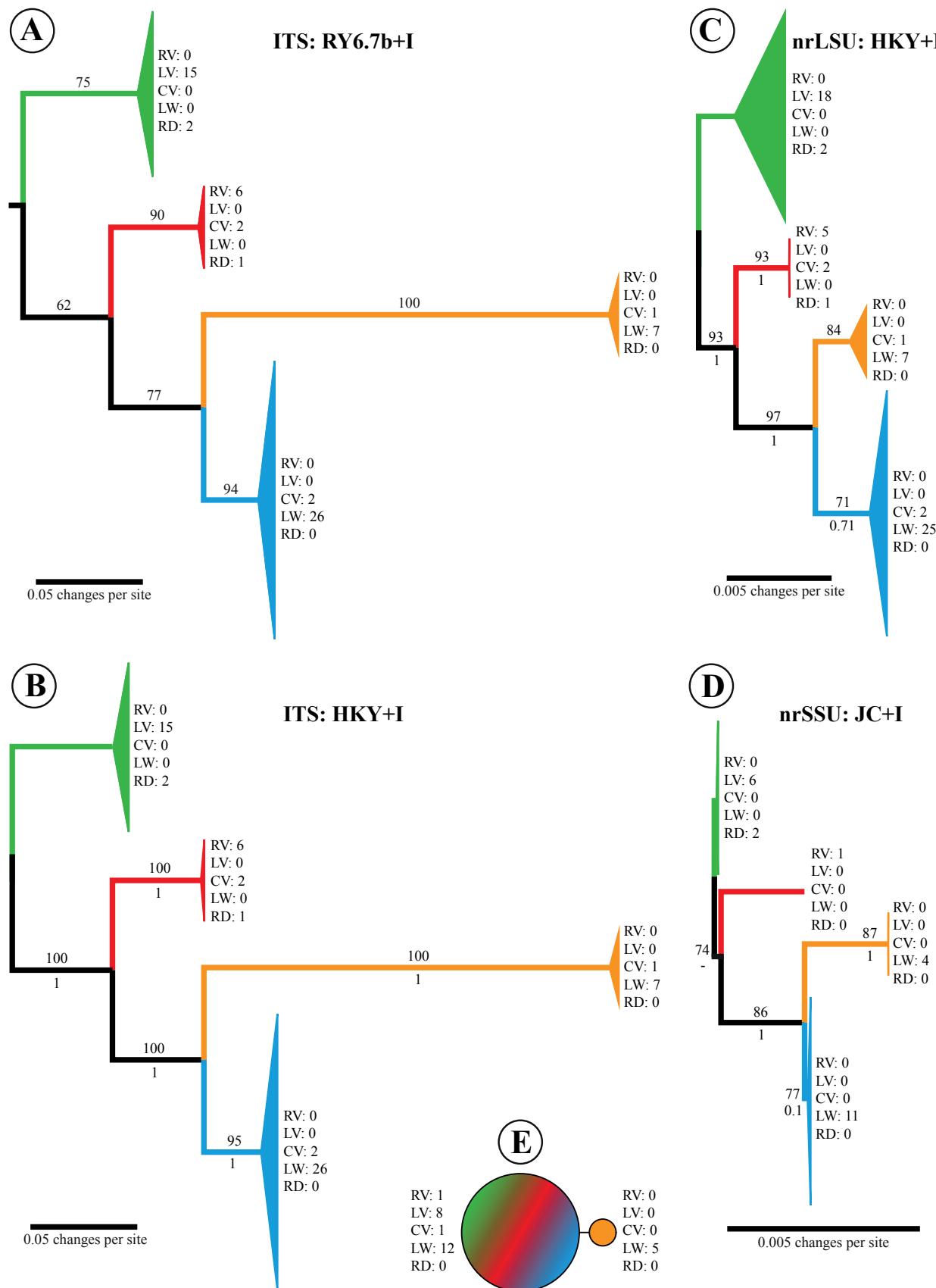


Fig. 3. Inferred phylogenies for each marker based on (A) ITS data under a RY6.7b time-irreversible model; (B) ITS data under a HKY+I model; (C) nrLSU data under a HKY+I model; (D) nrSSU data under a JC+I model. Branch support is indicated (upper: bootstrap proportion in ML analysis, lower: posterior probabilities in the Bayesian inference). Crown groups have been transformed into triangular cartoons, the width of which represents the branch length from the most recent common ancestor to the tip of the highest branch, and the height of which is proportional to the number of included individuals. The nrSSU data is represented by a haplotype network (E), the size of the filled circles being proportional to the number of individuals and a line representing one mutational step. The groups marked here in green, red, orange, and blue are topologically congruent at the level of individual. Clades and haplotypes have been annotated with the number of individuals in the five positions on the hosts (see Fig. 2 for an explanation to abbreviations).

DISCUSSION

Four species of *Coreomyces*

The four major clades (green, red, orange, blue) in Fig. 3 are identified and interpreted here as species, because (1) Poisson tree process modelling on the ITS and nrLSU trees indicates that the clades represent independent species, (2) they are topologically completely congruent across markers (although with different degrees of resolution), and (3) they correlate with ecological parameters, primarily the position on the host.

All species were present in the entire study area, from Uppland in the north to Skåne and Denmark in the south (Table 1). Species were also often mixed in the same host populations, and sometimes co-occurred on the same host individuals. The nomenclatural issues were not the focus of this study and assigning correct names, if available, will have to be the focus of a future research. However, thalli growing in three of the positions (CV, LV, and LW) are previously reported from Europe (Santamaria *et al.* 1991, Majewski 1994, De Kesel & Werbrouck 2008, Majewski 2008). The remaining RD and RV positions have not previously been explicitly reported in the literature. It remains unclear, however, whether they have been observed before, as early descriptions of *Coreomyces* tend to be vague about the exact positions on the host (Thaxter 1902, 1905, Spegazzini 1915, 1917, 1918, Thaxter 1931).

Specificity to host, position and sex

The *Coreomyces* taxa found in this study did not show any strict specificity to host species, as they occurred on five or seven corixid species belonging to two or three different but closely related genera within the subfamily Corixinae. This observation is in accord with available information on host preferences in the genus (e.g., Thaxter 1931, Majewski 1994). For instance, *Coreomyces corisae*, the species with the widest known host range, is known to parasitize a range of species belonging to at least four genera (Spegazzini 1918, Thaxter 1931, Santamaria *et al.* 1991, Majewski 1994, Santamaria 2003). Host ranges may, however, turn out to be narrower once species circumscriptions have been investigated by molecular means. Although we did not observe any strict host specificity, the effect sizes from the multinomial logistic regression suggest that some switches between host genera are associated with substantially increased odds for some of the clade memberships. The total effect of host genus is non-significant, however, and larger samples are needed to possibly detect subtle effects of this parameter.

Contrary to the strict position specificity reported for *Coreomyces* by Majewski (1973, 1994), our results indicate that none of the four species is restricted to only a single position on its host. Instead, each species inhabits two or three different positions, although one of them tends to be much preferred over the other(s). The latter observations explain why both the multinomial logistic regression and the log-linear analysis suggest a strong interaction between position on the host and clade membership. These findings are in agreement with Goldman & Weir (2012) and Goldmann *et al.* (2013). The former study demonstrated the presence of position specificity in *Chitinomyces*, with positions and morphology being different in male and female hosts. The latter investigation pointed to substantial morphological differences between conspecific thalli of *Hesperomyces* in different positions on the host, although it

was not clear whether these phenotypes were also correlated with host sex.

All *Coreomyces* species in our study were found on both male and female corixid hosts (Table 1). The log-linear analysis indicates that there is an interaction between host sex and the position on the host, suggesting positions are not entirely identical in males and females. This result is probably explained by individuals in positions CV (orange, blue, and red clades) and RD (red and green clades) being known only from male hosts in our data. Other positions appear to be less unequally distributed between male and female hosts. The multinomial logistic regression did not reject the null hypothesis of no effect of host sex on clade membership, nor did the stepwise log-linear model selection indicate that the interaction between host sex and clade membership is needed to explain the observed data. However, the multinomial logistic regression does suggest that host sex confers increased odds for membership in two of the clades. Sex-of-host specificity may be a non-existent extreme in a continuum, where instead weak preferences for one host sex may turn out to be frequent.

Dispersal and positioning of thalli

In this study we encountered positions present in both host sexes (LW, LV, RV) and those that are seemingly male-specific in our data (CV and RD), which is in agreement with earlier studies of position specificity (Goldmann & Weir 2012, Goldmann *et al.* 2013). These studies demonstrated that occurrences of the same species in the same dorsal position of both females and males is explained by mixed hetero- and homosexual mounting by males. Additional occurrences on the ventral side or on the legs in males are more easily explained, as they represent contact surfaces during heterosexual mounting. In corixid mating, males position themselves on the back of the females, or males in case of homosexual mounting (Popham 1961, Aiken 1982). Peters (1962) provided a detailed description of corixid mating behaviour. After mounting, the male swings his abdomen to the left and forces it under the female abdomen and finally becomes curled around the body of the female. This manoeuvre could account for the LV and LW positions on the left side of the body and possibly also the RV position on the right. The remaining, possibly male-specific positions (CV and RD), are more difficult to explain. An intriguing clue to how this could come about may be found in a peculiarity of the male corixid morphology. They are not bilaterally symmetric, both dextral and sinistral forms existing in many species (Schilthuizen 2013), the former normally being much more common. In sinistral males, the abdomen and the copulatory apparatus are reversed compared to dextral males, and consequently they instead wrap their abdomen around the right side of the female (Peters 1962). It could be speculated that rare occurrence of sinistral males in combination with homosexual mounting may explain the CV and RD positions.

Other kinds of behaviour associated with mating may also affect the exact positioning of the thalli. Jansson (1979), for example, indicated that the copulating animals to some extent change positions when they need to ascend to the water surface to breathe and that this requires some struggling. Agonistic behaviour should also be considered. Candolin (2004) documented males of *Sigara falleni* trying to hinder each other's mounting attempts, while Jansson (1973, 1979) recorded a nudging behaviour among *Arctocoris* males trying to

outcompete each other. Non-social behaviour like cleaning was suggested by Huldén (1983) to result in the fungus occupying precise positions similar in both sexes. We regard this theory as unlikely, however, as we would then expect the fungus to inhabit the host limbs. This does not rule out that they may be accounted for by some male behaviour that actively transmits thalli from positions that are not sex-specific, as suggested by Benjamin (1971).

Although behaviour may explain occurrence patterns, there is also the possibility of passive infection through the host substrate. This has been suggested as an important mode of dispersal under terrestrial conditions (Arwidsson 1946, Lindroth 1948). In the case of *Laboulbenia slackensis*, however, active transmission was found to be far more important, whereas passive transmissions were independent of soil characteristics and often unsuccessful owing to the short life-span of the spores (De Kesel 1995). In the case of *Coreomyces*, the aquatic environment possibly favours spore longevity and consequently increases the chance of infection. However, the distinctly non-random positions of the thalli on the host indicate that indirect transmission of spores may not be important. Indirect transmission through a contaminated host substrate is also likely to be unfavourable in small host populations and when spore production is modest, as is the case in many *Laboulbeniales* (Huldén 1983).

Concluding remarks

This study has demonstrated that there are four species of *Coreomyces* in our sample, that they prefer but are not strictly specific to certain positions on the host, and that there may or may not be weak preferences on the part of the fungus regarding host sex and genus. These findings lead to further questions of importance to understand the evolution of *Laboulbeniales*: How do species boundaries arise to start with, when host preferences are not discontinuous? How does position specificity arise and how is it related to substrate requirements? How does mating take place in the fungus, if at all? Genomic data as well as controlled observations of host behaviour under laboratory conditions may help solve these and other questions.

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AUTHORS' CONTRIBUTIONS

H.S. did the sampling and laboratory work. S.E. did the statistical and phylogenetic analysis. Figures were made by S.E. and H.S. Species determinations of the host material were made by J.B. The text was written jointly by H.S., Å.K. and S.E. with valuable input from J.B. Å.K. and S.E. supervised the project.

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