DNA sequencing of the Internal Transcribed Spacer (ITS) to reconstruct speciation events within the Mytilinidiaceae (Pleosporomycetidae, Dothideomycetes, Ascomycota)

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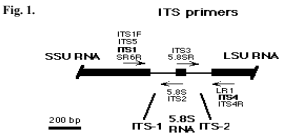
Rationale: The specific aim of the research program is to expose biology students to state-of-the-art scientific methodologies, namely gene amplification, DNA sequencing, and bioinformatics, to address questions in evolutionary mycology, specifically in the phylogenetic reconstruction of ascomycetous fungi. Essentially, we are interested in whether morphological features historically used in the delineation of species (e.g., spore morphology) are evolutionarily or phylogenetically informative in the context of sequence-based phylogenies derived from gene geneaoligies using both coding and noncoding genomic regions. Information relating to fungal biology and phylogeny can be found at the Tree of Life (http://tolweb.org/fungi). Specific information on the two bitunicate ascomycete families that we focus on, namely the Hysteriaceae & Mytilinidiaceae (Eumycota, Ascomycotina, Dothideomycetes, Pleosporomycetidae), can be found online at http://www.eboehm.com/ and at http://tolweb.org/Hysteriaceae.

Fig. 1.



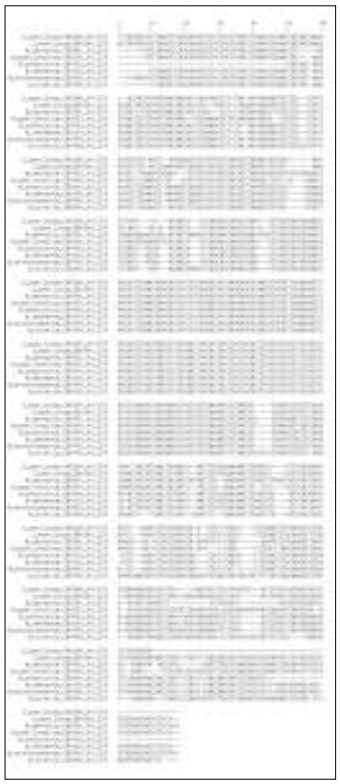


Fig. 2. DNA sequence alignment of the ITS1 – 5.8S – ITS2 regions of the fungal ribosomal operon, for eight species belonging to the family Mytilinidiaceae. See Table 1 for designations.

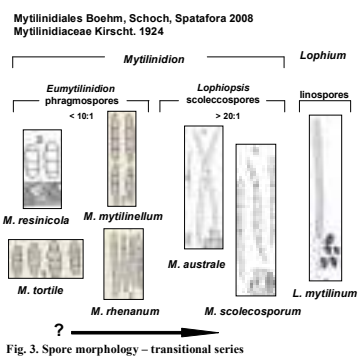


Fig. 3. Spore morphology – transitional series

Left to right: Ziphora Sam, Adade Kouevi, Eric Boehm, Carlos Garcia, Eunice Nkansah & Tariq Walker

Fig. 4. These fungi possess miniature bivalve shell-shaped or conchate fruiting bodies, reminiscent of bivalve mollusks. E.g., fungal genus *Mytilinidion* (Mytilinidiaceae) from the Latin *Mytilus*, a genus of mussels in the Mytilidae.

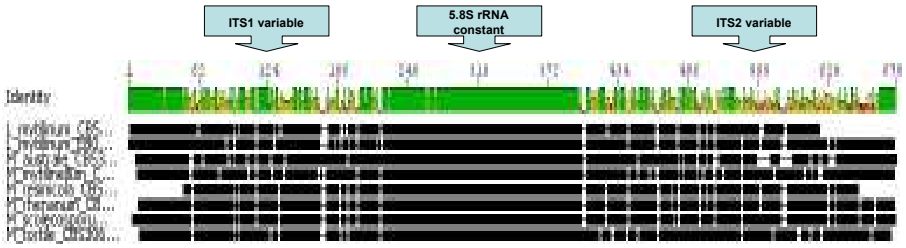


Fig. 5. Graphical representation of the DNA sequence alignment presented in Fig. 2, of the ITS1 – 5.8S – ITS2 regions of the fungal ribosomal operon, for eight species belonging to the family Mytilinidiaceae. See Table 1 for designations.

Materials & Methods: Fungal specimens were obtained from local collections, as well as from the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands (Table 1). Genomic DNA was recovered using the DNeasy Plant Mini Kit (Qiagen Inc.), following the instructions of the manufacturer, but using sterile white quartz sandand a Kontes battery-powered pestle grinder in 1.5 ml microfuge tubes. The ITS region (Fig. 1) was amplified and double-strand sequenced using the forward primer ITS1 (TCCGTAGGTGAACCTGCGG) and the reverse primer ITS4 (TCCTCCGCTTATTGATATGC). Final concentrations for 50 mL PCR amplification reactions were as follows: 1 mM of each forward and reverse primer, 2 mM $MgCl_2$, 200 mM dNTP, 1X GoTaq Flexi Reaction Buffer (Promega), 1.25 U GoTaq polymerase (Promega), and 2 mL template DNA diluted tenfold. PCR reaction parameters were as follows: a 95°C pre-melt for 3 min., and 35 cycles of 95°C for 20 sec., 54°C for 30 sec., and 72°C for 60 sec., followed by a final extension at 72°C for 10 min. Amplicons were column purified and outsourced to Macrogen USA Inc. for sequencing.

Results: The students were able to PCR amplify and sequence the fast evolving, hypervariable, non-coding ITS region from eight species in the family Mytilinidiaceae (Table 1). The alignment of all eight DNA sequences (Fig. 2), and their graphical representation (Fig. 5), indicates that the ITS1 and ITS2 regions were hypervariable, whereas the 5.8S rRNA coding sequence was not. The ITS phylogenetic tree generated from the eight aligned sequences (Fig. 6) proved to be identical with that obtained from a recent study (Boehm *et al.* 2009), in which four coding nuclear genes (nuSSU, nuLSU, TEF1 & RPB2) were sequenced for a subset of the same isolates. The congruence between coding genes and the non-coding ITS – for a total of five genomic regions sampled – supports the premise that an accurate evolutionary snapshot of speciation events has been obtained for this family. Furthermore, it was also found that species with similar spore morphologies, ranging from phragmospores to scolecospores to linospores (Fig. 3) – previously considered closely related – in fact proved not to be (Fig. 6).

Table 1. Fungal isolates used in this study

Mytilinidiales Boehm, Schoch & Spatafora 2008		
Mytilinidiaceae Kirschst. 1924		
***Lophium mytilinum* Pers. : Fr.**	**CBS 269.34 / AFTOL 1609**	**ML Lohman, No. 191, *Pinus*, MI, USA (1934)**
	CBS 114111	**K & L Holm & O Constantinescu, *Pinus sylvestris*, Uppland, Sweden (1988)**
	EB 0248 (CBS 123344)	**E Boehm, *Pinus strobus*, French Hill Rd., St. Lawrence Co., NY (2004)**
***Mytilinidion mytilinellum* (Fr.) Zogg**	**CBS 303.34**	**ML Lohman, No. 281, *Larix larcina*, MI,USA (1934)**
***Mytilinidion rhenanum* Fuckel**	**CBS 135.45**	**NCTC #6434 (1945)**
***Mytilinidion tortile* (Schw.) Sacc.**	**CBS 306.34**	**ML Lohman, *Juniperus virginiana*, MI,USA (1930)**
***Mytilinidion resinicola* Lohman**	**CBS 304.34**	**ML Lohman, No. 260, *Larix larcina*, MI,USA (1930)**
***Mytilinidion scolecosporum* Lohman**	**CBS 305.34**	**AH Smith & ML Lohman, *Pinus strobus*,WI, USA (1930)**
***Mytilinidion australe* Lohman**	**CBS 301.34**	**AH Smith & ML Lohman, *Pinus*, LA, USA (1931)**

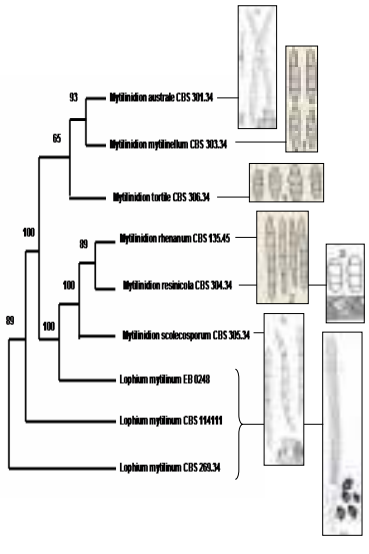


Fig. 6. Molecular Evolution – ITS phylogeny

Discussion: It is often assumed in evolutionary biology that organisms that share common morphological features – i.e., that look alike – are in fact quite closely related. That is, morphology is generally a good predictor of genetic relatedness. This can be tested by comparing morphology to molecules, as we have done here. Fungi classified in the family Mytilinidiaceae share a similar conchate fruitbody, shaped very much like a miniature bivalve mollusk (Fig. 4). They differ however in the shape of their spores, which forms the basis for their current classification. Given the natural transition series in spore morphology (Fig. 3), it was assumed that short phragmospores (e.g., *M. resinicola* & *M. tortile*) preceded the elongate phragmospore (e.g., *M. mytilinellum* & *M rhenanum*), which in turn preceded the scolecospore (e.g., *M. australe* & *M. scolecosporum*), culminating finally in the linospore seen in the genus *Lophium* (arrow in Fig. 3). Lohman (1932) proposed two subgenera for the genus *Mytilinidion*: (1) subgenus *Eumytilinidion* for phragmosporous species, with a spore ratio of length to width of 10:1 or less (e.g., *M. mytilinellum*, *M. tortile*, *M. rhenanum* & *M. resinicola*), and (2) subgenus *Lophiopsis* for scolecosporous species with a ratio of approximately 20:1 (e.g., *M. australe* & *M. scolecosporum*), proposed to form a transitional series to connect *Mytilinidion* with the heretofore somewhat isolated genus *Lophium* (Lohman 1932). Surprisingly, sequence data provided here for the ITS, as well as for other genes (Boehm *et al*. 2009), clearly indicate that within the genus *Mytilinidion* spore shape is not a synapomorphic character state. Thus, scolecospores have evolved at least twice within the family, as evidenced by separate clades containing *M. australe* & *M. scolecosporum* (Fig. 6). Also, short phragmospores of *M. resinicola* & *M. tortile* do not segregate from the elongate, presumably transitional, phragmospores of *M. mytilinellum* & *M rhenanum* (Fig. 6). The lack of support for subgenus *Lophiopsis*, and the lack of spore homogeneity within a clade, thus illustrates a case of convergent evolution in spore morphology within this family. Lastly, the genus *Lophium* is seen here as ancestral to the genus *Mytilinidion*, with linospores preceding phragmospores & scolecospores in the Mytilinidiaceae.