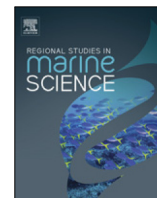




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journal homepage: www.elsevier.com/locate/rsmaMorphological and phylogenetic analyses of *Nia vibrissa*, a marine Basidiomycota collected in Portuguese watersEgídia Azevedo^{a,b,*}, Margarida Barata^{b,c}, Maria Filomena Caeiro^{a,c}^a Centro de Estudos do Ambiente e do Mar (CESAM), Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal^b Centro de Ecologia, Evolução e Alterações climáticas (CE3C), Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal^c Departamento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

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ABSTRACT

This study presents morphological and phylogenetic characterizations of *Nia vibrissa* specimens detected on *Fagus sylvatica* baits, after six months of incubation in moist chambers at the laboratory. The baits had been submerged at Cascais marina, Portugal, during a survey carried out in 2006–2008. Morphological observations evidenced differences in basidiocarp color and in the morphology of the peridial hairs, varying from straight to curved and with bifurcate to non-bifurcate ends. Morphological variability has often been reported, associated to the suggestion that *N. vibrissa* is a species complex. We addressed this subject through the evaluation of pairwise distances and phylogenetic analyses applying Bayesian and Maximum Likelihood methods, to multi-sequence alignments involving the large subunit (LSU) of the nuclear ribosomal DNA. Even though the six *N. vibrissa* isolates under analysis consistently clustered together with high support values and most of their pairwise distances ranged between 0 and 3%, one of them presents a higher distance value (4%) relative to other two isolates. These results recommend the need of further genetic evaluations, involving more isolates and gene regions, to answer the question addressed in this study: is *N. vibrissa* a species complex?

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1. Introduction

Basidiomycota are rare in marine environments where, according to Jones et al. (2015), 21 species were identified in 17 genera, including as last additions, four taxa from less saline waters (man-grove habitats): *Henningsomyces* spp., *Schizophyllum commune*, *Hyphoderma smabuci* and *Grammothele fuligo*.

Members of three genera from the family Marasmiaceae (*Nia*, *Calathella*, *Halocyphina*) are the most frequently detected in studies of marine mycology (Jones et al., 2015). In surveys carried out in Portugal, only species of the genus *Nia* were detected: *N. globispora* on *Spartina maritima* baits submerged in the estuary of Mira river (Barata et al., 1997; Barata, 2006) and on drift stems collected in sandy beaches (Sridhar et al., 2012) and also *Nia vibrissa* on baits of *S. maritima* submerged in Mira River (Barata, 2006) and baits of *Fagus sylvatica* submerged at Cascais marina (Azevedo et al., 2010, 2011).

N. vibrissa is widespread in marine environments colonizing a variety of submerged drift or intertidal woody substrates. This

species was originally detected on beech wood shavings submerged during several months at Biscayne Bay, Florida, having been classified as deuteromycete (Moore and Meyers, 1959). Later, Doguet (1967, 1968) observed basidia and clamp connections and Brooks (1975) demonstrated the existence of dolipore septa. These characters indicate that *N. vibrissa* is a homobasidiomycete, though not revealing its taxonomic position.

N. vibrissa produces minute, white, yellow, pink to dark yellow, superficial fruit bodies with peridial hairs, ovoid or ellipsoidal hyaline basidiospores with four lateral and one apical appendages (Kohlmeyer and Kohlmyer, 1979; Binder et al., 2001). This fungus differentiates reproductive structures in culture and Schimpfhauser and Molitoris (1991) reported the fact that some strains differentiate in culture either hairy or smooth fruit bodies with no peridial hairs.

Jones and Jones (1993), Binder et al. (2001) and Jones et al. (2009), based on the high morphological diversity observed on basidiocarp color and peridial hairs suggested the existence of a multi species complex for *N. vibrissa*. Since then, all authors agreed that further studies are required to address this subject.

Based on analyses of partial sequences of nuclear (nuc) and mitochondrial (mt) small subunits (SSU) and large subunits (LSU) of ribosomal DNA (rDNA) regions, Binder et al. (2001) evaluated the phylogenetic position of *N. vibrissa*. These authors showed that *N.*

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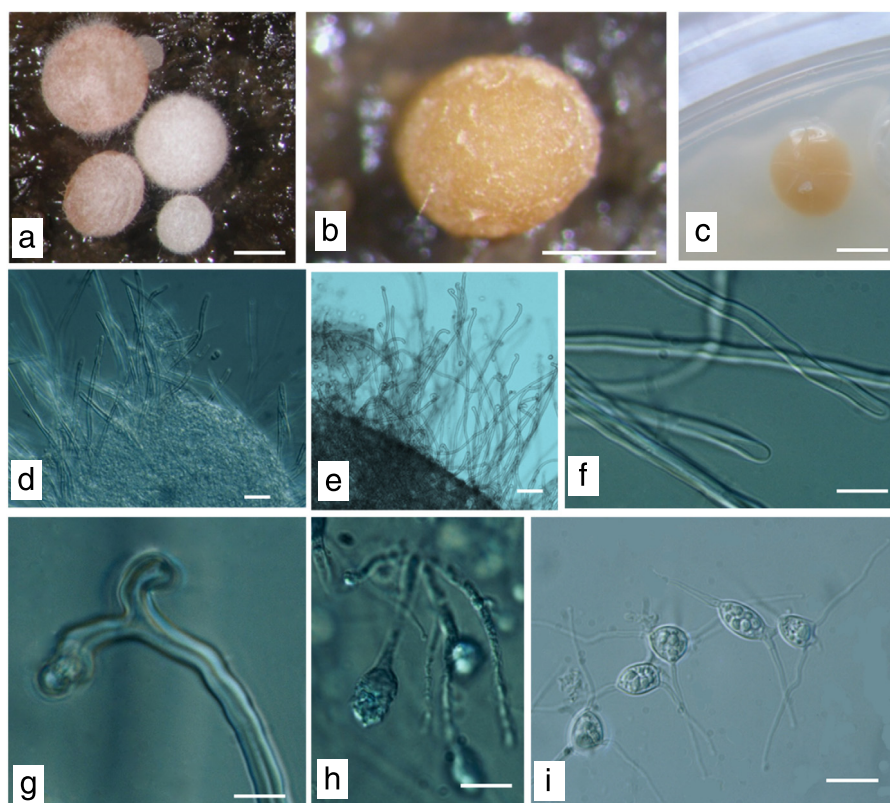


Fig. 1. Morphological features of *Nia vibrissa*. a, b—Basidiocarps on *Fagus sylvatica* baits. c—Basidiocarp on culture medium. d, e—Basidiocarp wall with straight and curled peridial hairs. f—Straight peridial hairs. g—Bifurcated curled peridial hairs. h—Immature basidia. i—Basidiospores with appendages. Scale bars: a = 1 mm; b = 10 mm; c = 1 mm; d, e = 5 μ m; f, g, i = 10 μ m; h = 40 μ m.

vibrissa (one sequence) is a member of a named euagarics clade, clustering with the cyphelloid fungus *Henningsomyces candidus* (one sequence) whose basidiocarps are minute cup shaped forms.

Hibbet and Binder (2001) assessed the evolution of marine mushrooms based on the same regions referred above, including in their analyses four marine species (*Calathella mangrovei*, *Halocyphina villosa*, *N. vibrissa*, *Physalacria maipoensis*), one freshwater (*Limnoperdon incarnatum*) and 40 terrestrial species. This former study confirmed the placement of *N. vibrissa* in the euagarics clade. *N. vibrissa* (one sequence) and *H. villosa* (one sequence) clustered with *C. mangrovei* in a clade strongly supported by bootstrap value, all closely related with two terrestrial species (*Cyphellopsis anomala* and *Favolaschia intermedia*). These results were confirmed in LSU and combined LSU/5.8S phylogenetic analyses (Bodensteiner et al., 2004) where a well-supported group denominated *Nia* clade included three clades, one of them consisting of *N. vibrissa*, *H. villosa* and *C. mangrovei* (one sequence from each species). An identical *Nia* clade was obtained with a multilocus rDNA analysis, by Binder et al. (2006).

Marine fungi play diverse roles in ecosystems, allowing a number of biotechnological applications. This applies to isolates of *N. vibrissa*, evidencing involvement in wood decay (Schimpfhauser and Molitoris, 1991; Jones and Jones, 1993; Binder et al., 2001) and in the removal of hydrocarbon compounds of waters (Reyes et al., 2012).

Starting with a collection of morphologically distinct specimens of *N. vibrissa* detected on wood baits previously submerged at Cascais marina (Azevedo et al., 2010, 2011) and considering the availability of sequences of this fungus on databases, this work aims to evaluate the correspondence between morphological and genetic variability within *N. vibrissa*. Is *N. vibrissa* a species complex? This subject has been addressed by phylogenetic analyses

involving the LSU of the nuc rDNA region, to morphologically distinct representatives of this species, whose pairwise distances were also compared, for the same region.

2. Materials and methods

2.1. Morphological analysis

Fagus sylvatica baits collected at Cascais marina during a survey of our team (Azevedo et al., 2010, 2011) were subjected to indirect observations (after 5 mo of incubation in moist chambers at room temperature) under a stereomicroscope (Wild M8). Where basidiocarps of *N. vibrissa* were detected on the surface of the wood, fruit bodies were carefully removed with a needle and observed under the light microscope (Leitz Larbourz S with Normarski) in slides done with seawater as mounting media.

Microscopic characterizations of diagnose characters presented in Fig. 1 and Table 1, followed the current guidelines of marine mycology. Identifications were made using the dichotomous keys of Kohlmeyer and Kohlmyer (1979), Kohlmeyer and Volkmann-Kohlmeier (1991), Hyde and Sarma (2000), Jones et al. (2009). Photographs of microscopic characters were obtained with a Leica Wild MPS 52 camera using Fujichrome RTP-135, 64T Tungsten. Macroscopic features were obtained with a Nikon Coolpix 5000 camera and color annotations in macroscopical basidiocarp descriptions were based on Munsell soil color charts (<http://www.vcsu.edu/cmsfiles/327/b2fc4f5ebb.pdf>, last access: 8/17/2017).

The macromorphology of *N. vibrissa* colonies was performed following the procedures described by Sidrim and Moreira (1999), comprising the observation of color, rate of growth, margin and

Table 1Morphological characterization of the Portuguese isolates of *Nia vibrissa*.

Isolates	Basidiocarp	Dimensions (mm)	Peridial hairs
	Color (according to Munsell soil color charts)		
<i>Nia vibrissa</i> FCUL090707CF3	Brownish yellow (10YR 8/6)	2.0	Straight with bifurcate curled ends
<i>Nia vibrissa</i> FCUL070108CF9	Brownish yellow (10YR 8/6)	2.1	Straight
<i>Nia vibrissa</i> FCUL170907CF3	Light Orange (7.5YR 8/6)	1.9	Bifurcate with curled ends
<i>Nia vibrissa</i> FCUL179707CF6	Brownish yellow (10YR 8/6)	2.0	Straight
<i>Nia vibrissa</i> FCUL170907CF9_1	Brownish yellow (10YR 8/6)	2.0	Straight with bifurcate curled ends
<i>Nia vibrissa</i> FCUL170907CF9_2	Light pink (2.5YR 8/3)	2.0	Straight

pigment diffusion into corn meal agar (CMA, Fluka, India), made with 50% sea water.

Single spore cultures were obtained and preserved as described by Azevedo et al. (2010).

2.2. DNA extraction

A small piece of a basidiocarp was picked up with a sterile needle and crushed in a 100 µL solution containing 90 µL of dilution buffer from Phire direct PCR Plant Kit (Finnzymes, now Thermo Fisher Scientific, Massachusetts, USA) with chitinase (Nzytech, Lisboa, Portugal) at 0.05 units/mL. After overnight incubation at 60 °C, this mixture was incubated for 3 h at 60 °C with 0.2 mg/mL proteinase K, followed by 10 min at 98 °C. A 5 min centrifugation at 12 000 g was performed to pellet insoluble materials; supernatants containing DNA were stored at –20 °C.

DNA from fungal biomass of *N. vibrissa* isolates was extracted with Nucleospin Plant DNA extraction Kit (Machery–Nagel, Germany), following the instructions of the manufacturers (Azevedo et al., 2011).

2.3. PCR amplification and sequencing

PCR reactions performed according to Phire direct PCR Plant Kit (Finnzymes, now Thermo Fisher Scientific, Massachusetts, USA) procedures in 25 µL reaction mixtures containing 1 µL of template DNA, were amplified in a Tpersonal cycler (Whatman Biometra, Göttingen, Germany). Partial nuc LSU sequences were amplified with the primer set LR0R/LR5 (Vilgalys and Hester, 1990) under the following conditions: 98 °C for 3 min; 35 cycles of 98 °C for 10 s, 62 °C for 5 s, 72 °C for 20 s; 72 °C for 1 min.

Aliquots from the PCR reactions were analyzed on 0.7% agarose gels stained with ethidium bromide. After purification with Jet quick DNA Clean up Kit (Genomed GmbH, Lohne, Germany) following the manufacturer instructions, PCR products were sequenced in both directions at StabVida (Caparica, Portugal) using the same primers.

2.4. Phylogenetic analysis

The nucleotide sequences from the Portuguese isolates were confirmed as ribosomal DNA by homology searches using BLAST (<http://www.ncbi.nlm.nih.gov>). Multiple sequence alignments executed with Muscle were manually corrected and the number of nucleotides of all sequences adjusted to the nucleotide length of the smallest representative to maximize genetic similarities.

The final LSU alignment, 463 nucleotides long, comprises six Portuguese isolates of *N. vibrissa* and 14 sequences of marine and terrestrial Basidiomycota retrieved from databases, including three *Nia vibrissa* isolates and *Auricularia auricula-judae* as out-group taxon (Table 2). A matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach generated with Mega 7.0 (Kumar et al., 2016) is shown in Table 3.

Phylogenetic trees were inferred by Maximum Likelihood (ML) and Bayesian methods performed, respectively, with Mega 7.0

Table 2

Taxa and GenBank and NBRC.

Taxon	Isolate identity	Sequence ID
<i>Auricularia auricula-judae</i>	MW 446	AF291289
<i>Calathella mangrovei</i>	1-30-01Jones	AF426954
<i>Cyphellopsis anomala</i>	PB318	AY570998
<i>Cyphellopsis anomala</i>	PB333	AY571000
<i>Digitatispora marina</i>	3027C	KM272362
<i>Halocyphina villosa</i>	NBRC 32086	AB455965 ^a
<i>Halocyphina villosa</i>	NBRC 32087	AB455966 ^a
<i>Henningsomyces candidus</i>	PB338	AY571008
<i>Haloaleurodiscus mangrovei</i>	TMIC 34914	AB176452
<i>Fistulina hepatica</i>	DSH 93–183	AF261592
<i>Mycaureola dilseae</i>	BM17/85	DQ093774
<i>Nia vibrissa</i>	FCUL090707CF3	MG597150
<i>Nia vibrissa</i>	FCUL070108CF9	MG597151
<i>Nia vibrissa</i>	FCUL170907CF3	MG597152
<i>Nia vibrissa</i>	FCUL179707CF6	MG597153
<i>Nia vibrissa</i>	FCUL170907CF9_1	MG597154
<i>Nia vibrissa</i>	FCUL170907CF9_2	MG597155
<i>Nia vibrissa</i>	M200 REG	AF334750
<i>Nia vibrissa</i>	NBRC 32089	AB455967 ^a
<i>Nia vibrissa</i>	NBRC 32090	AB455968 ^a

^a Identification Numbers (ID) from the LSU sequences selected for this study.

(Kumar et al., 2016) and BEAST V2.4.8 software package (<https://www.beast2.org/>).

The evolutionary history inferred by using the ML method was based on the Hasegawa–Kishino–Yano model and the bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates were collapsed. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.5565)). Codon positions included were 1st + 2nd + 3rd + Noncoding.

For Bayesian analysis, BEAUti was used to generate an xml file with the input information executable in BEAST V2.4.8. The selected substitution model was HKY, which assumes an estimated proportion of invariant sites and four gamma distributed rate categories to account for the rate heterogeneity across sites. The tree priors were set to Yule specifications, log normal was employed for main parameters and MCMC options were configured to run fifty million generations and saving trees every 1000 generations. TreeAnnotator was used to summarize the posterior sample of trees to produce the maximum clade credibility tree and to specify the percentage of discarded trees. The first 10% trees were removed (burn-in) and a majority rule consensus tree was generated from the remaining. The posterior probability limit was set in 0.5. Tracer V1.6 (<http://tree.bio.ed.ac.uk/software/tracer>) was used to provide a useful summary statistics on the results of the analysis. The graphical representation of the tree (Fig. 2) was performed with

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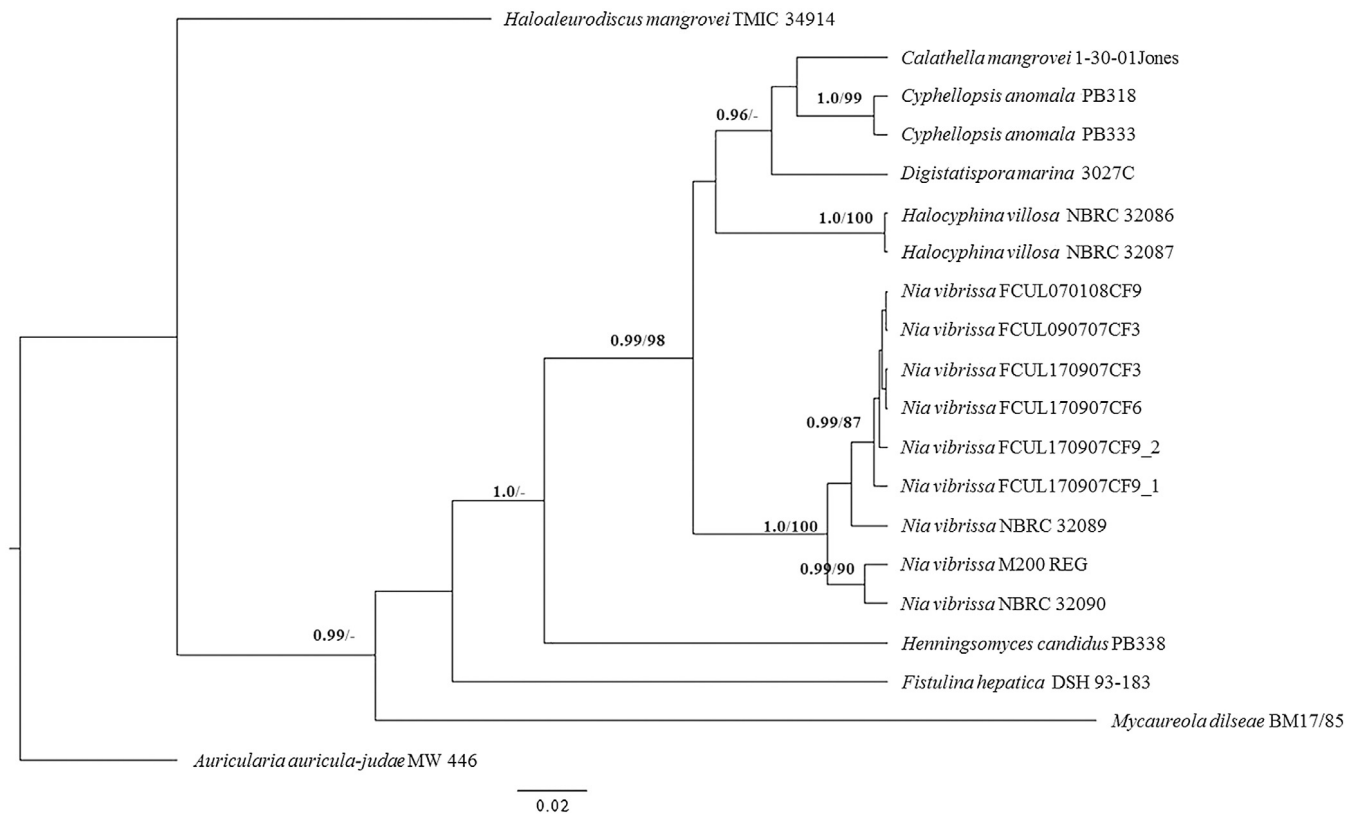


Fig. 2. Phylogenetic tree derived from Bayesian analysis based on nuc rDNA LSU data set. Posterior probability (PP) values from Bayesian analysis followed by bootstrap (BS) values from maximum likelihood analysis are added to the left of a node (PP/BS).

FigTree V1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>). Only significant values of posterior probability (PP > 0.95) and bootstrap (BS > 60) are shown.

3. Results

3.1. Morphological characterization

The microscopic characterization of the Portuguese *Nia vibrissa* specimens of this study is presented in Fig. 1 and Table 1. Morphological differences were found in basidiocarp color varying from light pink to brownish yellow (respectively 2.5YR 8/3 and 10YR 8/6 on Munsell soil color charts, 2010) and on peridial hair surface ranging from straight to bifurcate with curled ends.

Single spore colonies on 50% seawater CMA achieved 5 cm in 20 d at room temperature, presented sparse aerial mycelium with white color, plane relief and no pigment diffusion. Yellow globose basidiomata were differentiated within eight wk at room temperature (18 °C).

3.2. Phylogenetic analysis

The *N. vibrissa* sequences selected for phylogenetic analyses correspond to isolates collected in different geographic locations: six isolates are from Cascais marina, Portugal (wood baits), M200 REG from the west coast of Turkey, NBRC 32089 from Japan (sea foam) and NBRC 32090 from Singapore (wood in sand). The other sequences subjected to the phylogenetic analyses correspond to the marine Basidiomycota listed in Jones et al. (2015) that were found in databases and to terrestrial taxa placed close to *N. vibrissa* in previous studies (Binder et al., 2001; Hibbet and Binder,

2001; Bodensteiner et al., 2004; Binder et al., 2006). According to those criteria, from a previous list of 24 sequences (outgroup included), the following four sequences were removed due to pairwise distance values over 41%, between them and all the others (data not shown): *Favolaschia intermedia* L-13421-Sp; *Mycaureola dilseae* BM17/85; *Physalacria maipoensis* AF426970; *Schizophyllum commune* DQ071814.

The ML and Bayesian analyses applied to nuc LSU rendered identical results and, once the corresponding trees were similar, the tree generated with BEAST was selected to be presented in Fig. 2, where only significant PP and BS values are displayed.

Both phylogenetic analyses showed a highly supported clade (PP = 1, BS = 100) where the sequences of the six Portuguese isolates of *N. vibrissa* cluster together and with *N. vibrissa* NBRC 32089 (PP = 0.99, BS = 87), separately from the other group (PP = 0.99, BS = 90) consisting of the other two sequences of *N. vibrissa* under analysis (NBRC 32090, M200 REG). From the same highly supported node (PP = 1, BS = 97), also segregate the clades. *Halocyphina villosa* (two sequences, PP = 1, BS = 100) and *Cyphellopsis anomala* (two sequences, PP = 1, BS = 100), the last one clustering with *Calathella mangrovei* and *Digitatispora marina* with a significant PP value (0.96). *Hemingsomyces candidus* PB338 segregates in a sister branch (PP = 1). All the sequences referred above, *Fistulina hepatica* DSH93-183 and *Mycaureola dilseae* BM17/85(2) share the same origin (PP = 0.99). Other two sequences segregate separately: *Haloaleurodiscus mangrovei* TMIC 34914 and *Auricularia auricula-judae* MW446 (outgroup).

The pairwise distances matrix generated by the MCL approach targeting the same LSU alignment (Table 3) displays, for sequences of the same species, distances equal or lower than 3% (underlined in the table). This applies to *N. vibrissa* sequences, where distances

of 0% are found between the Portuguese isolates, exception for the isolates FCUL170907CF9_1 and FCUL170907CF9_2, exhibiting 1% distance. Distances of 2 and 3% are found between each Portuguese isolate and the others. Exception (distances of 4%, in bold type in the table) is *N. vibrissa* NBRC 32089 when compared both with *N. vibrissa* NBRC 32090 and *N. vibrissa* M200 REG. Also evidenced in bold type, are pairwise distances of 4% found between sequences of different species (*C. mangrovei* with both *C. anomala* PB318 and *D. marina*).

4. Discussion

The morphological diversity observed in peridial hairs was considered important to address the question whether or not *N. vibrissa* is a species complex, the answer to this question previously considered dependent upon further studies by several authors (Jones and Jones, 1993; Binder et al., 2001; Jones et al., 2009). This study, involving a group of six new isolates, morphologically characterized and subjected to phylogenetic analyses, is a contribution for the discussion of this taxonomic issue.

Our isolates reveal variability on basidiocarp color and dimensions, probably related to different stages of maturation, and on morphology of peridial hairs, varying from straight to bifurcate with curled ends.

Morphological comparison between our isolates and *N. vibrissa* isolates included in the phylogenetic analyses was only possible for *N. vibrissa* M200 REG whose peridial hairs were described as slightly curved, tips not bifurcated (Binder et al., 2001).

Despite the morphological differences found among *N. vibrissa* isolates, the phylogenetic analyses indicate a close genetic identity, once all isolates under analysis constitute highly supported clades. However, when looking at pairwise distances, the same level of variability can be found between sequences from different species and within the *N. vibrissa* clade. This result still keeps the first question addressed in this study as an open subject.

In what concerns the phylogenetic placement of *N. vibrissa*, our results are partially in accordance with previous studies (Hibbet and Binder, 2001; Bodensteiner et al., 2004; Binder et al., 2006), which showed a close relationship with *Halocyphina villosa* and its placement in a robust clade with a terrestrial taxon (*Cyphellopsis anomala*). However, this study does not confirm the proposed close relationship with *Favolaschia intermedia* and *Schizophyllum commune*. Sequences from these species were actually removed from the final alignment, due to the high values found for pairwise distances between them and all other sequences, also resulting in phylogenetic trees with generalized low support values.

Since highly supported by PP and BS values, this study also allows establishing a close relationship among the clades *Nia vibrissa*, *Halocyphina villosa*, *Cyphellopsis anomala* and two taxa represented by single sequences: *Calathella mangrovei* and *Digitatispora marina*. Noteworthy is the fact that 10% is the maximum value of pairwise distances found between pairs of sequences from those taxa. Values between 13 and 29% are found for pairwise distances involving the other taxa, in accordance with their placement in the phylogenetic trees.

5. Conclusion

This study contributed to redefine the *Nia* clade, as only consisting of *Nia vibrissa* sequences.

Considering that this study only targeted partial sequences of the nuc LSU, further phylogenetic methodologies involving other species of *Nia* and sequences of other regions, nuc rDNA ITS region included, are needed to address the genetic variability of *N. vibrissa*, and to better understand its relationships with marine and terrestrial Basidiomycota.

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