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DNA barcodes for marine fungal identification and discovery

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ARTICLE INFO

Article history:
Received 1 May 2012
Revision received 24 February 2013
Accepted 7 May 2013
Available online 5 July 2013
Corresponding editor:
Felix Bärlocher

Keywords:
Barcoding gap
DNA barcode
Internal transcribed spacer
Species boundary
5.8S rRNA
18S rRNA
28S rRNA

ABSTRACT

We employed DNA barcodes for identification of fungal species in marine sediments. Sediments were collected seasonally along the Southeast coast of India from which a culturable fungal library was constructed. All cultured species were morphologically documented using microscopical analysis. A maximum population density of 19.3 × 10^3 CFU/g was recorded in monsoon and minimum of 3 × 10^3 CFU/g in premonsoon season. Two-way analysis of variance suggests that the fungal community varied significantly between the seasons ($F = 9.543$, $P < 0.001$) and at various depths sampled ($F = 4.655$, $P < 0.05$). In total, 54 fungal species belonging to 13 different families were documented and all species were sequenced for internal transcribed spacer genes. Each species was represented by at least two specimens constituting a total of 171 specimens for DNA barcoding. Twelve species of a marine fungi were sequenced for the first time. Branching patterns of phylogenetic tree strongly supported the sequence variations within and between all species barcoded. Based on the pairwise distance model we suggest barcode gaps of 15 %, 21 %, 30 %, 35 % and 51 % for genera, family, order, class and phyla respectively.

Introduction

Fungi are an indispensable part of life in the biosphere as they have many functional roles in different ecosystems. Obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat. Facultative marine fungi are those from freshwater or terrestrial habitats able to grow, and possibly also sporulate, in the marine environment (Kohlmeyer & Kohlmeyer 1979). Knowledge of fungal biodiversity has become of prime importance from practical and scientific standpoints. Marine fungi are heterotrophic microorganisms occurring commonly in a wide variety of substrata as saprotrophs and parasites (Raghukumar 1990). All groups of fungi including filamentous higher fungi, yeasts, thraustochytrids and zoosporic fungi, occur as saprotrophs in large particulate detritus and also as parasites in plants and animals in the marine environment. However, the prevalence of these groups of fungi varies greatly in different marine ecosystems. There are approximately more than 100 000 species of described fungi (Blackwell 2011). The issue of the number of fungal species on earth has come to the fore since extrapolations from several independent data sets point to at

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1754-5048/$ – see front matter
http://dx.doi.org/10.1016/j.funeco.2013.05.003

least 1.5 million species of fungi. Of these, only 5–6 % are known (Hawksworth 1991, 2001; O'Brien *et al.* 2005; Kirk *et al.* 2008).

Morphological identification of fungi often requires cultivation of the target organism, which is time consuming and, in many cases, poses major problems (O'Brien *et al.* 2005), and the analysis of spore morphology as a key criterion in species description requires expertise. Therefore, molecular methods, such as DNA barcoding, may be the only rapid way to identify such organisms (Hebert *et al.* 2003). A DNA barcode is defined as a short and easily PCR amplifiable DNA fragment for identification of organisms to the species level. DNA barcoding must ideally be feasible for non-experts, accurate, rapid, cost-effective, culture-independent and universally accessible (Frézal & Leblois 2008). The aim of a barcode system is to be applicable for all kingdoms of eukaryotic life and to simplify recognition of cryptic species. The identification of fragments of organisms or different life cycle stages will become possible, where morphological identification is not realizable (Gilmore *et al.* 2009). This is a general problem for microorganisms, which live inside and outside of host systems, forming a mutualistic or parasitic association with a host. Barcoding can also facilitate the search for new species.

The first official barcode accepted by the Consortium for the Barcode of Life (CBOL) consists of approx. 650 bp of the cytochrome oxidase subunit I (COX1) gene in the mitochondrial DNA, and it is mainly used for animal species identification (Hebert *et al.* 2003). The mitochondrial DNA of plants has a low substitution rate, and a two-locus barcode from the chloroplast region, using the *matK* and *rbcL* genes consequently was proposed by the CBOL plant working group (Hollingsworth *et al.* 2009). The two-locus barcode successfully separated different species with a success rate of approx. 72 %, which did not increase significantly when taking into account all seven markers (*matK*, *rbcL*, *rpoC1*, *rpoB*, *psbK-psbI*, *trnH-psbA*, *psbK-psbI*) analysed together. *COX1* works well for most animal species (Ward *et al.* 2005; Prasannakumar *et al.* 2011); however some drawbacks have been detected within the fungi. In the genus *Penicillium*, *COX1* seems to be a likely candidate for species identification (Seifert *et al.* 2007), although it has considerable size variations within the genus. The *COX1* gene of fungi varied between 1 548 bp and 22 kb, similar to the barcoding region within *COX1* gene which ranged from 642 bp to 12.3 kb (Seifert *et al.* 2007; Seifert 2009). Only one *COX1* gene was published for just one glomeromycotan species with a length of 2 200 bp (based on NC_12056, Lee & Young 2009). However, the length could be varied as seen in other parts of the mitochondrial genome in the *Glomeromycota*. The mitochondrial large subunit (mtLSU), for example, has different sizes and introns even among closely related species (Raab *et al.* 2005; Börstler *et al.* 2008). Multiple copies (paralogous) of the *COX1* gene have been found in the genus *Fusarium* and proved inadequate for species level identification (Gilmore *et al.* 2009). A similar situation was observed within the complex group of *Aspergillus niger* (Geiser *et al.* 2007). Lang & Hijri (2009) reported a *COX1* intron in *Glomus diaphanum* with a high sequence similarity to both a plant sequence and a *Rhizopus oryzae COX1* intron. This intron may result from a lateral gene transfer from fungi to plants (Vaughn *et al.* 1995; Seif *et al.* 2005; Lang & Hijri 2009). Small ribosomal DNA sequences helped resolve the morphological similarity between two different fungal species, but sometimes its application in species identification is questionable. For example, rDNA sequences could not delineate closely related species like in the genus *Ambispora*, members of which have dimorphic spores (Walker *et al.* 2007). Hence, some scientists advocated the use of multiple gene sequences for fungal species identification (Amanda *et al.* 2010). Recent studies using molecular markers for fungal species identification typically use nuclear ribosomal genes such as the small subunit (SSU), the ITS region (ITS1-5.8S-ITS2) or the partial large subunit (including two variable sub-regions called D1 and D2) (Brock *et al.* 2009). For most fungi the ITS has become the default marker for species identification (Seifert 2009) and has been found superior to COI sequences (Dentinger *et al.* 2011). However, yeasts represent an exception, as the LSU is already widely used for species identification (Seifert 2009). The length of the ITS varies within fungi from 270 bp for yeasts to 973 bp for *Dothideomycetes*, but the indels and the length variations make the ITS difficult to align (Seifert 2009). This study aims to create a preliminary barcode library for the mycoflora in marine sediments and to test the efficacy of ITS gene in species level identification and resolution.

Materials and methods

Description of study area

Sediments were collected along the southeast coast of India (Table 1). A total of 10 stations were sampled.

Sediment collection and fungus isolation

Samples were collected during the postmonsoon season (Jan., Feb. and Mar.), summer (Apr., May and Jun.), premonsoon (Jul., Aug., Sep.) and the monsoon season (Oct., Nov. and Dec.). Sediment samples were collected using the Van-Veen grab, and the central portion was sub-sampled aseptically using a glass corer (2 cm diameter), and were always processed within 8 hr of collection. One gram of the sediment was serially diluted in filter sterile seawater and 0.5 ml of inocula was plated on to Rose Bengal Agar (HiMedia, India) supplemented with chloramphenicol 100 mg 1^{-1}, and Malt Extract Agar medium (HiMedia, India) supplemented with 0.1 g streptomycin and 10 000 units of penicillin/100 ml to inhibit bacterial growth (Nadimuthu 1998; Raghukumar *et al.* 2001, 2004). The two agar media differ in nutrient composition and required two different antibiotics which were highly effective in inhibiting bacterial growth in their respective media (Nadimuthu 1998; Raghukumar *et al.* 2001, 2004). All the sample aliquots were analysed in duplicates. Plates were incubated for one week to 15 d at 27 °C or as soon as the colonies appeared prior to spore formation, to avoid over estimation due to autoinoculation (Raghukumar *et al.* 2001). Two replicates were maintained for all the stations. The colonies formed on the plates were then counted and expressed as CFU g^{-1} of dry sediment weight. All the colonies were subsequently picked up, sub-cultured and maintained in slants for further studies.