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Barcoding and species recognition of opportunistic pathogens in *Ochroconis* and *Verruconis*

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ABSTRACT

The genera *Ochroconis* and *Verruconis* (*Sympoventuriaceae*, *Venturiales*) have remarkably high molecular diversity despite relatively high degrees of phenotypic similarity. Tree topologies, inter-specific and intra-specific heterogeneities, barcoding gaps and reciprocal monophyly of all currently known species were analyzed. It was concluded that all currently used genes viz. SSU, ITS, LSU, *ACT1*, *BT2*, and *TEF1* were unable to reach all 'gold standard' criteria of barcoding markers. They could nevertheless be used for reasonably reliable identification of species, because the markers, although variable, were associated with large inter-specific heterogeneity. Of the coding protein-genes, *ACT1* revealed highest potentiality as barcoding marker in mostly all parts of the investigated sequence. SSU, LSU, ITS, and *ACT1* yielded consistent monophyly in all investigated species, but only SSU and LSU generated clear barcoding gaps. For phylogeny, LSU was an informative marker, suitable to reconstruct gene-trees showing correct phylogenetic relationships. Cryptic species were revealed especially in complexes with very high intra-specific variability. When all these complexes will be taxonomically resolved, *ACT1* will probably appear to be the most reliable barcoding gene for *Ochroconis* and *Verruconis*.

Introduction

The genus *Ochroconis* was introduced by de Hoog & von Arx (1973) for melanized anamorphic fungi that were characterized by septate conidia with rhexolytic conidium liberation, a rather unique feature in ascomycetous fungi. The genus was phenotypically different from the neighboring genus *Scolecobasidium* by having elongate rather than T- or Y-shaped conidia. Members of the two genera were characterized by rust-brown to olivaceous colonies (Ellis 1971) and were judged

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to be closely related. Samerpitak et al. (2014) revised the taxonomy of both genera using nuclear ribosomal (nuSSU, nuLSU, and ITS) and protein coding (actin: *ACT1*, β-tubulin: *BT2*, translation elongation factor 1-α: *TEF1*) genes and confirmed an affinity of species with differentially shaped conidia. However, these authors also noted that the original material defining *Scolecobasidium* was ambiguous and therefore the genus was considered no longer valid. The authors recognized thirteen species in *Ochroconis*, and a new genus, *Verruconis* was introduced which was located at a significant phylogenetic distance from *Ochroconis*. In addition, *Verruconis* species were thermophilic, and the genus comprised the human opportunistic neurotroph, *Verruconis gallopava*. Machouart et al. (2014) investigated higher relationships of these fungi using conserved genes (nuSSU, nuLSU, mtSSU, and *RPB2*) and found that both *Ochroconis* and *Verruconis* belonged to the dothidealean order *Venturiales* in the family *Sympoventuriaceae*.

A remarkable feature of the *Sympoventuriaceae* was noted to be large phylogenetic distances among and high molecular variability within species. Only a fraction of this variability could be explained by the existence of unrecognized taxa or incomplete taxon sampling. Giraldo et al. (2014) described three new species, *Ochroconis icarus*, *Ochroconis ramosa,* and *Ochroconis olivacea*, during an investigation of *ochroconis*- and *verruconis*-like strains from clinical samples. Samerpitak et al. (2015a) proposed a new species, *Ochroconis globalis*, which was phylogenetically close to but morphologically different from *Ochroconis tshawytschae*. Crous et al. (2014) described *Ochroconis macrozamiae*, a sister species of *Ochroconis gamsii*. Three further phylogenetically different species were described as *Ochroconis bacilliformis*, *Ochroconis phaeophora,* and *Ochroconis robusta* (Samerpitak et al. 2015b).

Several new species were established, yet significant distances remained between most of the species notwithstanding the phenotypic uniformity of the two genera. Several species appeared to have degrees of molecular heterogeneity that are much larger than observed in most other ascomycetous fungi. Samerpitak et al. (2014) revealed that not only ITS, but even nuLSU, which is usually invariant between closely related ascomycetes, showed significant sequence variation for species recognition. A suitable barcode marker for identification of these fungi, however, requires that intra-specific is superior over inter-specific variation to meet a stringent pairwise distance (K2P) based barcoding concept. The present study aimed to evaluate six genes for their use as barcoding markers for routine identification of members of the *Sympoventuriaceae*.

Materials and methods

DNA preparation and sequencing

Sixty one strains of *Ochroconis*, *Verruconis* and related genera (Table S-1) were cultured and extracted for DNA according to Badali et al. (2008), Najafzadeh et al. (2009) and Feng et al. (2014). Six markers, viz. partial nuSSU, D1/D2 region of nuLSU, ITS, and the partial genes actin (*ACT1*), β-tubulin (*BT2*) and translation elongation factor 1-α (*TEF1*) were amplified using these primers; NS1, NS24 and Oli04 for nuSSU (Hendriks et al. 1989; White et al. 1990; Gargas & Taylor 1992), ITS4, ITS5, V9G and LS266 for ITS (White et al. 1990; Masclaux et al. 1995; de Hoog & Gerrits van den Ende 1998), LROR and LR7 for nuLSU (Vilgalys & Hester 1990), ACT-512F and ACT-783R for ACT1, EF1-728F and EF1-986R for TEF1 (Carbone & Kohn 1999), and Bt2a and Bt2b for BT2 (Glass & Donaldson 1995). The primer sequences and PCR conditions were shown in Table S-2. PCR reactions contained 1 μl of 100 pg to 100 ng of DNA template, 0.25 μl of 10 pM primer, 1.25 μl PCR buffer, 0.5 μl of 1 mM dNTP, 1 μl of 25 mM $MgCl_2$, 0.7 μl DMSO and 0.25 unit of *Taq* polymerase (Flexi-Taq®; Promega, Leiden, The Netherlands), and water was added to a final volume of 12.5 μl. For sequencing, 0.1–0.2 pg of template was sequenced using the Big dye terminator cycle sequencing RR mix protocol (Thermo Fisher Scientific), and PCR was performed in 25 cycles of 96 °C 10 s, 50 °C 5 s and 60 °C 4 min. Sequences were edited and adjusted by SEQMAN program, Lasergene software (DNASTAR, Madison, WI, USA). The length and guanine-cytosine content (G + C %) of each gene were analyzed using BioEdit v. 7.0.5.2. and MEGA6 (Tamura et al. 2011). nuLSU, nuSSU, ITS, and *TEF1* datasets were aligned using the web-based program MUSCLE and *ACT1* and *BT2* with Mafft (http://www.ebi.ac.uk). All aligned sequences were adjusted and edited using BioEdit v. 7.0.5.2.

Phylogenetic assessments

All single-gene datasets, viz. nuSSU, ITS, nuLSU, *ACT1, BT2,* and *TEF1,* and a six-genes concatenated were phylogenetically analyzed as described by Samerpitak et al. (2014). In brief, phylogenetic trees with the best models were reconstructed using maximum likelihood in MEGA6. Bootstrap analyses with 100 replicates were performed. Tree visualization and editing were done with MEGA6. Gene sequences of *Scolecobasidium excentricum* CBS 469.95 were used as outgroup in phylogenetic analyses.

Inter-specific and intra-specific distances via the best model estimation and barcoding gaps

Inter-specific and intra-specific distances for each gene were calculated by estimation of evolutionary divergence over sequence pairs between and inside species and conducted in MEGA6. Each gene was analyzed using its best model searched by MEGA6, as shown in Table 2. The average distances of each dataset were calculated by Microsoft Excel 2010. The barcoding gaps were calculated by Microsoft Excel 2010 as following; Barcoding gap = (lowest inter-specific distance)−(highest intra-specific heterogeneity).

Sliding window analysis

Sliding windows for each gene marker were inferred via the SPIDER (Species identity and evolution in R; http://spider.r-forge.r-project.org/) package in R statistical software, employing the pairwise distance (K2P as default) functions 'slideAnalyses' and 'slideBoxplots' as implemented in the package; with 'library' dependencies 'ape', 'pegas', 'adegenet', and 'ade4'. The sliding window 'walk' was pre-defined with 100 bp overall