Fungi associated with the skin of a southern right whale (*Eubalaena australis*) from South Africa

DESRAY REEB[1], PETER B. BEST[1], ALFRED BOTHA[2], KAREN J. CLOETE[2], MEREDITH THORNTON[1] & MARNEL MOUTON[2]

[1]Mammal Research Institute, University of Pretoria, c/o Iziko South African Museum, PO Box 61, Cape Town 8000, South Africa

[2]Department of Microbiology, University of Stellenbosch, Private Bag X1, MATIELAND, Stellenbosch, South Africa

Abstract

Cutaneous fungi are known to affect parts of the outermost skin layers of mammals, including the epidermis, stratum spinosum and stratum corneum, as well as mucocutaneous membranes, genitalia or external ears. Relatively little is known about the fungal infections of Mysticete cetaceans and studies are needed to determine the fungal diversity associated with these marine mammals. This case report was aimed at identifying the fungi associated with the skin of a diseased neonatal southern right whale (*Eubalaena australis*) found stranded in the Western Cape Province of South Africa. Initial physical examination on site revealed hyperplasia of the follicular and epidermal epithelium. Preliminary examination of skin biopsies using scanning electron microscopy indicated that the skin was colonized by fungal hyphae. Isolation methods yielded a number of fungal isolates of which the identity was determined using standard morphology and rDNA sequence data. These analyses confirmed colonization of the cutaneous layers by species belonging to the

genera *Fusarium, Chaetomium* and *Penicillium*. Moreover, all isolates were capable of degrading keratin indicating that skin may act as a natural substrate for these fungi. This study is the first report of the association of these fungi with southern right whale skin.

Keywords: Cutaneous fungi, *Fusarium, Chaetomium*, *Penicillium,* southern right whale, fungal diversity

Correspondence: Marnel Mouton, Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa. Tel: +27 21 8085857. Fax: +27 21 8085846. Email: marnel@sun.ac.za

Introduction

In the field of clinical pathology, four main types of fungal infections are recognized: superficial colonization (without tissue response), cutaneous infection (skin, eyes, sinuses, oropharynx, external ears and vagina), subcutaneous infection and deep infection (localized or disseminated) (De Hoog et al. 2000; Wagner and Sohnle 1997). In case of superficial colonization, the fungus grows on the compounds associated with the mammal skin including lipids and keratinous materials such as hair, without provoking an immune response. These fungi are known as commensals. Others can simply land on the mammal skin without growing or utilizing any host products as nutrients and are known as contaminants. Dermatophytes (fungi classified in *Epidermophyton, Microsporum* or *Trichophyton*) are generally regarded as commensals due to the fact that they are dependent on keratin as their source of carbon and nitrogen. Cutaneous mycoses include both dermatophytoses of the skin and non-dermatophyte cutaneous infections and pertains to muco-cutaneous membranes, living outermost skin, genitalia, as well as external ears (De Hoog et al. 2000; Matsumoto 2005).

Several mechanisms protect mammalian skin against colonization by pathogenic micro-organisms and their subsequent invasion into the epidermis or dermis. These include the physical barrier provided by hair, the epidermis, desquamation, the humidity of the skin, pH, niche filling by resident micro flora, exposure to ultraviolet light and the presence of inhibitory substances on the skin surface (Cove et al. 1992; Wagner and Sohnle 1997). The latter are secreted by sebaceous and sweat glands and may include mediators of specific humoral immunity (Gebhart and Kersten 1992). In cetaceans, however, the external glabrous body is characterized by the absence of hair, sebaceous and sweat glands (Parry

1949; Sokolov 1982; Yablokov et al. 1974). A study on three delphinid species (*Stenella attenuata, Delphinus delphis* and *Phocoena* phocoena) by Meyers and Seegers (2004) demonstrated that the enzyme lysozyme and the peptide group ß-defensins are products of the thick integument acting as non-specific defense against bacteria, fungi, algae and ecto-parasites.

Fungi form part of the normal skin micro flora of marine mammals (Buck 1984; Haldiman et al. 1985; Henk and Mullan 1996; Migaki and Jones 1983). However, research conducted on mammals showed that fungi may also be opportunistic and secondary invaders of skin tissue (Migaki and Jones 1983). Moreover, animals that are immuno- (Moeller 1997) or integumentarily compromised (Miller et al. 2002), malnourished, on long-term antibiotic or immunosuppressive agent treatment, or suffering from co-morbid diseases are generally more susceptible to fungal infection (Fowler and Cubas 2001; Reidarson et al. 2001). Interestingly, fungal diseases have also been documented to cause death in captive and beach-cast marine mammals (Buck et al. 1987; Higgins 2000; Mayer 1988; Migaki and Jones 1983; Sweeney and Ridgway 1975; Sweeney et al. 1976; Stroud and Roffe 1979; Tangredi and Medway 1980). However, infections from pathogenic fungi are less common or documented in free-ranging cetaceans, since diseased animals generally die at sea, which hampers further investigations (Dhermain et al. 2002). In southern right whales specifically, scant literature exists on the incidence of mycotic dermatitis. One case of zygomycosis in a stranded calf has been reported (Best and McCully 1979) that presented with multiple suppurative granulomatous lesions in the left epaxial muscle as a result of colonization by a representative of the genus, *Mucor*. A yeast infection caused by *Candida zeylanoides* has recently been documented in a beached southern right whale neonate (Mouton et al. 2009).

On 16 September 1999, the corpse of a beached 4.84 m long male southern right whale (SRW) neonate was reported from Voëlklip beach, Hermanus, South Africa (34° 25'S 19° 17.4'E). It was believed to have stranded overnight and was examined the next day. The calf suffered from a skin condition, possibly mycotic dermatitis although its body condition was excellent (code 2: fresh carcass of Pugliares et al. 2007), with no signs of autolytic bloating or exfoliation of the outer layers of skin. The eyes were noted as small and sunken. On gross inspection, the skin appeared unusually formed in places, seemingly lacking epidermal covering, and harbouring a heavy infestation of orange-colored cyamids, *Cyamus erraticus*. Other features noted were that the heart was flaccid and the pericardium contained several litres of straw-coloured fluid, while the bladder was grossly distended. Histological analysis of renal and cardiac samples from another calf with similar post-mortem features indicated renal congestion and interstitial myocardial oedema. The contents of the stomach, intestine and rectum were noted as an opaque reddish-brown fluid, greenish-yellow mucus and light greenish faeces respectively. The umbilicus was unhealed but granulating, the tracheoles of the lungs contained frothy air and an excised section of lung floated in water. These latter features plus the lack of meconium and the heavy external parasite infestation indicated that the animal must have been alive for some time (at least a few days) before stranding. These observations were very similar to the previously studied SRW neonate where *C. zeylanoides* was isolated from all the sampling sites (Mouton et al. 2009). The aim of our present study was to investigate the fungal diversity associated with the skin of the stranded neonate and compare those results to the previous findings.

Materials and Methods

This work was carried out under annual permits issued to PBB in terms of the Sea Fishery Act of South Africa, 1988 (Act no. 12 of 1998), dated 10th March 1998, and the Marine Living Resources Act of South Africa, 1998 (Act no. 18 of 1998), dated 29th January 1999 and 27th January 2000, respectively.

Sampling. Skin samples (approximately 5 cm^2 and 3 cm deep) were taken from five equally-spaced lesional areas along the mid-dorsal, lateral and mid-ventral surface, numbered 1 to 5 posteriorly from the neck region. Prior to cutting, each sampling site was first sterilized with 80 % ethanol to exclude post-mortem contaminants. The samples were placed in sterile foil strips and frozen at −20 ^{o}C on return from the field.

Scanning electron microscopy. For scanning electron microscopy, samples were stored in 25% (v/v) glutaraldehyde solution for a minimum of three days and a maximum of six days, after which they were placed in buffer containing 2.5% (v/v) glutaraldehyde, 0.1M NaH_2PO_4 and 0.2M Na_2HPO_4. The samples were subsequently stored at −5 ^{o}C, until further analyses. Frozen samples were dehydrated in a graded ethanol (EtOH) (Merck AG ethanol, Merck & Co., Inc., Whitehouse Station, NJ) series (30%, 50%, 70%, 80%, 90%, 100%), each solution being changed at 2h intervals for 2.5 hrs per concentration. The samples were subjected to two additional washes in absolute ethanol, for 2.5 hours each. The samples were subsequently critical-point dried from 100% EtOH in CO_2, mounted and coated with gold-palladium in a sputter coater and viewed using a JEOL JSM-5200 Scanning Microscope (JEOL-USA, Inc., Peabody, MA, USA) operating at 15kV.

Isolation and culturing of fungi. For isolation, frozen skin samples were plated out on a range of microbial growth media, including Biolab Malt Extract Agar (Biolab Diagnostics (Pty) Ltd., Wadeville, RSA), half strength Difco Potato Dextrose Agar (Becton Dickenson, Sparks, USA), Sabouraud Glucose Agar (Ronald, 1993), as well as Bird Seed Agar (Kurtzman and Fell 2000). These growth media are routinely used to cultivate a wide range of fungal taxa. After 2 - 7 days of incubation at 25°C, fungal colonies started to develop on some of the skin samples, which were subsequently selected for purification via repetitive culturing on Biolab Malt Extract Agar at 25°C. The fungal isolates were examined morphologically using light microscopy. To test for the ability to utilize keratin as a source of carbon and nitrogen, the fungal isolates were inoculated onto keratin azure medium according to the method of Scott and Untereiner (2004) and incubated at 25°C. Additionally, isolates were also inoculated onto Malt Extract Agar (MEA) adjacent to a sterile whale skin section and incubated at 25°C to support the keratin azure experiment.

Fungal genomic DNA extraction. To screen for non-culturable fungi, genomic DNA was extracted directly from frozen skin samples according to the method described by Hoffman and Winston (1987), whilst genomic DNA from fungal isolates was extracted using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Cat. Nr: D6005, USA). Fragments of the nuclear rDNA gene complex were amplified using the polymerase chain reaction (PCR). These included the D1/D2 (600 – 650 bp) region of the large subunit (LSU, 26S) rDNA, as well as the internal transcribed spacer region (ITS1, *ca.* 113 to 121 bp), 5.8S rDNA gene (*ca.* 161 bp) and ITS2 region (*ca.* 222 to 230 bp) (Lloyd-MacGilp et al. 1996). A similar procedure was followed for control reactions, where an equal volume of milliQ water was added to the reaction mixture instead of the template DNA. Universal fungal primer pairs were

used in the PCR including F63 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and LR3 (5'-GGT CCG TGT TTC AAG ACG G-3') (Fell et al. 2000) for the 26S fragment, as well as ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'; White et al. 1990) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) for the ITS1/5.8S/ITS2 fragment. In order to estimate the quantity of PCR product, the amplification products were separated electrophoretically on 1% (wt/vol) agarose gels, containing ethidium bromide (1 μg/ml) at 90 mV for 1h. DNA fragments were immediately purified by means of a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals (Pty) Ltd., S.A.) following the manufacturer's protocol (Roche instruction manual, vol. 2, Sept. 1999; Roche Molecular Biochemicals).

Sequencing and data analysis. Sequencing of the rDNA fragments was performed employing a Hitachi 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) using F63/LR3 and ITS1/ITS4 as sequencing primers. The fungal isolates were initially identified using the sequences to perform a BLAST search on the NCBI database for each of the isolates. The results of these searches were used to identify species with high and lower similarity on Genbank, for further use in compiling the datasets.

Multiple consensus sequences (500 – 600 bp) were aligned using ClustalX version 2.0.10 with default settings and final manual editing was performed with Sequence Alignment Editor Version 2.0a11. The *Chaetomium* sequence dataset included partial 28S sequences (Untereiner et al. 2001) of our four isolates and closely related *Chaetomium* spp., as well as other more distant species (from GenBank) and outgroups included *Daldinia concentrica* and *Xylaria curta*. A separate sequence dataset was compiled for the ITS1, 5.8S, ITS2 region of the *Penicillium* sp.

including species with high similarity, as well as more distant spp. and the outgroup included *Chaetomium globosum* and *Phialophora europae*. The datasets were analyzed using Parsimony Analysis in PAUP 4.0b10 (Swofford 2002). Using heuristic searches, the 'Multrees' option was activated while performing tree bisection reconstruction (TBR) branch swapping to find the most parsimonious tree. Gaps were treated as missing data. Bootstrap support for nodes was evaluated from 1000 heuristic searches. Groups with a bootstrap frequency of 50% and higher were included in the consensus trees.

Results

Examination of the skin using scanning electron microscopy, revealed a mat of fungal mycelia, yeast-like cells, as well as bacterial colonies covering the skin. A scanning electron micrograph showing the hyphal growth is presented in Figure 1A and B. However, no characteristic features could be recognized enabling species identification.

The range of skin sections on growth media yielded four fungal isolates from four different sampling sites on the animals' body. These isolates were identified using morphological characteristics as well as sequencing results of the LSU and the ITS1/5.8S/ITS2 regions of the fungal rDNA and results are presented in Table I. These include *Chaetomium murorum* (W47, W49), *C. globosum* (W48) and *Penicillium coprophilum* (W50).

To test for the ability to utilize keratin as a source of carbon and nitrogen, the fungal isolates were inoculated onto keratin azure medium (Scott and Untereiner 2004), where we found that all the isolates were able to release the blue dye into the

underlying clear medium indicating their ability to utilize keratin. Moreover, isolates were able to colonize the sterile whale skin section on the MEA and even formed sexual fruiting bodies on these (Fig. 1C).

After a few days of incubation at 25°C on MEA, prior to the appearing of fruiting bodies, light microscopy of the *Chaetomium* isolates revealed the presence of unusual densely packed and also irregular jig-saw-shaped cells (textura intrica / epidermoidea) (Fig. 1D). These cells later form the peridium (fruit body wall) in these ascomycetes and were later recognized as the structured observed in the scanning electron micrographs (Fig. 1B). One to two weeks later, light microscopy of the *C. murorum* (W47 and W49) isolates revealed violet-blue to black spherical to ovoidal ascomata. The ascomatal setae were long, flexuous often recurved at the apex, smooth, verrucose or spinulose. Asci were clavate containing eight ascospores. The ascospores were ellipsoidal to broadly fusiform, brown with a distinct apical pore and darker longitudinal bands. The *C. globosum* isolate (W48) was identified by its spherical to ovoidal ascomata with brown peridium. Numerous brownish, unbranched, septate and coiled ascomal hairs could be observed. The asci were clavate, containing 8 ascospores. Brown ascospores were limoniform with an apical germ pore. The identities of the *C. murorum,* as well as *C. globosum* isolates, could be confirmed using partial sequences of the LSU ribosomal DNA (500 – 600 bp, 28S) (Spatafora et al., 1998). The phylogenetic tree (Fig. 2) shows that the two *C. murorum* isolates group closely with a *C. murorum* isolate (MUCL 40179; CBS 163.52) (Genbank nr.:AF286405.1) from an unknown substrate (96 % parsimony bootstrap). Other closely related species include a *Chaetomium / Thielavia* sp. from porcupine dung (MUCL 40242) (Genbank nr.:AF286412.1), *Zopfiella ebrosia* (Genbank nr.:AY346305.1) and *T. cephalothecoides* from mouse dung (MUCL

40270) (Genbank nr.:AF286413.1) (95 % parsimony bootstrap). The *C. globosum* isolate grouped with other *C. globosum* isolates (97 % parsimony bootstrap) including species from canine skin (Genbank nr.:AB292591.2), cow dung (Genbank nr.:AY346272.1) and stored cotton (Genbank nr.:AF286403.1).

The *Penicillium* isolate (W50) was characterized microscopically by an abundance of terverticillate mononematous conidiophores. Conidia were observed to be smooth-walled, ovoid to ellipsoidal produced in chains from flask-shaped phialidic conidiogenous cells, narrowing towards the collulum. No sexual phase could be observed. Colonies were characterized by a dark brown reverse color on MEA, as well as a brown diffusible color on MEA. Colony diameter after one week on CYA was 21 – 24 mm at 30°C and 29 – 33 mm at 25°C. Using sequencing data from the internal transcribed spacer 1 region, the 5.8S rDNA gene and the internal transcribed spacer region 2, this species was identified as *Penicillium coprophilum*. This was confirmed with a phylogenetic analysis where this *Penicillium* isolate grouped closer to the *P. coprophilum* spp. than any of the other close related species such as *P. concentricum, P. chrysogenum* and *P. commune.*

Genomic DNA extracted directly from skin from the neonate's body and the internal transcribed spacer region of the rDNA was amplified with PCR using fungal specific primers. Sequence analyses of the ITS1/5.8S/ITS2 regions of the rDNA revealed the presence of *Fusarium oxysporum* (Genbank accession nr.:GQ241936).

Discussion

Mycological and systematic analyses of fungal isolates from skin samples revealed the presence of fungi belonging to the genus *Chaetomium* that are well known to

occur in marine environments (Grishkan et al. 2003). Although infections caused by *Chaetomium* spp. have not yet been reported in marine mammals, *Chaetomium* spp. are well known to be causative agents of infections in humans: *C. globosum*, for example, has been related to cases of onychomycosis, cutaneous lesions and was isolated from the pleural fluid of a patient with leukemia (De Hoog et al. 2000). In addition, an infection of the subcutaneous tissue and dermis of the chest and abdominal area in a Chinese patient was attributed to *C. murorum* (De Hoog et al. 2000). Other infections caused by *Chaetomium* spp. include brain abscesses and peritonitis (De Hoog, et al. 2000; Guppy et al. 1998; Rock 1998; Sutton et al. 1998). *Penicillium coprophilum* was also isolated from the cutaneous tissues of the calf although this species is not a documented pathogen. However, the keratin azure experiment clearly proved the ability of this species to utilize keratin which would explain its presence on the whale skin. Fungi belonging to the genus *Penicillium* are commonly considered as contaminants but may cause infections, particularly in immuno-compromised hosts (Wong et al. 2001). In addition to their infectious potential, nearly all *Penicillium* spp. are active producers of mycotoxins (Pitt et al. 2000). This genus is widespread and represents the most abundant group of mesophilic fungi from temperate soil, decaying vegetation, and many species are airborne explaining their ubiquity (De Hoog et al. 2000).

Results from molecular analyses of genomic fungal DNA directly from skin samples indicated the presence of *Fusarium oxysporum*. The occurrence of *Fusarium* infections or fusariosis have been recorded in captive marine mammals (Higgins 2000), whilst an increased frequency of opportunistic infections in humans and in animals such as reptiles, turtles, pinnipeds and dolphins were reported over the years (Cabañes et al. 1997; Frasca et al. 1996), including evidence of cutaneous

nodules presumed to be caused by *Fusarium oxysporum* in a stranded Atlantic white-sided dolphin (*Lagenorhynchus acutus*) and pygmy sperm whale *(Kogia breviceps)* along the northeast coast of the USA (Frasca et al. 1996). Moreover, similar nodules were found in a captive beluga (Bowenkamp et al. 2001).

Many marine mammal studies have proposed a linkage of immuno-suppression with contaminants, environmental stress, including anthropogenic impacts, and disease among marine mammals (Aguilar and Borrell 1994; Hutchinson and Simmonds 1994; Ross et al. 1996a, 1996b). When an animal is faced with a stressor, a variety of neurochemical and hormonal changes occur with notable physiological consequences that have been well-described (Burchfield 1985; Chrousos and Gold 1992; Guyton and Hall 1996). The latter may also increase the probability of infection from pathogenic agents (Solomon et al. 1985). This case study is the first report of the presence of the *Chaetomium* spp. and *P. coprophilum* with the skin of a southern right whale.

Schaeffer et al. (1998) suggested that literature on pathology in marine mammals may contribute as a tool in assessing the stress and health of an ecosystem. Recognizing alterations in health, assessing and monitoring the effects of stress on marine mammal populations might provide an understanding of underlying causes of mortality among marine mammals (Fair and Becker 2000). Our previous study documented a yeast infection in a similar beached southern right whale neonate (Mouton et al. 2009) whereas this study provides evidence of the presence of four more fungal species associated with the skin of this second beached neonate. We believe that documenting our findings will contribute to the knowledge of fungi

associated with southern right whale skin, thereby expounding the role of these opportunists in this unique ecological niche.

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Figure and Table captions

Table I. Fungal isolates from different skin sections of the southern right whale neonate cultured on different growth media and identified using morphology, as well as sequencing data from the 26S and ITS regions of the ribosomal gene cluster.

Figure 1A – D. Micrographs of fungi associated with the southern right whale neonate skin. (A & B) Scanning electron micrographs showing fungal growth on the whale skin. (B) Scanning electron micrograph of an early formation of a fruiting body. (C) Fruiting bodies (ascomata) of *C. globosum* on a section of whale skin. (D) A light micrograph of the unusually formed cells of *C. globosum* starting to form the textura intrica / epidermoidea of the peridium (fruit body wall).

Figure 2. Maximum parsimony phylogenetic tree based on rDNA partial large subunit sequences (± 500 bp) of whale skin fungal isolates and selected sequences from Genbank. Numbers above branches indicate bootstrap values (> 50%) from 1000 replicates.

Tables

Table I. Fungal isolates from different skin sections of the southern right whale neonate cultured on different growth media and identified using morphology, as well as sequencing data from the 26S [a] and ITS regions [b] of the ribosomal gene cluster.

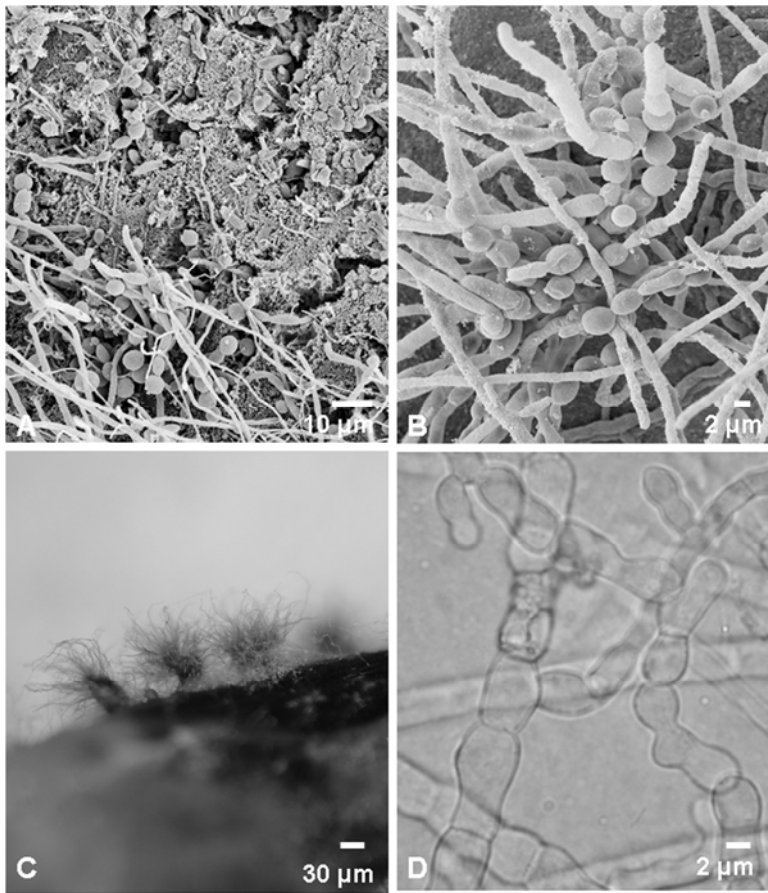| Isolate nr.	Sampling site	Isolation medium	Identity	Accession nr.
W47	Mid-ventral 4	Bird Seed Agar	*Chaetomium murorum*	GQ241932 [a]
W48	Mid-dorsal 2	Malt Extract Agar	*Chaetomium globosum*	GQ241934 [a]
W49	Right lateral 3	Half strength Potato Dextrose Agar	*Chaetomium murorum*	GQ241931 [a]
W50	Right lateral 5	Sabouraud Glucose Agar	*Penicillium coprophilum*	GQ241935 [b]

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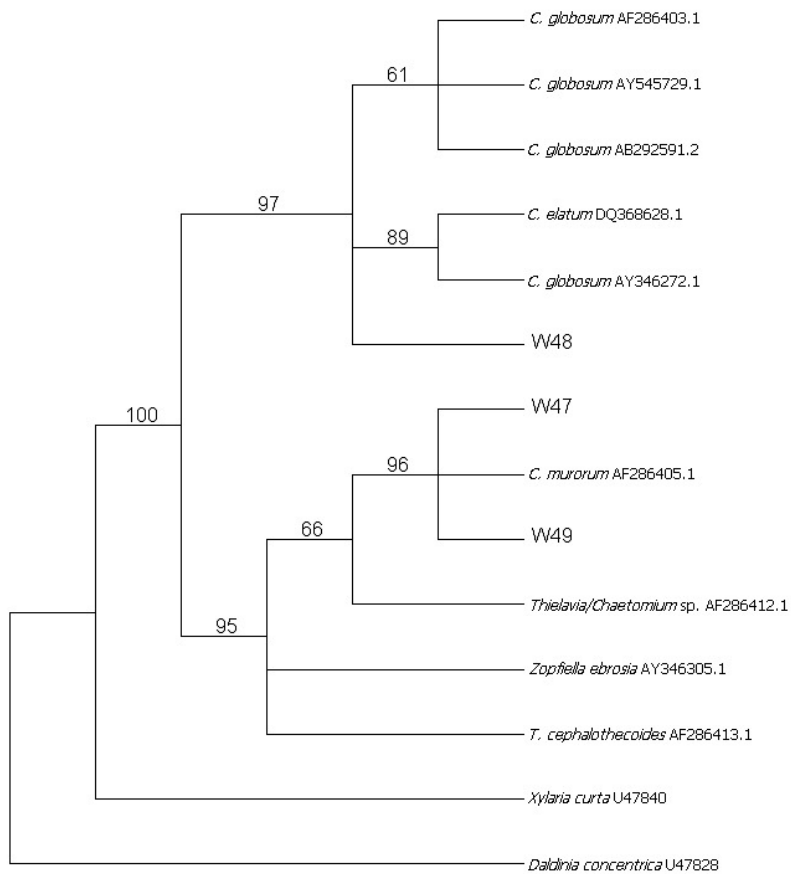
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