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ARTICLE

Harorepupu aotearoa (*Onygenales*) gen. sp. nov.; a threatened fungus from shells of *Powelliphanta* and *Paryphanta* snails (*Rhytididae*)

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Abstract: A cleistothecial fungus, known only from the shells of giant land snails of the family *Rhytidae*, is described as a new genus and species within *Onygenales*, *Harorepupu aotearoa* gen. sp. nov. Known only from the sexual morph, this fungus is characterized morphologically by a membranous ascoma with no appendages and ascospores with a sparse network of ridges. Ribosomal DNA sequences place the new species within *Onygenales*, but comparison with the known genetic diversity within the order linked it to no existing genus or family. It is the first species of *Onygenales* reported from the shells of terrestrial snails. This fungus has been listed as Critically Endangered in New Zealand and has been previously referred to as '*Trichocomaceae* gen. nov.' in those threat lists.

Key words: *Gastropoda* snail shell phylogeny *Trichocomaceae* Gondwana

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INTRODUCTION

Few fungi have been reported from the shells of terrestrial snails compared to aquatic snails (Říhová *et al.* 2014). In a survey of fungi associated with empty shells of *Cepaea hortensis*, Říhová *et al.* (2014) reported 27 species, mostly common soil fungi. They found few potentially keratinolytic species and concluded that the fungi they detected were likely to be accidental colonisers rather than specialist shell decomposing fungi. Snail shells have a layer of calcium carbonate covering a core of conchiolin, a keratin-like compound very resistant to decay (Ormsby *et al*. 2006, Goffer 2007).

Říhová *et al*. (2014) mentioned a report on the NZFungi database (http://nzfungi2.landcareresearch.co.nz/) of a species of *Trichocomaceae* reported from shells of *Powelliphanta* and *Paryphanta* species in New Zealand. These snails are members of the family *Rhytididae* (*Mollusca*; *Gastropoda*; *Pulmonata*), the thick shells of which are composed almost entirely of conchiolin with only thin outer layers of calcium carbonate (Ormsby *et al*. 2006). Hitchmough (2002) listed this fungus as 'Undescribed genus, *Trichocomaceae'* and accorded it a Nationally Critical threat status. The same fungus has been mentioned in Department of Conservation reports (e.g. Anon. 2007, Miller & Holland 2008).

The tentative NZFungi identification of the fungus on *Powelliphanta* and *Paryphanta* as *Trichocomaceae* was based on the macroscopic appearance of the ascomata and ascospore morphology. An asexual morph has not been observed. *Trichocomaceae* is a family in *Eurotiales*, some species of which have sexual morphs similar to those of *Onygenales*, the two orders being most easily distinguished morphologically by their asexual morphs (Currah 1994). Currah (1994) notes that amongst these fungi, the keratin degrading species are restricted to the families *Onygenaceae* and *Arthrodermataceae* within *Onygenales*. Of these two families, the fungus on *Powelliphanta* and *Paryphanta* is morphologically similar to *Onygenaceae sensu* Currah (1985). Two fungi reported from cultures derived from *Cepaea* shells by Říhová *et al*. (2014) were identified using DNA sequences as *Onygenales*, but the sequences for these are not available.

In this paper we describe the fungus associated with *Powelliphanta* and *Paryphanta* shells as a new genus within *Onygenales incertae sedis*, its phylogenetic position being based on SSU, ITS and LSU sequences. We compare it with the known genetic diversity within the order.

MATERIALS AND METHODS

Morphology

Fungarium specimens were rehydrated in 3 % KOH and the hymenial elements examined microscopically in either 3 % KOH or 3 % KOH mixed with Lugol's iodine solution. Vertical sections were cut at a thickness of about 10 µm using a freezing microtome and mounted in lactic acid. Material for scanning electron microscopy (SEM) was obtained by placing a mass of dried ascospores onto carbon tape on a stub, then sputter coating with gold. Photomicrographs taken on a Jeol Neoscope JCM-5000 (Landcare Research). Specimens have been deposited in PDD.

Molecular analyses

For DNA extraction, three separate extractions were done from three different ascomata from PDD 105262. DNA was extracted and amplified using a REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, USA), following the manufacturer's protocol except that the ascomata were ground in 30 µL extraction solution with a plastic pestle. Amplification primers for ITS were ITS1F and ITS4 (White *et al.* 1990; Gardes & Bruns 1993), for LSU were LR0R and LR5 (Bunyard *et al*. 1994; Vilgalys & Hester 1990), and for SSU were NS1 and NS4 (White *et al*. 1990).

Additional sequence data of SSU, LSU and ITS were downloaded from GenBank (Table 1). Sequences of each gene were aligned with MAFFT 7.122b (Katoh & Standley 2013) and trimmed with BioEdit (Hall 1999). Alignments were deposited in TreeBASE (www.treebase.org/treebase/), study accession number 17085. Molecular phylogenies were constructed using Bayesian inference (BI) and maximum likelihood (ML). To select the most appropriate model of sequence evolution, jmodeltest 2.1.1 (Darriba *et al*. 2012; Guindon & Gascuel 2003) was applied on each alignment (ITS, SSU, LSU). The GTR + I + G model was selected for ITS, SSU, and LSU according to the Akaike information criterion (AIC). The SSU and LSU matrices were concatenated with SeaView (Gouy *et al*. 2010). BI analyses were performed with MrBayes 3.2 (Ronquist & Huelsenbeck 2003). Three independent Markov chain Monte Carlo (MCMC) runs were performed simultaneously. Each MCMC ran for 3 x 10^6 generations for the SSU+LSU analysis and the ITS analysis, sampling every 500 generations until convergence (standard deviation of split frequency < 0.01). The first 25 % of trees were discarded as burn-in while the remaining trees combined with a 50 % majority rule consensus. ML analyses were performed with phyML 3.0 (Guidon *et al*. 2010) running inside SeaView (Gouy *et al.* 2010) with the following options: GTR model; aLRT branch support; empirical nucleotide equilibrium frequencies; optimized invariable site; optimized across site rate variation with 8 rate categories; NNI tree searching operations; BioNJ starting tree with optimized tree topology.

TAXONOMY

Harorepupu P.R. Johnst., H.D.T. Nguyen, D.C. Park, & Hirooka, **gen. nov**.
MycoBank MB811561

Etymology: From the Māori words harore = fungus, and pūpū = snail (fem.).

Diagnosis: Ascomata globose, sessile, membranous, solitary or in small, confluent groups; asci subclavate, wall undifferentiated; ascospores hyaline, oblong-elliptic, ornamentated with anastomosing ridges.

Type: *Harorepupu aotearoa* P.R. Johnst. *et al.* 2015 .

Harorepupu aotearoa P.R. Johnst., H.D.T. Nguyen, D.C. Park, & Hirooka, **sp. nov.**
MycoBank MB811562
(Fig. 1)

Etymology: The species epithet is from the Māori word for the country of origin.

Diagnosis: Ascomata 0.8–1.2 mm diam, white to pale yellow; asci 13–16 × 7.5–8.5 µm, 8-spored; ascospores 4.2–5.4 × 2–3.1 µm (average 4.8 × 2.6 µm), oblong-elliptic, ends rounded, sparse network of narrow, ridge-like ornamentations.

Type: **New Zealand**: *Nelson*: Golden Bay, Wainui Falls Tr., on empty shell of *Powelliphanta* sp., 16 May 2014, *P.R. Johnston* FUNNZ 2014/0999 (PDD 105262 – holotype).

Description: *Ascomata* 0.8–1.2 mm diam, globose, sessile, membranous, surface slightly woolly but with no distinctive appendages, white to pale yellow; Opening by irregular cracks, revealing the dry, powdery, bright yellow spore masses inside; wall 80–100 µm thick, comprising tightly tangled hyphae 4–6 µm diam, walls thin, mostly hyaline, outer 3–4 rows of cells sometimes with pale yellow walls; outermost layers of hyphae sometimes with ends free; peridial appendages lacking. *Asci* 13–16 × 7.5–8.5 µm, clavate with a narrow, foot-like base and rounded apex, wall thin, undifferentiated, 8-spored, contents orange-brown in Lugol's iodine. *Ascospores* 4.0–5.5 × 2–3 µm (average 4.8 × 2.6 µm), oblong-elliptic, ends rounded, ornamented with sparse network of narrow, anastomosing ridges, hyaline to pale yellow, 0-septate. Asexual morph not seen.

Additional specimens examined: **New Zealand**: *Nelson*: vic. Karamea, Kohaihai, Nikau Walk, on empty shell of *Powelliphanta* sp., 11 May 1994, *P.R. Johnston* (PDD 74629); vic. Karamea, Oparara Basin, Moria Gate Track, on empty shell of *Powelliphanta* sp., 10 May 2006. *T. Atkinson* FUNNZ 2006/1066 (PDD 92048); vic. Westport, Charming Creek Walkway, on empty shell of *Powelliphanta* sp., 10 May 2006 *A. Wilson* FUNNZ 2006/0160 (PDD 89035). *Northland*: Waipoua Forest, on empty shell of *Paryphanta* sp., 2001, *E. Horak*, (PDD 74625).

RESULTS

DNA sequences from all three ascomata from PDD 105262 were identical. They have been accessioned as GenBank KP683349, KP683350, and KP683351.

Phylogenetic analyses with the combined SSU + LSU sequences was performed to determine the higher taxonomic placement of *Harorepupu aotearoa*. After removing ambiguously aligned regions, SSU and LSU alignments were both 1300 base pairs long and contained a total of 257 (20 %) and 421 (32 %) parsimony informative characters respectively. Both the BI analysis (Fig. 2) and ML analysis (not shown) placed *H. aotearoa* in an isolated position in *Onygenales*. To determine whether we could place it in a well-supported family in *Onygenales*, we then performed phylogenetic analyses of the ITS region, with an

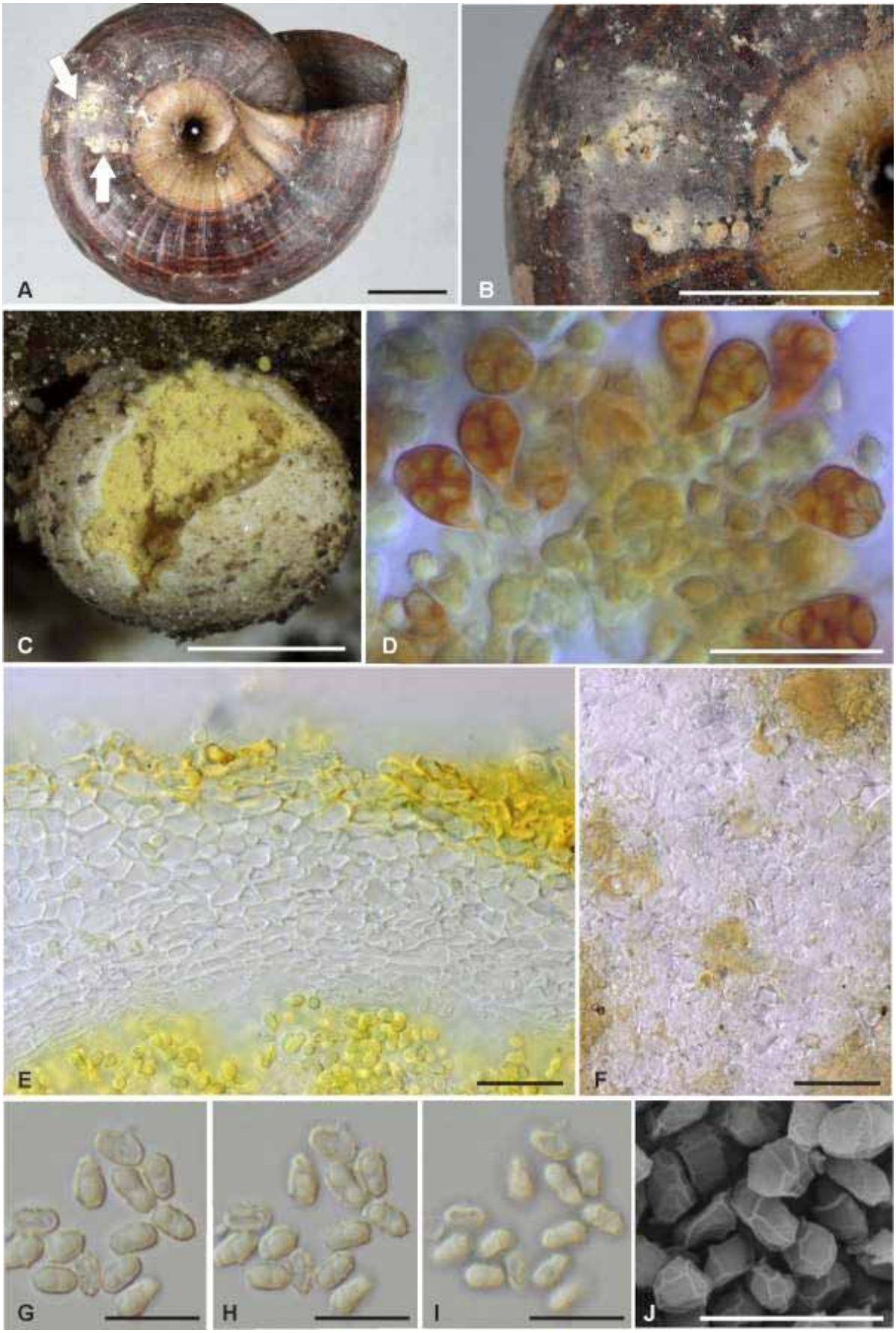

Fig. 1. *Harorepupu aotearoa*. **A.** Ascomata on shell, arrows indicate groups of ascomata on host shell (PDD 105262). **B.** Detail from A. **C.** Ascoma with wall breaking to expose powdery mass of yellow spores inside (PDD 74629). **D.** Asci in 3 % KOH plus Lugols iodine (PDD 74629). **E.** Ascomatal wall in vertical section (PDD 89035). **F.** Surface of ascoma (PDD 105262). **G–I.** Ascospores under light microscope, at three planes of focus (PDD 105262). **J.** Ascospores under SEM (PDD 105262). Bars: A, B = 10 mm; C = 0.5 mm; D, G–J = 10 µm; E–F = 20 µm.

Table 1. Species, culture, or voucher numbers, and GenBank accession numbers of isolates used in the phylogenetic analyses.

Genus and species	Strain Number	18S	28S	ITS
Roccellographa cretacea	AFTOL-ID 93	DQ883705	DQ883696	—
Dendrographa decolorans	DUKE 47570	NG_013155	NG_027622	—
Ramularia endophylla	AFTOL-ID 942 = CBS 113265	DQ471017	DQ470968	—
Dothidea insculpta	CBS 189.58 = AFTOL-ID 921	NG_016493	NG_027643	—
Arachnomyces glareosus	CBS 116129	FJ358341	FJ358273	—
Arachnomyces kanei	UAMH 5908	AF525308	—	—
Arachnomyces minimus	CBS 324.70	FJ358342	FJ358274	—
Capronia pilosella	AFTOL-ID 657	DQ823106	DQ823099	—
Cyphellophora laciniata	AFTOL-ID 1033	EF413618	EF413619	—
Exophiala pisciphila	AFTOL-ID 669	DQ823108	DQ823101	—
Caliciopsis orientalis	AFTOL-ID 1911 = CBS 658.74	DQ471039	DQ470987	—
Caliciopsis pinea	AFTOL-ID 1869	DQ678043	DQ678097	—
Monascus purpureus	AFTOL-ID 426	DQ782881	DQ782908	—
Xeromyces bisporus	CBS 236.71	FJ358355	FJ358291	—
Byssochlamys nivea	CBS 100.11	FJ358345	FJ358279	—
Penicillium javanicum	AFTOL-ID 429	EF413620	EF413621	—
Aspergillus amstelodami	DAOM 222011 = ATCC 16464 = CBS 518.65	JN938999	JN938912	JN942872
Chaenothecopsis savonica	Tibell 15876	U86691	AY796000	—
Mycocalicium polyporaeum	ZWGeo60Clark	AY789361	AY789362	—
Stenocybe pullatula	Tibell 17117	U86692	AY796008	—
Sphinctrina turbinata	AFTOL-ID 1721	EF413631	EF413632	—
Ajellomyces capsulatus	ATCC 26032	AF320009	—	—
Ajellomyces capsulatus	CBS 136.72	—	AB176497	—
Ajellomyces capsulatus	UAMH 7141	—	—	AF038353
Ajellomyces dermatitidis	ATCC 18187	—	AY176704	—
Ajellomyces grisea	CBS 128.88 = UAMH 5409	AB075361	—	—
Ajellomyces grisea	UAMH 6836	—	AY176721	AY527404
Histoplasma capsulatum var. *duboisii*	H147	—	—	AB055247
Arthroderma ciferrii	AFTOL-ID 428	EF413624	EF413625	—
Arthroderma racemosum	UAMH 3367 = ATCC 18910 = CBS 423.74 = IMI 135822	—	—	HQ825139
Ctenomyces serratus	CBS 187.61	FJ358347	FJ358282	AJ877222
Epidermophyton floccosum	CBS 230.76	Z34923	—	—
Keratinomyces ceretanicus	CBS 269.89	—	—	AJ877224
Microsporum audouinii	CBS 109478	GU733362	—	—
Microsporum audouinii	ATCC 10216	—	EF078482	—
Microsporum ferrugineum	CBS 427.63	—	—	AJ252336
Trichophyton equinum	CBS 112198	—	—	EF043275
Trichophyton rubrum	CBS 118892	JX431933	JX431933	—
Trichophyton rubrum	UAMH 8547	—	—	AF170471
Ascosphaera apis	CBS 402.96	FJ358343	FJ358275	—
Ascosphaera apis	ATCC MYA-4451	—	—	FJ172293
Ascosphaera colubrina	CBS 160.87	FJ358344	FJ358276	U68320
Ascosphaera duoformis	ARSEF 5140	—	HQ540518	—
Ascosphaera subglobosa	A.A. Wynns 5004	—	HQ540517	—
Ascosphaera subglobosa	DAOM 188973	—	—	HQ540521
Eremascus albus	CBS 975.69	FJ358348	FJ358283	—
Arachniotus littoralis	CBS 454.73	FJ358340	FJ358272	—
Arachniotus ruber	UAMH 3543	AY177296	—	—
Gymnascella aurantiaca	CBS 655.71	AB015772	AB040684	—

Table 1. (Continued).

Genus and species	Strain Number	18S	28S	ITS
Gymnoascus confluens	IMI 100873 = UAMH 3565	—	—	AJ315837
Gymnoascus desertorum	CBS 634.72	—	—	AJ315838
Gymnoascus petalosporus	CBS 252.72 = UAMH 1712	AB015773	AB040685	—
Gymnoascus reesii	CBS 259.61	FJ358349	FJ358284	—
Kraurogymnocarpa trochleospora	CBS 591.71 = ATCC 18900 =UAMH 10101	—	AB075344	KF477238
Rollandina hyalinospora	CBS 548.72 = UAMH 3155 = NRRL 2881	AB015775	AB040687	—
Nannizziopsis barbata	UAMH 11185	—	—	JF323871
Nannizziopsis hominis	UAMH 7859	—	—	KF477215
Nannizziopsis infrequens	UAMH 10417	—	—	AY744467
Nannizziopsis obscura	UAMH 5875	KF466865	—	—
Nannizziopsis vriesii	UAMH 3527	—	—	KF477198
Nannizziopsis vriesii	ATCC 22444 = UAMH 3713 = CBS 407.71 = IMI 149994	AY304510	AY176715	—
Paranannizziopsis californiensis	UAMH 10693	KF466867	—	—
Paranannizziopsis crustacea	UAMH 10199	KF466868	—	—
Onygena equina	TU101989	—	—	UNITE-UDB018096
"Paracoccidioides" sp.	No name	—	—	HQ413323
Amaurascopsis perforata	FMR 5489	AJ315171	—	—
Amaurascopsis reticulata	IFO 9196	—	—	AJ271434
Amaurascopsis reticulata	CBS 392.61	—	—	AJ271418
Amauroascus aureus	ATCC 18654 = = CBS 593.71 = NRRL 12,184 = UAMH 3157	—	AY176705	—
Amauroascus mutatus	CBS 181.70	—	—	AJ271567
Amauroascus niger	IFO 32599 = ATCC 22339 = UAMH 3544	—	—	AJ133434
Aphanoascella galapagosensis	UAMH 11703	—	JQ864082	JQ864081
Aphanoascus arxii	CBS 466.88	—	—	AJ315843
Aphanoascus foetidus	CBS 452.75	—	—	AJ439448
Aphanoascus fulvescens	NBRC 31723 = ATCC 36140 = IFO 31723	JN941600	JN941548	—
Aphanoascus reticulisporus	IMI 336466	—	—	AJ439441
Apinisia graminicola	CBS 721.68	AB015781	AY176709	—
Apinisia racovitzae	CBS 151.65	—	—	AJ271429
Arachnotheca glomerata	CBS 348.71	—	AB075352	—
Ascocalvatia alveolata	ATCC 22147 = CBS 777.70 = UAMH 6475	—	AY176710	—
Auxarthron reticulatum	UAMH 2006	—	—	AJ271568
Auxarthron umbrinum	UAMH 3952	—	—	AY177309
Auxarthron zuffianum	CBS 219.58	—	AY176712	—
Auxarthronopsis bandhavgarhensis	NFCCI 2185 = CBS 134524	JQ048939	JQ048938	HQ164436
Byssoonygena ceratinophila	ATCC 64724 = FMR 785	—	AB075353	—
Chlamydosauromyces punctatus	UAMH 9990	AY177297	—	—
Chrysosporium parvum	UAMH 1067	U29390	—	—
Coccidioides immitis	ATCC 7366	—	AY176713	—
Coccidioides immitis	CBS 166.51	—	—	EF186783
Coccidioides posadasii	IFM 4935	—	—	AB232883
Emmonsia crescens	UAMH 3008	—	—	AF038334
Emmonsia parva	UAMH 130	—	—	AF038333
Emmonsia pasteuriana	UAMH 9510	—	—	EF592152
Emmonsia sp.	UAMH 10539	—	—	EF592156
Emmonsia sp.	UAMH 7101	—	—	EF592154
Emmonsia sp.	FDBC2	—	—	JQ247333

Table 1. (Continued).

Genus and species	Strain Number	18S	28S	ITS
Kuehniella aurea	CBS 593.71	—	AB075360	—
Lacazia loboi	No name	AF238301	—	—
Malbranchea cinnamomea	CBS 960.72	GU733363	—	—
Malbranchea cinnamomea	CBS 343.55	JQ067912	JQ067903	—
Malbranchea dendritica	UAMH 2731 = ATCC 34527 = CBS 131.77 = IMI 211199 = NCMH 367	AY124496	—	—
Malbranchea gypsea	IFM 47365	—	AB359425	—
Onygena equina	ATCC 22731 = IFO 31785 = CBS 947.70	—	AY176717	—
Ophidiomyces ophiodiicola	UAMH 6642	KF466869	—	—
Paracoccidioides brasiliensis	R-2878	AF227151	—	—
Paracoccidioides brasiliensis	Pb18	—	—	AF322389
Polytolypa hystricis	UAMH 7299	—	AY176718	AY527405
Harorepupu aotearoa	PDD 105262	KP683351	KP683349	KP683350
Renispora flavissima	UAMH 4140 = ATCC 38503	U29393	AY176719	—
Uncinocarpus queenslandicus	IFM 47370	—	—	AB361646
Uncinocarpus reesii	UAMH 160	L27991	—	—
Uncinocarpus reesii	ATCC 34533 = UAMH 3880 = CBS 121.77	—	AY176724	—
Uncinocarpus reesii	UAMH3881 = ATCC 34534 = CBS 120.77	—	—	JF451137
Pseudospiromastix tentaculata	CBS 184.92	AB075362	AY176722	AY527406
Spiromastix asexualis	UTHSC DI-13-1	—	KJ880031	KJ880032
Spiromastix princeps	IMI 169642	—	—	AJ315840
Spiromastix warcupii	AFTOL-ID 430	DQ782882	DQ782909	DQ782848
Pyrgillus javanicus	AFTOL-ID 342	DQ823110	DQ823103	—
Granulopyrenis seawardii	CBS 109025 = AFTOL-ID 2013	EF411059	EF411062	—
Dermatocarpon luridum	AFTOL-ID 2277	EF689833	EF643750	—
Placidiopsis cinerascens	AFTOL-ID 2284	EF689842	EF643759	—
Polyblastia melaspora	AFTOL-ID 1356	EF689854	EF643770	—
Geoglossum nigritum	AFTOL-ID 56	AY544694	AY544650	—
Trichoglossum hirsutum	AFTOL-ID 64	AY544697	AY544653	—
Cladonia caroliniana	AFTOL-ID 3	AY584664	AY584640	—
Lecanora concolor	VR 2-IX-00/17	AY640993	AY640954	—
Mitrula elegans	WZGeo47Clark	AY789334	AY789335	—
Pseudogymnoascus pannorum var. *pannorum*	CBS 108.14	AB015785	AB040703	—
Thelebolus ellipsoideus	AFTOL-ID 5005	FJ176840	FJ176895	—
Myriodontium keratinophilum	DUMC 134.08	—	—	EU925387
Myriodontium keratinophilum	MEA-B4-D	—	—	JX869561
Ascobolus crenulatus	AFTOL-ID 181	AY544721	AY544678	—
Hypocrea americana	AFTOL-ID 52	AY544693	AY544649	—
Chaetomium globosum	15-5973	AY545725	AY545729	—
Xylaria hypoxylon	spat03-03	AY544692	NG_027599	—

alignment of 876 base pairs in length that contained 456 (52 %) parsimony informative characters. *H. aotearoa* is sister to *Nannizziopsiaceae* but lacking strong statistical support, where the aLRT branch support was only 0.74 in the ML analysis (data not shown) and the posterior probability is only 0.58 in the BI analysis (Fig. 3). All phylogenetic analyses show that *H. aotearoa* represents an isolated lineage within *Onygenales*.

DISCUSSION

Although *Harorepupu aotearoa* has never been grown on artificial media, we obtained DNA sequence data from dried specimens. Our comprehensive LSU and SSU phylogenetic tree showthat this fungus is a member of *Onygenales* and that is distantly related from any recognized onygenalean fungi. In our ITS tree, *H. aotearoa* was sister to the *Nannizziopsiaceae*

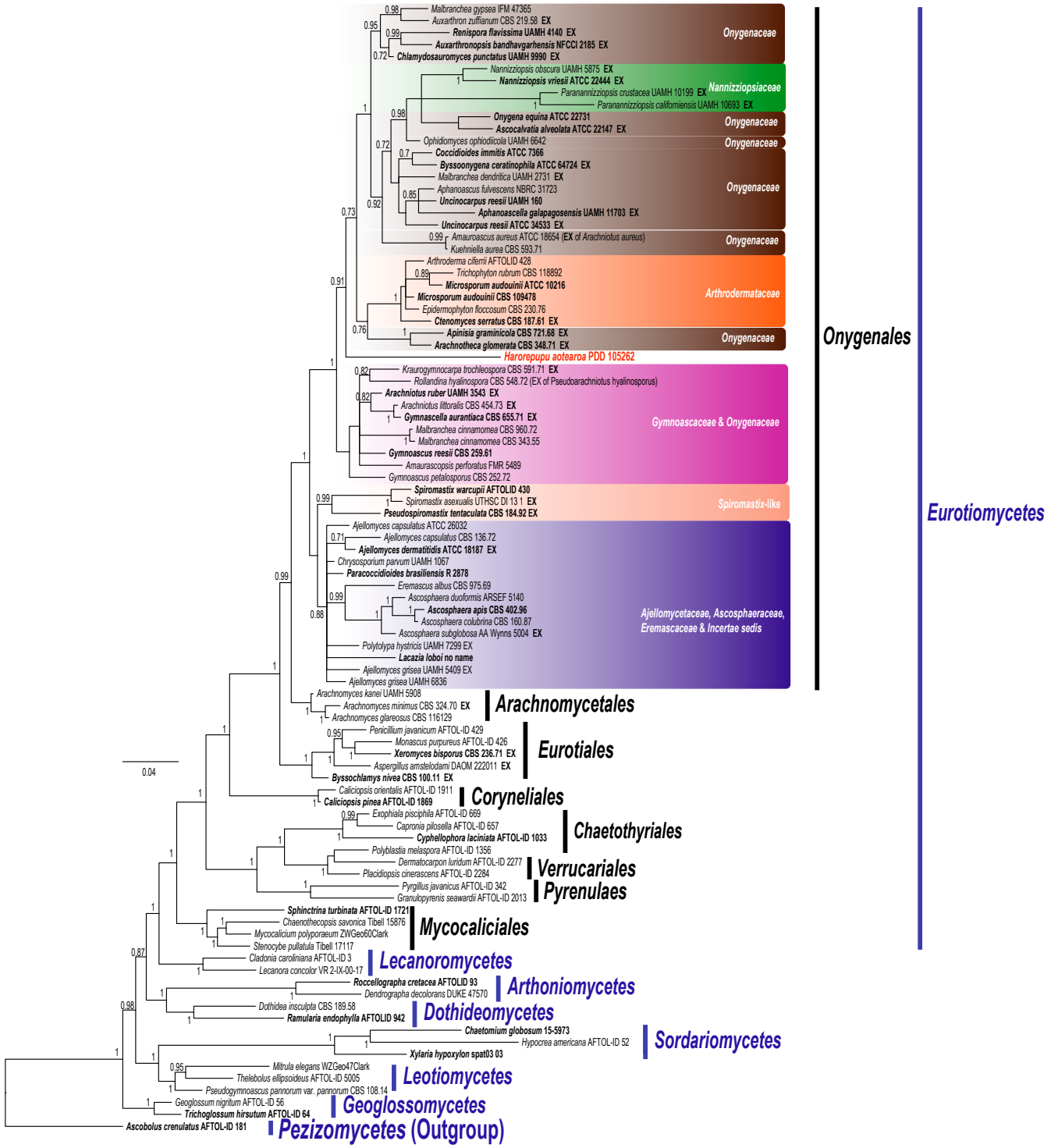

Fig. 2. 50 % majority rule consensus tree from Bayesian inference analysis of SSU and LSU sequences. Posterior probabilities greater than 0.7 shown above the edges. Taxa labelled EX are represented by sequences from ex-type cultures; bold type indicates the type species of genera.

clade but with low support in the BI analysis. The family *Nannizziopsiaceae* was described by Stchigel *et al*. (2013) on the basis of D1/D2 phylogenetic data, host range, morphology, and colony odour. Based on sexual morphology, historically taxonomically important for the group, species in *Nannizziopsiaceae* differ from our fungus in having ascomata with peridial appendages and ascospores that appear smooth under the light microscope (Currah 1985). The future discovery of additional species of *Harorepupu*, and of any asexual morph, could help clarify its position within the order. For now, however, we prefer to treat it as *incertae sedis* within the order rather than introduce a new family name for this single genus.

The biology of *Harorepupu aotearoa* is not understood, but as all collections are on empty shells of members of the family *Rhytididae*, it may be restricted to this substrate. If this is the case, threats to the snail population will present a threat to the fungus population. At present, with increased

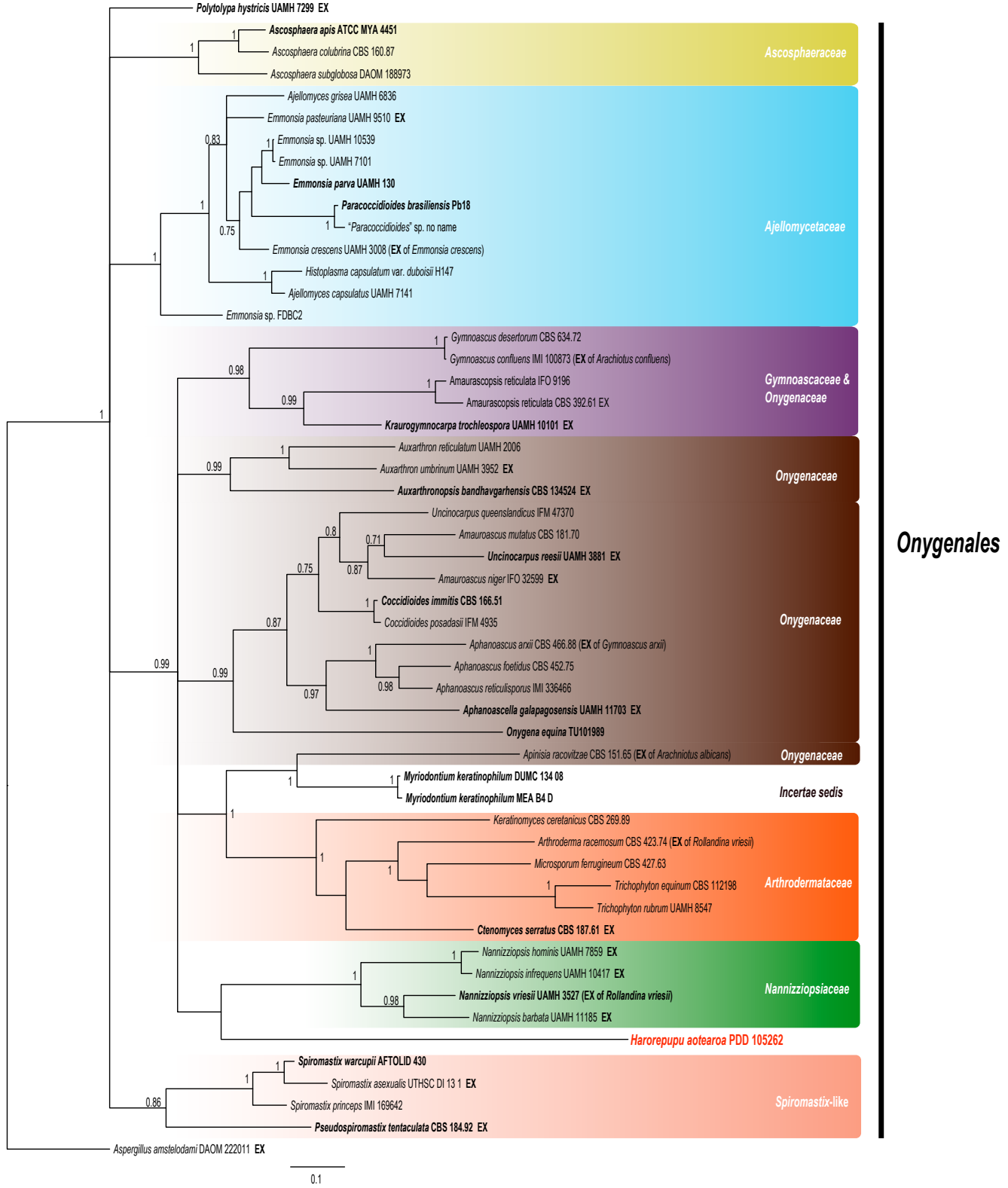

Fig. 3. 50 % majority rule consensus tree from Bayesian inference analysis of ITS sequences. Posterior probabilities greater than 0.7 shown above the edges. Taxa labelled EX are represented by sequences from ex-type cultures; bold type indicates the type species of genera.

predation and disturbance resulting in larger numbers of dead *Rhytididae* shells on the forest floor, this fungus may temporarily be more common than usual.

Members of the family *Rhytididae* are distributed across many regions linked geologically to Gondwana. Although *Harorepupu* is at present known only from New Zealand, additional material, and perhaps more species, may be expected on the shells of these snails in other regions.

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