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Molecular characterization and lesions associated with *Diomedenema diomedeae* (Aproctoidea: Desmidocercidae) from grey-headed albatrosses (*Thalassarche chrysostoma*) on Subantarctic Marion Island

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ABSTRACT

The Prince Edward Islands are Subantarctic islands in the southwest Indian Ocean that are of global importance as seabird nesting sites, and are breeding grounds for five species of albatrosses (Procellariiformes: Diomedeidae). In March–April 2016 numerous chicks of one of these species, the grey-headed albatross (*Thalassarche chrysostoma*), were found dead at colonies on Marion Island (46°57′S 37°42′E), the larger of the two Prince Edward Islands. Affected chicks were weak, prostrated, apathetic, had drooping wings, and many eventually died while sitting on the nest. Five carcasses were necropsied, and samples were obtained for pathological and parasitological analysis. Four chicks appeared to have died from starvation, and one died due to air-sac helminthiasis, with extensive hemorrhage in the air sacs and multifocal pyogranulomatous air-sacculitis. The air sac parasites were identified as *Diomedenema diomedeae* (Aproctoidea: Desmidocercidae). Phylogenetic analysis of the nuclear *18S rRNA* gene and mitochondrial *COI* gene confirmed that *D. diomedeae* belongs to the suborder Spirurina and showed that it is most closely related to the Diplotriaenidae (superfamily Diplotriaenoidea), a family of parasites that infect the air sacs and subcutaneous tissues of a variety of bird species. To our knowledge this is the first record of the occurrence of a nematode in the respiratory tract of an albatross and the first study to provide DNA sequences for a species of the superfamily Aproctoidea.

1. Introduction

Albatrosses (Procellariiformes: Diomedeidae) are the world's most endangered family of seabirds (Croxall et al., 2012), and yet there is limited information on the pathogens and parasites affecting these birds (Weimerskirch, 2004; Quintana, 2011). The Prince Edward Islands are Subantarctic islands in the southwest Indian Ocean that are of global importance as seabird nesting sites (Williams et al., 1979). These islands being breeding grounds for five species of albatrosses (Procellariformes: Diomedeidae): wandering albatross (*Diomedea exulans*), grey-headed albatross (*Thalassarche chrysostoma*), Indian yellow-nosed albatross (*T. carteri*), dark-mantled sooty albatross (*Phoebetria fusca*), and light-mantled sooty albatross (*P. palpebrata*) (Ryan et al., 2009; Schoombie et al., 2016). Although no studies have examined the occurrence of metazoan parasites in albatrosses on these islands, it is reasonable to assume that they are infected by the same species that have been recorded elsewhere considering the circumpolar distribution of these birds (Barbosa and Palacios, 2009; Quintana, 2011).

In April–May 2016, chicks of grey-headed albatrosses were found dead at colonies on Marion Island, the larger of the two Prince Edward Islands. Due to the history of avian cholera outbreaks (Cooper et al., 2009) and the recent occurrence of mouse scalping (Dilley et al., 2016) and avian pox in seabirds at Marion Island (Schoombie et al., 2018), this mortality led to concern and prompted a parasitological and pathological investigation.

In this study we report the results from the post-mortem examination of five grey-headed albatross chicks, including the identification of the nematode *Diomedenema diomedeae* in association in the air sacs of one bird. *D. diomedeae* had not been recorded since its original description by Johnston and Mawson (1952) and belongs to the

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Fig. 1. A grey-headed albatross (*Thalassarche chrysostoma*) chick at Grey-headed Albatross Ridge on Marion Island with drooping wings. A chick sitting with a normal posture can be seen in the background. Photo: P.G. Ryan.

superfamily Aproctoidea (class Chromadorea, order Spirurida, suborder Spirurina), a group of parasites for which there are no publicly-available DNA sequences. We obtained sequences of the nuclear small ribosomal subunit RNA gene and the mitochondrial cytochrome c oxidase subunit I gene of this parasite, and employed Bayesian analyses to infer the evolutionary relationship of this parasite to other spirurid worms.

2. Materials and methods

During four nest checks between 3 April and 26 May 2016 of all accessible colonies on Grey-headed Albatross Ridge and along the coast to Rook's Hut on Marion Island (see Schoombie et al., 2016), grey-headed albatross chicks were observed to be weak, prostrated, and apathetic, with drooping wings (Fig. 1). Over time an increasing number of chicks became too weak to lift their heads. At the time, the affected colonies were also heavily impacted by mouse attacks on chicks (Dilley et al., 2016). However, many chicks died without any impact from mice or predators.

Five of these “dead on nest” chick carcasses were collected for post-mortem examination from Grey-Headed Ridge (46°57′18″ S, 37°42′29″ E; cases 1, 2, 4 and 5) and Rooks Bay West (46°57′50″ S, 37°40′34″ E; case 3). All chicks were near fledging, with only a few patches of downy feathers remaining. Case 3 was seen alive a few hours before it died, and appeared very weak, lethargic and with drooping wings. The remaining cases were not observed before death. Carcasses were still in *rigor mortis* when collected, but due to field constraints the carcasses had to be kept at ambient temperature (2–10 °C) until necropsies could be conducted 11 h (case 1) or 72 h (cases 2 and 3) after carcass retrieval, or carcasses had to be frozen for later examination (cases 4 and 5).

Samples of organs and tissues were fixed in 10% neutral buffered formalin for 48 h, then transferred to 70% ethanol. Parasites were collected in 70% ethanol. Tissues were then embedded in paraffin and 3–5 µm sections were obtained, stained with hematoxylin-eosin and examined under light microscopy. Parasites (19 females and two males)

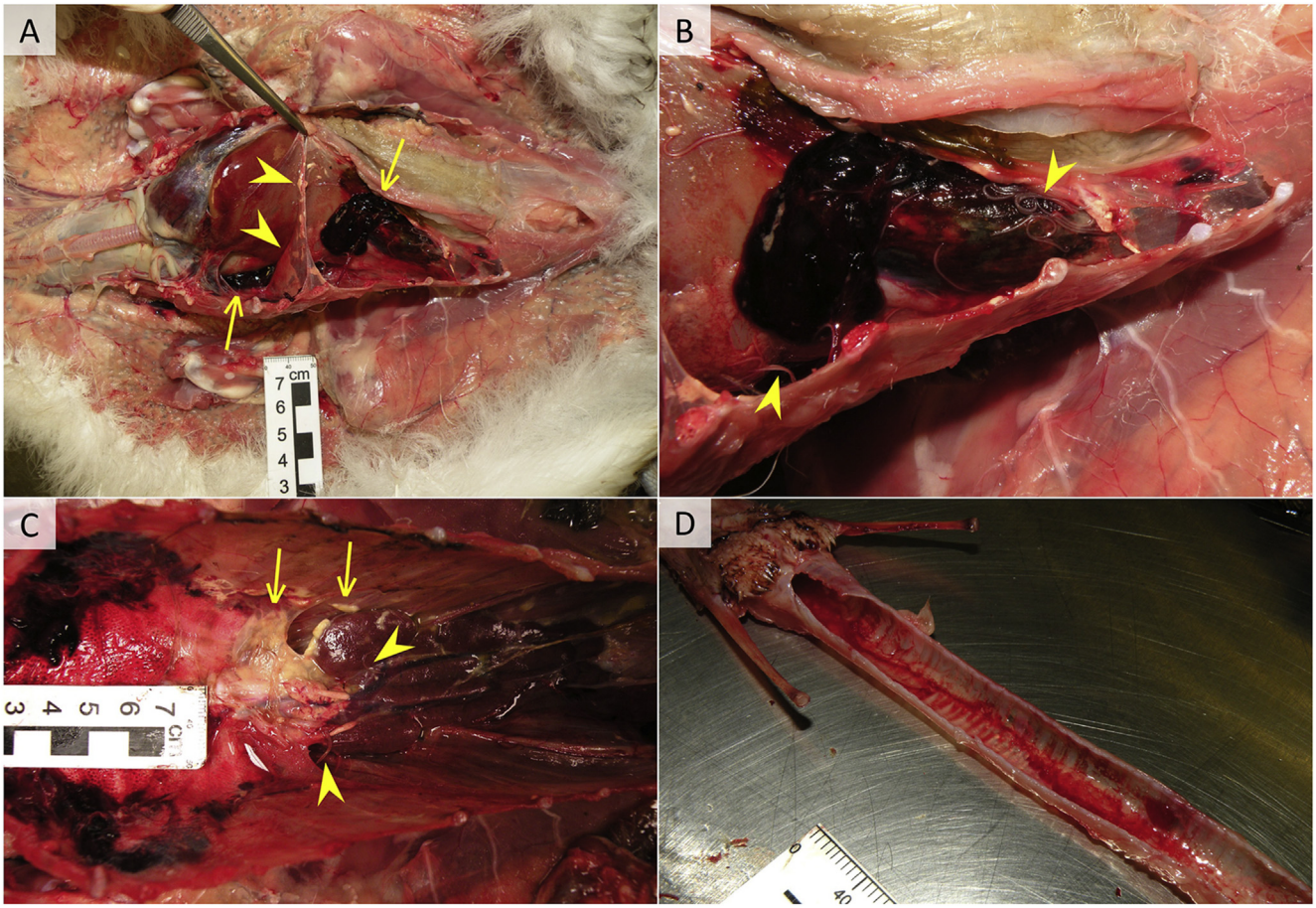

Fig. 2. Lesions associated with *Diomedenema diomedeae* infection in a grey-headed albatross chick (*Thalassarche chrysostoma*). Legend: (A) blood clots (arrows) and masses of pus (arrowheads) in the right thoracic air sac; (B) close-up of the blood clot and nematodes (arrowheads) in the right thoracic air sac; (C) nematodes (arrowheads) and masses of pus (arrows) in the abdominal air sacs; (D) tracheal hemorrhage.

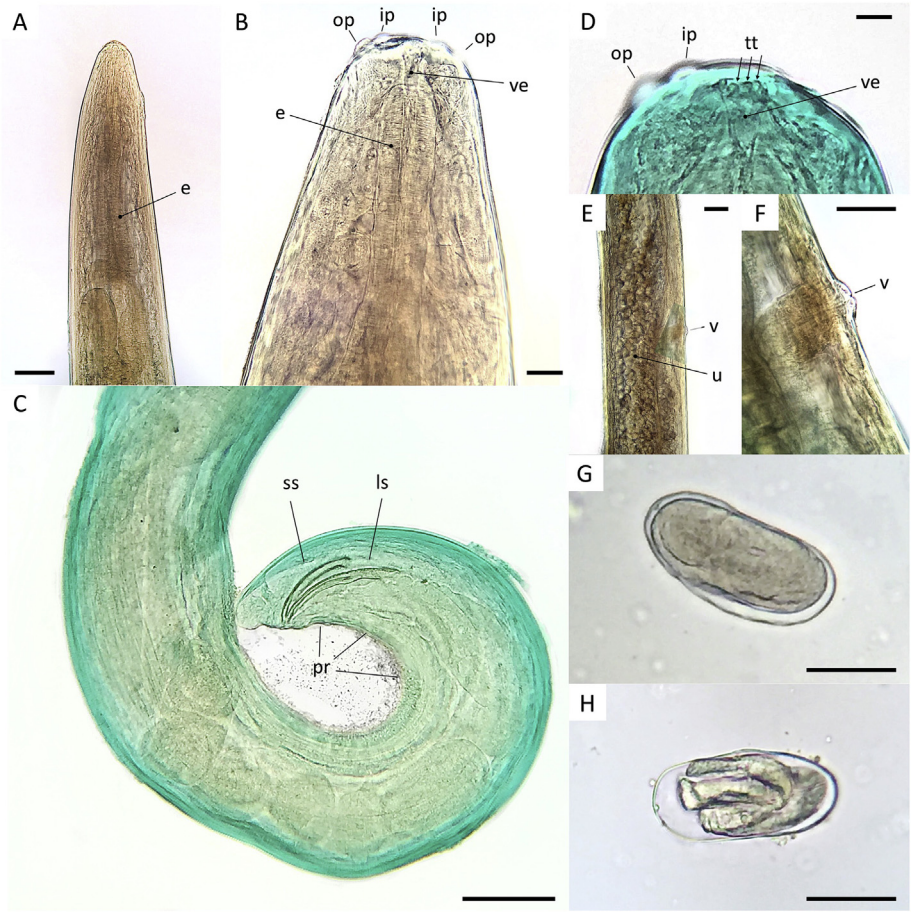

Fig. 3. Morphological characteristics of *Diomedenema diomedeae*. (A) Female, lateral view of the cephalic end: esophagus (e). (B) Female, dorsal view of the cephalic extremity: outer papilla (op), inner papilla (ip), vestibulum (ve), esophagus (e). (C) Male, lateral view of the posterior end (fast green counterstaining): large spicule (ls), small spicule (ss), precloacal papillae (pr). (D) Female, dorsal view of the cephalic end (fast green counterstaining): tricuspid tooth (tt), outer papilla (op), inner papilla (ip), vestibulum (ve). (E,F) Female, lateral view: uterus (u), vulva (v). (G,H) Eggs. Scale bars: (A) 125 µm, (B) 30 µm, (C) 150 µm, (D) 15 µm, (E) 100 µm, (F) 50 µm, (G,H) 30 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were examined under a stereomicroscope for morphological identification (Anderson et al., 2009; Bain et al., 2014).

DNA was extracted from the parasites (one male and one female) with the DNEasy Blood and Tissue kit (#69504, Qiagen, Hilden, Germany) following the manufacturer's instructions. Two gene fragments were amplified: part of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene using a cocktail of M13-labeled primers for nematodes (Prosser et al., 2013), and part of the small ribosomal subunit RNA (*18S rRNA*) gene using primers NEMFG1 and CRYPTOR (Bimi et al., 2005). Amplicons were visualized on 0.8% agarose gels stained with Gel Red Nucleic Acid Gel Stain 10,000× in DMSO (Biotium, Hayward, CA). Amplicons were extracted from the gel, purified using the QIAquick gel extraction kit (Qiagen) then bidirectionally sequenced at the Georgia Genomics Facility (Athens, GA). Partial sequences of the *COI* and *18S rRNA* genes (712 bp and 1733 bp, respectively) were deposited in GenBank (accession codes MG805658 and MG805661).

Phylogenetic analyses of the two gene sequences were conducted with publicly-available sequences from spirurid nematodes (Supplementary Data S1). Sequences were aligned using Muscle (Edgar, 2004) as implemented in MEGA 7.0.26 (Kumar et al., 2016). Bayesian phylogenetic trees were produced using MrBayes 3.2.6 (Ronquist et al., 2012). The GTR + I+Γ model of nucleotide evolution was used for both genes as recommended by jModelTest 2.1.10 (Darriba et al., 2012). For each gene, two Markov chains were run simultaneously for 10 million generations with sampling every 500 generations; the first 25% of trees were discarded as a burn-in step and the remaining trees were used to calculate the posterior probabilities.

3. Results

A total of 24 “dead on nest” grey-headed albatross chicks were found over the study period, 0.67% of pre-fledgling chicks counted in 2016 (n = 3595). This is a minimum estimate because other carcasses may already have been scavenged by giant petrels (*Macronectes* spp.), brown skuas (*Catharacta antarctica*) or lesser sheathbills (*Chionis minor*), or showed signs of mouse predation.

Details of the necropsy and histopathological findings for the five collected carcasses are provided in Supplementary Data S2. In case 1, nematodes were present in large numbers in the right thoracic air sac (> 100 individuals), and right abdominal air sac (c. 80 individuals), but were also present in the left thoracic air sac (5–10 individuals). Masses of pus were present in the right thoracic and abdominal air sacs (Fig. 2a and b) and large blood clots were present in the right thoracic air sac (Fig. 2a,c). The ventro-lateral edges of the lungs were hemorrhagic (Fig. 2b), and the tracheal mucosa had extensive areas of hemorrhage, with blood clots adhered to the anterior third of the trachea (Fig. 2d). The most significant histological findings in this case were: moderate-severe focal but extensive pyogranulomatous air-sacculitis associated with extensive hemorrhage, mild diffuse granulocytic pneumonia, mild diffuse lung congestion, mild-moderate multifocal granulocytic splenitis, and mild multifocal granulocytic hepatitis. Respiratory failure due to air-sacculitis caused by helminthic infection was considered the primary cause of death, in association with malnutrition.

Cases 2 to 5 were in very poor body condition, with significantly atrophied pectoral muscles, lacking subcutaneous and visceral fat stores, and showing signs of liver atrophy. The stomachs did not contain fresh or partly digested food material, but instead contained small quantities of dark brown-black liquid and squid beaks. Varying degrees of infection by the hard tick *Ixodes uriae* were noted in cases 2, 3 and 5. Several small areas of superficial subcutaneous and muscular bruising and small puncture wounds were seen in all four cases, suggesting antemortem harassment by predators (possibly lesser sheathbills).

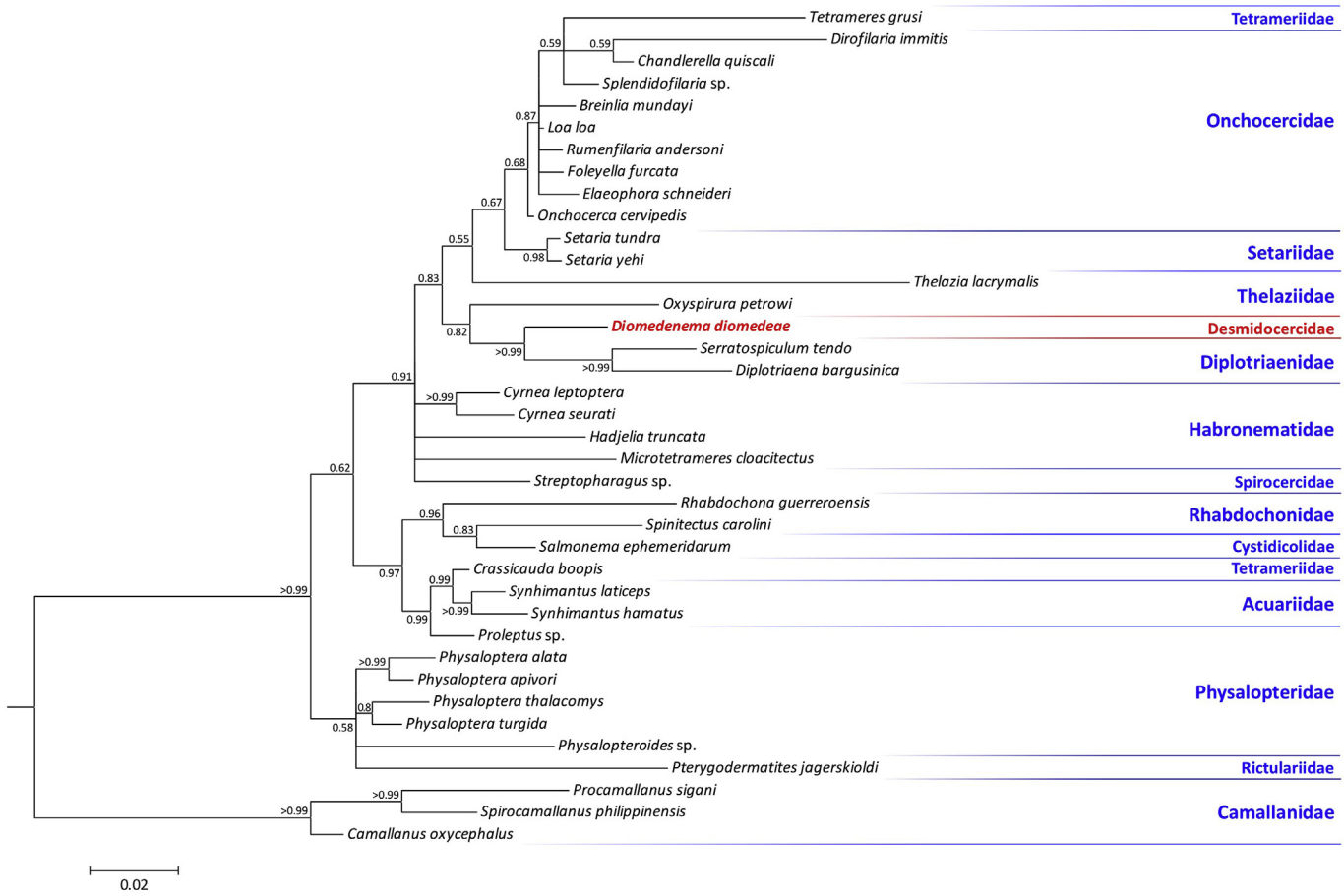

Fig. 4. Bayesian phylogenetic tree of *Diomedenema diomedeae* based on *18S rRNA* gene sequences of spirurid worms. Branch lengths are drawn proportionally to evolutionary distance (scale bar is shown). Numbers adjacent to nodes indicate posterior probabilities.

Histological examination did not reveal lesions that could be interpreted as suggestive of infectious or toxic processes. It was concluded that starvation was the apparent primary cause of death in these cases, in association with tick infection and ante-mortem harassment by predators.

The air sac nematodes from case 1 were identified as *Diomedenema diomedeae* following characters described in Johnston and Mawson (1952). Worms were filariform but relatively stout, with laterally compressed anterior ends, well-developed buccal capsules, two sharply tricuspid teeth, and body length > 10 mm (Figs. 2 and 3). Female lengths averaged (mean ± SD) 13.13 ± 1.34 mm (range: 11.13–14.87 mm; n = 19) and the vulva was located an average of 4.13 ± 0.35 mm from the cephalic end (range: 3.63–4.75 mm; n = 19). The spicules measured 155 µm and 240 µm in one male and 157 µm and 238 µm in another male (measurements taken at the longest points). Eggs averaged 59 × 26.7 µm (range: 53–63.1 × 25–30 µm; n = 35).

In the phylogenetic analysis of the *18S rRNA* gene (Fig. 4), *Diomedenema diomedeae* formed a strongly-supported clade (posterior probability > 0.99) with *Diplotriaena bargusinica* and *Serratospiculum tendo*, both of which in the family Diplotriaenidae (superfamily Diplotriaenoidea). In the phylogenetic analysis of the *COI* gene (Fig. 5), *Diomedenema diomedeae* was placed in a moderately-supported clade (posterior probability = 0.87) with *Cylicospirura petrowi* (superfamily Spiruroidea, family Spirocercidae) and *Ochoterenella* sp. (superfamily Filarioidea, family Onchocercidae); however, no species from the superfamily Diplotriaenoidea could be included in the *COI* gene analysis due to the lack of publicly-available sequence data.

4. Discussion

The superfamily Aproctoidea (class Chromadorea, order Spirurida, suborder Spirurina) are nematodes that parasitize the respiratory tract and subcutaneous tissues of birds (Anderson et al., 2009). This superfamily comprises two families, Aproctidae and Desmidocercidae (Zhang, 2011). Aproctidae has 59 recognised species in six genera (*Aprocta*, *Hovorkonema*, *Mawsonfilaria*, *Pseudaprocta*, *Squamofilaria*, and *Tetracheilonema*) that parasitize the air sacs, nasal passages, orbits and subcutaneous tissues of the head and neck of a broad variety of terrestrial birds (Anderson et al., 2009; Zhang, 2011). Desmidocercidae comprises four genera that infect the air sacs of fish-eating birds: *Desmidocerca*, *Desmidocercella* and *Skrjabinocercella* parasitize herons, cormorants and gulls, whereas *Diomedenema* parasitizes albatrosses and penguins (Barus et al., 1978; Anderson et al., 2009; Zhang, 2011; Knoff et al., 2017). *Diomedenema diomedeae* was described by Johnston and Mawson (1952) based on specimens recovered from the body cavity of a grey-headed albatross that washed ashore in South Australia, Australia. *Diomedenema tavaresi,* the only other species described from this genus, was described by Knoff et al. (2017) based on specimens recovered from the lungs of a Magellanic penguin (*Spheniscus demersus*, Sphenisciformes: Spheniscidae) that stranded in Rio Grande do Sul, Brazil. Neither of these species has been reported since their original description.

To our knowledge, this study provides the first DNA sequences for a species of the superfamily Aproctoidea. Our phylogenetic analyses of the *18S rRNA* and *COI* genes confirm that *D. diomedeae* belongs to the suborder Spirurina, and the analysis of the *18S rRNA* gene reveals that *D. diomedeae* is most closely related to the family Diplotriaenidae (the

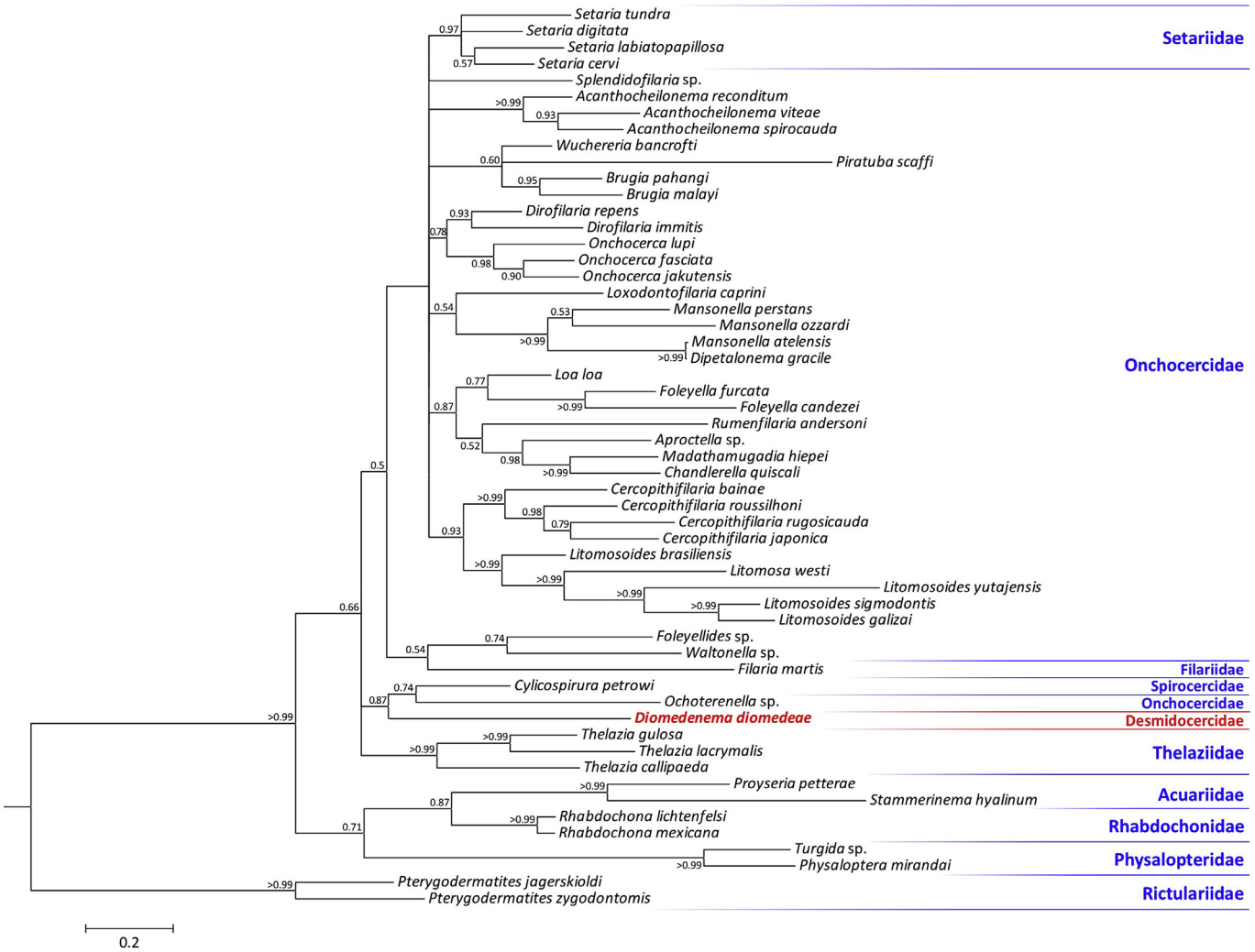

Fig. 5. Bayesian phylogenetic tree of *Diomedenema diomedeae* based on *COI* gene sequences of spirurid worms. Branch lengths are drawn proportionally to evolutionary distance (scale bar is shown). Numbers adjacent to nodes indicate posterior probabilities.

only family within the superfamily Diplotriaenoidea). This is consistent with the life history characteristics shared by the superfamilies Aproctoidea and Diplotrianoidea, since most of the species within these superfamilies are parasites of the air sacs of birds (Anderson, 2000). The transmission of Aproctoidea and Diplotrianoidea is thought to involve eggs migrating up the trachea into the buccal cavity, where they are swallowed and eliminated in the feces, where they are ingested by omnivorous intermediate host insects (Anderson, 2000; Bain et al., 2014). Species belonging to Desmidocercidae appear to specialize on fish-eating birds and it has been speculated that aquatic invertebrates may play the role of intermediate hosts with fish serving as paratenic hosts (Anderson, 2000).

Diomedenema diomedeae was originally described in the body cavity, not air sacs, of a grey-headed albatross (Johnston and Mawson, 1952). However, the air sacs of birds are complex and delicate structures that can easily be disrupted during necropsy, and thus the location of air sac worms can sometimes be incorrectly identified; it is also possible that worms migrate through adjacent tissues and accidentally enter the body cavity pre- or post-mortem (Anderson, 2000). Considering that *D. diomedeae* was present in large quantities within the air sacs but not in the body cavity of the specimen examined in this study, and that worm presence was associated with pathology in the respiratory system, we suggest these nematodes are parasites of the respiratory system and do not normally occur in the body cavity. This is corroborated by the fact that the only other species within the genus, *D. tavaresi*, was found in the lungs but not in the body cavity of its host (Knoff et al., 2017).

The only other record of a nematode in the respiratory tract of a procellariiform seabird is the record of immature nematodes in the air sac of a greater shearwater (*Ardenna gravis*; Procellariiformes: Procellariidae) (Bourgeois and Threlfall, 1979). The worms were thought to belong to the subfamily Dicheilonematinae (suborder Spirurina, superfamily Diplotriaenoidea, family Diplotriaenidae); however, no details were provided on the morphological criteria used to identify them. Considering the morphological similarities between the larval stages of Diplotriaenidae and Desmidocercidae (Anderson et al., 2009; Bain et al., 2014), it is possible that the worms found by Bourgeois and Threlfall (1979) could have been *Diomedenema* sp.

In this study, *D. diomedeae* was associated with severe air-sacculitis and hemorrhage, which was thought to have led to the death of the bird. These lesions are similar to those reported in birds of prey infected with other respiratory nematodes such as Syngamidae (*Cyathostoma*, *Syngamus*) or Diplotriaenidae (*Serratospiculum*), which may include large masses of parasites and necrotic tissue in the air sacs along with air sac epithelial hyperplasia, pyogranulomatous air-sacculitis, pneumonia and lung congestion (Bigland et al., 1964; Ward and Fairchild, 1972; Lavoie et al., 1999). No parasites were present in the trachea or lungs of the albatross, but there were extensive areas of hemorrhage on the surface of the trachea as well as diffuse mild granulocytic pneumonia, suggesting there was indirect irritation by nematodes and tissue debris. If the life cycle of *D. diomedeae* is analogous to that of

Diplotriaenidae (Anderson, 2000), the tracheal hemorrhage could also potentially be related to the migration of eggs or larvae.

Because of the small sample size examined in this study and because *D. diomedeae* was only recorded in one host individual, it is impossible to evaluate the prevalence of this parasite, its population-level effects or its genetic variability. It is unclear whether intense infections as documented in this study are common or instead represent unusual instances. Considering these limitations, it is clear that further studies are necessary to clarify the epidemiology and conservation significance of *D. diomedeae*.

From a demographic perspective, the relatively small number of "dead on nest" chicks had a limited impact on the breeding success at a population level. In the long-term study colony of grey-headed albatrosses at Marion Island (Ryan et al., 2007), breeding success was 47% for the 2015–2016 season, similar to the long-term average of 50% (22–73%, n = 20) for all seasons monitored since 1997. Although respiratory helminthiasis was determined to be the cause of death of one chick, this bird also was in poor body condition, and starvation appeared to be the primary factor leading to the death of the other individuals examined. We therefore suspect that parental abandonment or suboptimal feeding played a central role in the mortality of most grey-headed albatross chicks at the time. In this sense, *D. diomedeae* infection likely played a secondary role in the broader context of the mortality witnessed at the time.

Conflicts of interest

We declare there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.04.002.

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