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Detection of cryptic species of *Rugopharynx* (Nematoda: Strongylida) from the stomachs of Australian macropodid marsupials

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ABSTRACT

Sequences of the internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2) were determined for species of the genus *Rugopharynx* and *Rugonema labiatum*, nematodes from the stomachs of macropodid marsupials. Phylogenetic analyses of the aligned sequence data were conducted. The relationships provided molecular support for all species currently recognised, some of which are based on minor morphological differences and on multilocus enzyme electrophoretic data, but also indicated that additional, cryptic species exist within the genus. In addition, the genus *Rugonema* is placed as a synonym of *Rugopharynx*, its sole species becoming *Rugopharynx labiatum* n. comb. The molecular data provided some insights into the evolution of complex buccal capsule morphologies within the genus, but there was no evidence of co-evolution between the macropodid hosts and their parasites.

1. Introduction

The sacculated forestomachs of kangaroos and wallabies (family Macropodidae) commonly contain large numbers of nematodes belonging to the strongylid sub-family Cloacininae. For example, Vendl and Beveridge (2014) reported means and ranges in numbers of cloacinine nematodes in the stomachs of the red-necked wallaby, *Macropus rufogriseus*, the eastern grey kangaroo, *Macropus giganteus* and the swamp wallaby, *Wallabia bicolor*, as 60,800 (2000–210,000), 20,500 (7000–79,000) and 20,000 (3000–58,000), respectively. These data support earlier published figures for high intensities of infection in the western grey kangaroo, *Macropus fuliginosus*, the red kangaroo, *Macropus rufus*, and *M. giganteus* (Arundel et al., 1979; Beveridge and Arundel, 1979).

Although there is considerable species diversity in the stomach-inhabiting cloacinine nematodes (Spratt et al., 1991), members of the genus *Rugopharynx* are prominent representatives of these nematode communities. For instance, *Rugopharynx australis* dominates the gastric helminth community of *M. rufus*, with mean and maximum burdens of 47,000 and 266,000 nematodes, respectively (Arundel et al., 1979). In spite of the numerical significance of this genus in the gastric helminth communities of macropodids, continuing taxonomic studies are needed, as it is unlikely that all species have yet been described and the presence of cryptic species could potentially complicate the interpretation of ecological data published to date. The genus *Rugopharynx* was revised by Beveridge (1982), who recognised nine species. However, Beveridge (1982) noted that *R. australis* was potentially a complex of a number of species, differentiable only by very minor and often overlapping morphological characteristics. Multilocus enzyme electrophoretic (MEE) studies, combined with morphological evidence, indicated the existence of two new species, *R. sigma* and *Rugopharynx mawsonae*, both formerly confused with *Rugopharynx zeta* (Chilton et al., 1993; Beveridge et al., 1994), while an additional MEE investigation of *R. australis* by Chilton et al. (1996) provided evidence for at least seven species within this taxon. Subsequently, Beveridge and Chilton (1999) split *R. australis* into 10 species and resurrected *R. alpha* as a valid species. The latter revision was based on extrapolating the minor morphological differences identified in samples included in the earlier electrophoretic study across the entire species complex. In spite of this progress, there has been no attempt to independently verify the validity of the species erected to date using molecular methods. A closely related genus,

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Rugonema was erected by Beveridge (1999) for specimens formerly referred to as *R. australis* occurring in the stomach of the black-gloved wallaby, *Macropus irma*, from Western Australia, based on morphological differences in the labial collar. Because of this close association with *Rugopharynx*, *Rugonema labiatum* was also included in the present study.

Given the prevalence and abundance of this nematode genus in the stomachs of kangaroos and wallabies, and the diversity of species currently recognised based on minor morphological criteria, this study was undertaken to attempt to establish species boundaries within the genus based on sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA. These molecular target regions have proved to be highly informative for the specific identification of a range of strongylid nematodes, including taxa within the Cloacininae (Chilton, 2004), the subfamily to which *Rugopharynx* belongs.

2. Materials and methods

2.1. Collection, storage and preparation of nematodes

Nematodes were obtained from the stomachs of a range of kangaroos and wallabies (Fig. 1; Table 1), which had been collected as fresh road-kills or from road-kills frozen prior to examination. Host nomenclature follows Van Dyck and Strahan (2008). In instances where a nematode species occurred across a large geographical area, an attempt was made to include samples from different geographical regions of Australia, particularly any occurring on the island of Tasmania (Fig. 1). Australian state names are abbreviated as: NSW, New South Wales; Qld, Queensland; SA, South Australia; Tas, Tasmania; Vic, Victoria; WA, Western Australia. Nematodes were washed in saline, frozen in liquid nitrogen and stored at −80 °C until morphological and molecular studies were undertaken. Nematodes were then thawed, the head and tail of each worm removed, fixed in lactophenol and mounted permanently in polyvinyl lactophenol as voucher specimens. Nematodes were identified according to previous descriptions (Beveridge, 1982; Beveridge et al., 1994; Chilton et al., 1993; Beveridge and Chilton, 1999; Appan et al., 2004). Voucher specimens have been deposited in the South Australian Museum (SAM), Adelaide (Table 1). The mid-body region was used for genetic analyses.

2.2. Morphological methods

Species within the genus *Rugopharynx* were divided into three groups based on the morphology of the buccal capsule (Table 1). Nematodes with a simple, cylindrical buccal capsule were designated as type I (Fig. 2), while those with a buccal capsule divided into two sections were designated as type II. These buccal capsules were further subdivided into species with a buccal capsule divided in the mid region (*R. epsilon*) (type IIA) and those in which the division occurred in the anterior quarter (*R. rufogrisea*) (type IIB). Species with a buccal capsule divided into three segments were

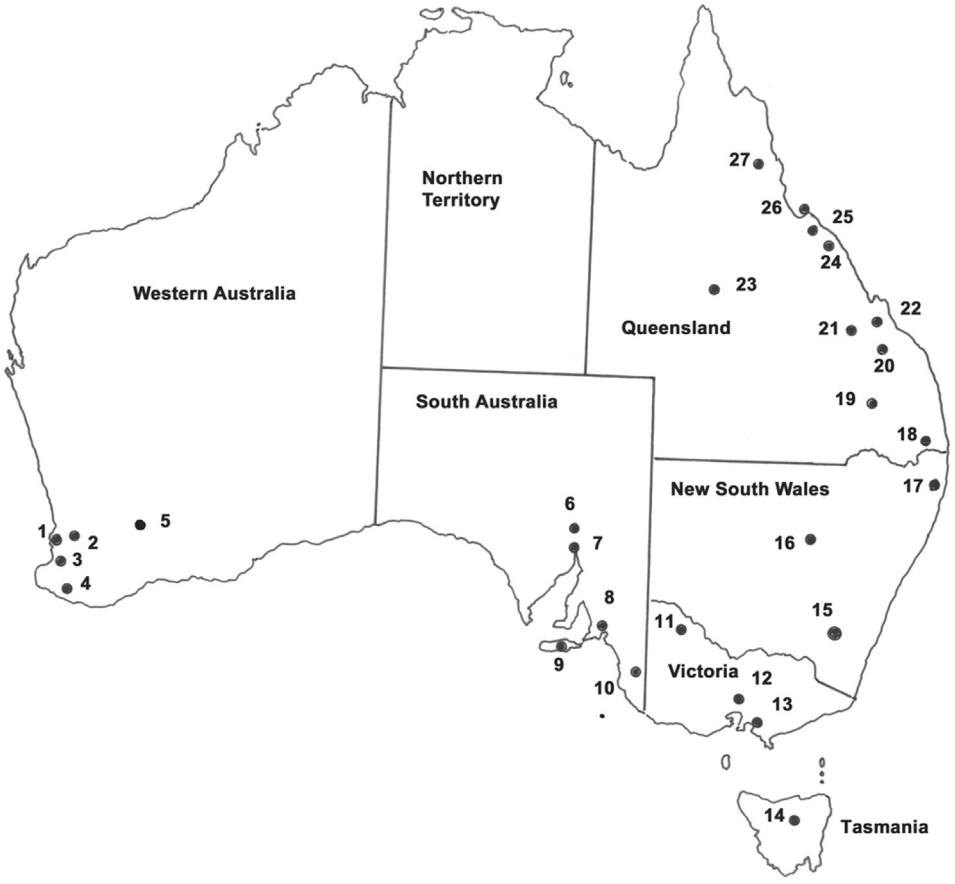

Fig. 1. Localities within Australia at which specimens of *Rugopharynx* used in this study were collected. Coordinates for each locality are provided in Table 1. 1, Lake Clifton; 2, Waroona; 3, Collie, Wellington Dam; 4, Perup River; 5, Kalgoorlie; 6, Wallerberdina Station; 7, Port Augusta; 8, Ashbourne; 9, Kangaroo Island; 10, Naracoorte; 11, Hattah Lakes National Park; 12, Yan Yean; 13, The Gurdies; 14, Launceston; 15, Emu Flat, Bondo State Forest; 16, Trangie; 17, Grafton; 18, Lamington National Park; 19, Miles; 20, Dawes; 21, Mt Sebastopol; 22, Rockhampton; 23, Winton; 24, Proserpine; 25, Bowen; 26, Magnetic Island; 27, Lake Barrine.