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MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF THE GENUS *GLOBOCEPHALOIDES* FROM MACROPODID MARSUPIALS IN AUSTRALIA

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To my grandparents

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Publications resulting from this research

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- Fazenda, I.P., Gasser, R.B., Carvalho, L.M. & Beveridge, I. (2009). Redescription of *Globocephaloides wallabiae* Johnston & Mawson, 1939 (Nematoda: Trichostrongyloidea) from macropodid marsupials in north-eastern Australia. *Acta Parasitologica*, *In Press*.

This research will be also presented in the *IX Iberian Congress of Parasitology* (September, 2009) in both abstract and poster formats.

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF THE GENUS *GLOBOCEPHALOIDES* FROM MACROPODID MARSUPIALS IN AUSTRALIA

Abstract

The genus *Globocephaloides* (Nematoda: Trichostrongyloidea) is a pathogenic group of parasitic nematodes present in the duodenum of kangaroos and wallabies (Marsupialia: Macropodidae) in Australia. *Globocephaloides* species (*G. trifidospicularis, G. macropodis* and *G. affinis*) have been poorly studied and thus, there are significant controversies regarding their systematics and population structures.

In the present study, single-strand conformation polymorphism (SSCP) and targeted sequencing of the internal transcribed spacers (ITS) of nuclear ribosomal DNA, were used to assess the genetic variation within and among *Globocephaloides* populations and individuals, from different host species and geographical origins. No or minor (0.2%) variation was detected among individuals of *G. trifidospicularis* and *G. affinis*. However, within *G. macropodis* populations there was a consistent heterogeneity in the ITS sequences (5.2 - 7.1%) between worms derived from two different host species (*Macropus agilis* and *M. dorsalis*).

Under light microscopy, these two *G. macropodis* genotypes differed by the length, arrangement and tip of the spicules, and by pattern of the bursal rays. Thus, they were considered to represent sibling species.

The molecular and morphological evidence culminated with the erection of a new species, namely *G. wallabiae*, and provided further insights into the host affiliation and geographical ranges of *Globocephaloides* spp.: *G. wallabiae* occurs mainly in *M. dorsalis* (north/east), *G. macropodis* in *M. agilis* and *Petrogale persephone* (north), *G. affinis* in *M. dorsalis* (north-east), and *G. trifidospicularis* in various *Macropus* species (south).

Moreover, in the present study, the phylogenetic analyses between the genus *Globocephaloides* and other trichostrongyloid genera, using divergent domains of the 28S rRNA genes, gave evidence for the exclusion of the genus *Globocephaloides* from the Herpetostrongylidae, with the suggestion of a new family, namely ‘Globocephaloididae’, within the Heligmosomoidea.

Keywords: *Globocephaloides*/Macropodid marsupials/Genetic variation/*G. wallabiae*/ Systematics

CARACTERIZAÇÃO MOLECULAR E MORFOLÓGICA DO GÉNERO *GLOBOCEPHALOIDES* EM MARSUPIAIS MACROPODÍDEOS NA AUSTRÁLIA

Resumo

O género *Globocephaloides* (Nematoda: Trichostrongyloidea) é um importante grupo de nemátodes patogénicos presente no duodeno de cangurus e wallabies (Marsupialia: Macropodidae) na Austrália. O estudo das espécies de *Globocephaloides* (*G. trifidospicularis, G. macropodis* e *G. affinis*) tem sido limitado, e por isso, existem numerosas controvérsias em relação à sua sistemática e às suas estruturas populacionais.

No presente estudo, as técnicas de análise de ácidos nucleicos, 'single-strand conformation polymorphism' (SSCP) e sequenciação-alvo da região ITS ('internal transcribed spacers') do DNA ribossomal foram usadas com o objectivo de analisar a variação genética entre indivíduos e populações de *Globocephaloides* provenientes de diferentes hospedeiros e áreas geográficas. Em ambas as espécies *G. trifidospicularis* e *G. affinis* pouca ou nenhuma variação foi encontrada (0.2%). Contudo, nas populações de *G. macropodis* foi detectada uma consistente heterogeneidade nas sequências ITS (5.2 - 7.1%) entre espécimes provenientes de dois hospedeiros distintos (*Macropus agilis* e *M. dorsalis*).

Recorrendo à microscopia óptica verificou-se que os dois genótipos de *G. macropodis* diferiam no comprimento, conformação e ponta das espículas, assim como, no padrão dos raios da bolsa copuladora. Como tal, foi considerado que os dois genótipos representavam duas espécies congéneres.

Os resultados da biologia molecular e do estudo morfológico determinaram o reconhecimento de uma nova espécie denominada *G. wallabiae* e, além disso, forneceram dados sobre a especificidade de hospedeiros e distribuição geográfica do género *Globocephaloides*. *G. wallabiae* ocorre principalmente no hospedeiro *M. dorsalis* (norte/este), *G. macropodis* no *M. agilis* e *Petrogale persephone* (norte), *G. affinis* no *M. dorsalis* (nordeste) e *G. trifidospicularis* em várias espécies do género *Macropus* (sul).

Adicionalmente, a análise das relações filogenéticas entre o género *Globocephaloides* e outros tricostrongilídeos, empregando os domínios do gene 28S rRNA, deu provas para a exclusão do género *Globocephaloides* da família Herpetostrongylidae, com a sugestão de uma nova família, denominada 'Globocephalidae', inserida na superfamília Heligmosomoidea.

Palavras-chave: *Globocephaloides*/Marsupiais macropodídeos/Variação genética/ *G. wallabiae* / Sistemática

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Abbreviations and symbols

♀	Female
♂	Male
A, C, G, T	Adenine, cytosine, guanine, thymine
BI	Bayesian inference
bp	Base pair
D	Pairwise differences in percentage
ddNTP	Dideoxyribonucleotide triphosphate
DGGE	Denaturating gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
e.g.	For example
EPG	Eggs per gram
EDTA	Ethylenediaminetetraacetic acid
ETS	External transcribed spacer
gDNA	Genomic DNA
GluCls	Glutamate-gated chloride channels
Hg	Haemoglobin
i.e.	That is
IGS	Intergenic non-transcribed spacer
ITS	Internal transcribed spacer
ITS-1	First internal transcribed spacer
ITS-2	Second internal transcribed spacer
ITS-2^{+}	ITS-2 plus flanking sequence
kb	Kilo base pair
L1	First larval stage
L2	Second larval stage
L3	Third larval stage
LM	Light microscopy
LSU	Large subunit
MEE	Multilocus enzyme electrophoresis
min	Minute
ML	Maximum likelihood
MP	Maximum parsimony
mya	Million years ago
n	Number
NJ	Neighbour joining
NSW	New South Wales
NT	Northern Territory
PCR®	Polymerase Chain Reaction
PCV	Packed cellular volume
PO	Per os
pp	Posterior probabilities
Qld	Queensland
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SA	South Australia
SAM	South Australian Museum
SC	Subcutaneous
SDS	Sodium dodecyl sulphate

SEM	Scanning electron microscopy
sid	Once a day
sp.	Species (singular)
spp.	Species (plural)
SSCP	Single-strand conformation polymorphism
ssDNA	Single-stranded DNA
SSU	Small subunit
TAS	Tasmania
TBE	Tris borate EDTA
TPP	Total plasma protein
Tris-HCl	Tris hydrochloride
VIC	Victoria
w/v	Weight per volume (mass concentration)
WA	Western Australia

Chapter 1
Literature review

1.1. Introduction

The phylum Nematoda represents one of the most varied and ubiquitous groups of organisms on earth, comprising free-living forms and parasites of plants and animals (Chitwood & Chitwood, 1974).

Parasitic nematodes affecting domestic animals have been studied due to their socio-economic and zoonotic importance. However, considerably less attention has been paid to roundworms of wild-life. For instance, the genus *Globocephaloides* Yorke & Maplestone, 1926 represents an important pathogenic group of parasitic nematodes present in the duodenum of macropodid marsupials in Australia. *Globocephaloides* infections are usually asymptomatic but can cause serious disease in juvenile hosts, leading to substantial mortalities, particularly under poor environmental conditions (Arundel, Barker & Beveridge, 1977).

Central to studying the diseases, ecology, epidemiology and systematics of parasites of macropods is the accurate identification of the causative agent(s). Traditional methods have been used widely for specific identification, but molecular tools using specific genetic markers (reviewed by Gasser, 2006) hold great promise for the genetic characterization of *Globocephaloides* species and the accurate diagnosis of the infections they cause, which also underpins a better understanding of its systematics, populations structures, geographical distribution and host affiliation.

The purpose of this chapter is: (i) to provide background information on specific issues (ecology, nutrition and helminth fauna) of kangaroos and wallabies, (ii) to review the salient literature on the genus *Globocephaloides* and highlight gaps in the knowledge of this important genus of nematodes, (iii) to provide an account of some of the molecular approaches used for the specific identification and delineation of closely-related nematodes, and finally (iv) to draw conclusions from the literature review, to set the research aims of the present study.

1.2 Host - kangaroos and wallabies (Marsupialia: Macropodidae)

Given that the focus of the present study is to characterize species of *Globocephaloides*, it is of major importance to review key aspects of the macropodid marsupial hosts.

The origins of marsupials date from approximately 135 million years ago (mya) (Richardson, 1988) when Australia, South America and Antarctica were still a single land mass called Gondwana. In the Late Cretaceous, these land masses separated gradually, and two marsupial clades were formed, the 'australidelphian' and the 'ameridelphian' (Woodburne & Case, 1996).

At this stage, marsupials and monotremes were the only mammals inhabiting Australia (Johnson, 2003), and thus a considerable evolutionary radiation occurred in these animals. One of the most successful and recent (~8-10 mya) branches is the Macropodinae Gray, 1821 (Flannery, 1989; Burk & Springer, 2000; Meredith, Westerman & Springer, 2008), which includes approximately 50 out of 62 species present in the family Macropodidae (Van Van Dyck & Strahan, 2008). Members of this family have evolved unique features (Fig. 1), but they are referred to as 'macropodids' due to their 'big foot'.

The diversification of macropodids among other marsupials was patent when grasses became the major component of Australian vegetation (Hume, 1999) and is comparable with the expansion of ruminants among the Artiodactyla (Janis, 1976), when grasslands spread throughout the world.

In the case of macropods, the success was firstly due to the adaptation to a grazing diet utilizing fibrous plant material (Beveridge & Spratt, 1996), and secondly due to an efficient bipedal hopping locomotion, which allowed speedy escape from predators and long distance travelling, with less energy consumption. In addition to these primary features, macropods are able to run, walk and climb; browse and dig; eat fruit and/or insects; and adapt to all sorts of habitats from arid to wet tropical regions (Hume, 1999; Van Dyck & Strahan, 2008).

The reproductive characteristics of kangaroos and wallabies also contributed to their rapid expansion. In the majority of macropodine species, the breeding season continues throughout the year and after approximately one month of gestation a single young is born (Fig. 2). Due to the high mortality rate of pouch-young, females have a post-partum oestrus and mate shortly after giving birth; the resultant quiescient blastocyst is held in lactation-controlled embryonic diapause to replace the born or dead young a month later.

There are few exceptions to this reproductive patron: *M. rufogriseus* (Tasmania) and *M. eugenii* have births strictly in late January-early February and *M. fuliginosus* does not exhibit embryonic diapauses (Johnson, 2003; Van Dyck & Strahan, 2008; Vogelnest & Woods, 2008).

Figure 1. Main characteristics of macropodid marsupials at each taxonomic level. (Based on Johnson, 2003; Van Dyck & Strahan, 2008; Vogelnest & Woods, 2008)

Systematics		General characteristics
Subclass	**Marsupialia**	*Marsupium* means 'pouch' and the young develops inside this structure (no placenta). The young is born in an incomplete state: minute, blind, hairless and partially developed hindlimbs.
Superorder	**Australidelphia**	Native to Australasia.
Order	**Diprotodontia**	Paired procumbent lower incisors; absence of lower canine teeth.
Superfamily	**Macropodoidea**	Hopping gait; pedal morphology: digit 4 greatly enlarged, digits 2 and 3 syndactylous, digit 1 absent. Foregut fermentation and embryonic diapause (except musky rat-kangaroo).
Family	**Macropodidae**	Powerful hindlimbs with slender hindfoot and long, powerful fourth toe (except rock-wallabies and tree-kangaroos).

Figure 2. Pouch-young of *Macropus giganteus*. This young was found during a necropsy of a road-killed female. (Original)

Even though macropods have been a highly successful group, after European settlement, approximately 210 years ago, their distribution and numbers have been significantly changing. Some species have taken advantage of human resources and have been considered as agricultural pests or nuisances competing with sheep and cattle for pastures (e.g., *Macropus giganteus* and *M. rufus*), whereas others have declined to become endangered (e.g., *Petrogale persephone*) or even extinct (e.g., *M. greyi*), due to the clearing of forests and introduction of feral herbivores/predators (Edwards, Dawson & Croft, 1995; Johnson, 2003; Van Dyck & Strahan, 2008).

In spite of their status, all macropodine species are endemic to Australia and/or New Guinea (Van Dyck & Strahan, 2008) and are currently protected native fauna under state or territories legislations (*Environment Protection and Biodiversity Conservation Act, 1999*).

Nevertheless, some species can be commercially harvested for meat and skins, such as *M. rufogriseus* in Tasmania, and *M. rufus*, *M. robustus*, *M. giganteus* and *M. fuliginosus* in Queensland (Australian Government, Department of the environment, water, heritage and the arts, 2009).
In the present thesis, only selected macropodine species, belonging to the genera *Macropus*, *Wallabia* and *Petrogale*, are discussed in the following sections.

1.2.1 Genus *Macropus*

The genus *Macropus* Shaw, 1790 comprises a total of 14 species within three subgenera: *Notamacropus*, *Macropus* and *Osphranter*. Because adults range in weight from 4 kg (*M. eugenii*) up to 92 kg (*M. rufus*), species of less than 20 kg are commonly called “wallabies”, and species of more than 20 kg are referred to as “wallaroos” and “kangaroos”. These two later designations are distinguished by habitat preferences with wallaroos tending to live in steep and hilly country, whereas kangaroos inhabit flat or undulating land.
Generally, kangaroos and wallabies are nocturnal, resting during the day time under cover in thick vegetation (e.g., shrub understorey), emerging between dusk and dawn to feed in open grasslands, woodlands or forests (Johnson, 2003; Van Dyck & Strahan, 2008).

1.2.1.1 Subgenus *Notamacropus* Dawson and Flannery, 1985

Notamacropus species (Fig. 4A) can be solitary (*M. rufogriseus)* or gregarious, forming groups up to 20 individuals (*M. agilis*). Except for *M. eugenii*, which has no social grouping, *Macropus* species usually aggregate into large “mobs” (i.e., groups) in feeding areas (Johnson, 2003; Van Dyck & Strahan, 2008).
These wallaby species are mainly dicotyledonous browsers (Dawson, 1989), feeding on native roots, leaf litter, flowers and fruits of a range of shrub and tree species (Van Dyck & Strahan, 2008).

1.2.1.2 Subgenus *Macropus* Shaw, 1790

This subgenus includes two of the most abundant species of kangaroos: *Macropus fuliginosus* (western grey kangaroo) and *Macropus giganteus* (eastern grey kangaroo) (Fig. 4B). In the past, they represented a single species, but after the disruption of their habitat, eastern and western populations evolved unique features. For example, *M. fuliginosus* developed resistance to sodium monofluoracetate produced by *Gastrolobium* spp. (plant), whereas *M. giganteus* adapted to heat-loss, possessing a larger body and shorter extremities (Van Dyck & Strahan, 2008).

Both species are predominantly monocotyledonous grazers (Dawson, 1989), but in drought periods they can also eat leaves of shrubs (Hume, 1999; Vogelnest & Woods, 2008). To counteract this abrasive diet of 99% of grasses, grey kangaroos have molar progression, a characteristic shared only with elephants, dugongs and manatees (Vogelnest & Woods, 2008). Grey kangaroos are gregarious and sociable (Fig. 3), with feeding mobs of up to 50 individuals (Johnson, 2003; Dyck & Strahan, 2008).

Figure 3. Part of a feeding mob of *Macropus giganteus* on Anglesea Golf Course, Victoria. (Original)

1.2.2. Genus *Wallabia*

The only living member of the genus *Wallabia* Trouessart, 1905 is *W. bicolor* (swamp wallaby) (Fig. 4C). It differs from *Macropus*, in its distinct reproductive, dental and behavioural characteristics. Moreover, *W. bicolor* has 11 and 10 chromosomes in the male and female, respectively, whereas wallabies in the *Macropus* genus have 16 chromosomes (Dyck & Strahan, 2008).

The swamp wallaby is solitary and its distribution is determined by the availability of adequate dense vegetation for shelter. It is a natural browser of forbs and shrubs (Hume, 1999), but it is also able to consume hypogeous fungi, ferns, grasses and seeds (Hume, 1999; Johnson, 2003; Dyck & Strahan, 2008).

1.2.3. Genus *Petrogale*

The genus *Petrogale* Gray, 1837 is a distinctive group of macropods ranging in weight from 1 to 12 kg. These macropods are commonly named ‘rock-wallabies’, because of their rocky-habitat preferences (Fig. 4C). They are social animals, living in colonies up to 100 individuals, and are mixed feeders (i.e., grazer and browser). Moreover, in drought periods they have been seen eating cow manure (Van Dyck & Strahan, 2008). These wallabies are nocturnal, resting in understorey or inside caves and crevices of rocky piles (Johnson, 2003).

Figure 4. Map of Australia showing the geographical distribution of the macropodid marsupials included in the present study. **A**. *Notamacropus* species: *M. agilis* (grey), *M. dorsalis* (yellow), *M. eugenii* (pink), *M. rufogriseus* (light blue). **B**. *Macropus* species: *M. fuliginosus* (dark blue), *M. giganteus* (green). **C**. *Wallabia bicolor* (orange) and *Petrogale persephone* (black dots). The common species name is indicated in brackets, followed by a briefly description of the geographical range and distinctive morphological characteristic. NSW = New South Wales; NT = Northern Territory; Qld = Queensland; SA = South Australia; TAS = Tasmania; VIC = Victoria; WA = Western Australia. (Based on Watts, 1998; Johnson, 2003; Van Dyck & Strahan, 2008).

A

Macropus agilis Gould, 1842 (agile wallaby) is the most abundant macropod in grassy woodlands of tropical coastal Australia (NT, Qld and WA) and New Guinea. Morphological feature: sandy brown body above and distinct light stripe from knee to hip.

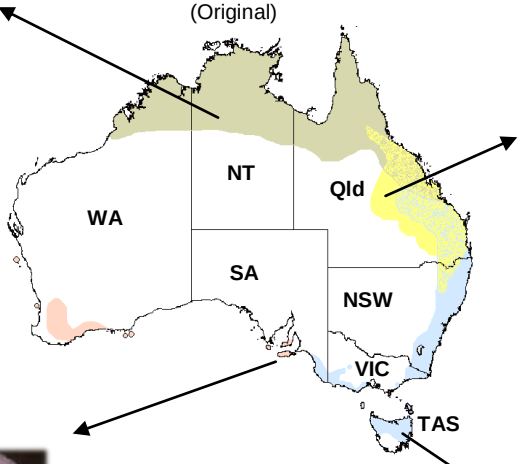
(Johnson, 2003)

Macropus dorsalis Gray, 1837 (black-striped wallaby) is common in forest and scrub of eastern Australia (Qld and north-eastern of NSW). Morphological feature: dark mid-dorsal stripe from neck to rump.

(Johnson, 2003)

(Watts, 1998)

(Original)



(Dyck & Strahan, 2008)

Macropus eugenii Desmarest, 1817 (tammar wallaby) has a limited distribution in the mainland (south-western of WA), but is still abundant in Kangaroo Is (SA) and other southern offshore islands in WA. Morphological feature: smallest wallaby; grizzled grey-brown above, becoming rufous on the sides of the body.

(Johnson, 2003)

Mainland

Tasmania (Original)

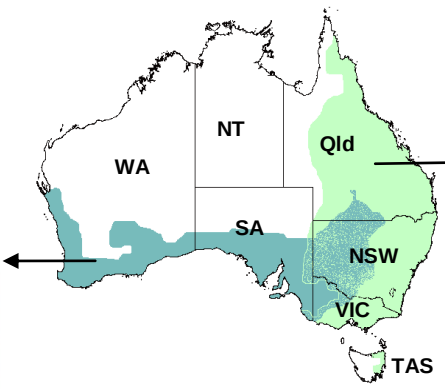
Macropus rufogriseus Desmarest, 1817 (red-necked wallaby) is especially abundant in forests of TAS and eastern Australia (coastal VIC and NSW). Morphological feature: reddish brown neck.

Figure 4. (Continuation)

B

(Dyck & Strahan, 2008)

(Original)



(Dyck & Strahan, 2008)

(Watts, 1998)

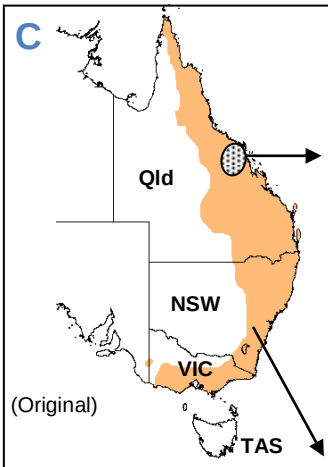
Macropus fuliginosus Desmarest, 1817
(western grey kangaroo)

(Watts, 1998)

Macropus giganteus Shaw, 1790
(eastern grey kangaroo)

Grey Kangaroos are endemic in Australia and have an extensive sympatry in woodlands, scrub and plains of SA, VIC, NSW and Qld. *M. giganteus* is also found in TAS. Distinct morphological feature: Western Grey Kangaroo has darker facial and body colour, and distinctive woolly fur; also, males from western species have strong curry-like odour.

C



(Original)

(Johnson, 2003)

Petrogale persephone Maynes, 1982 (Proserpine rock-wallaby) has a limited range, occurring only in Proserpine and Whitsundays area in Qld. Morphological feature: dark grey body with light mauve tinge.

(Johnson, 2003)

(Watts, 1998)

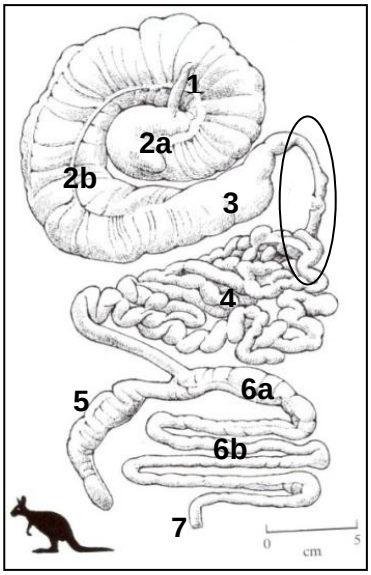
(Watts, 1998)

Wallabia bicolor Desmarest, 1804 (swamp wallaby) occurs in dense forest along coastal Qld, NSW and VIC. Morphological feature: dark body, yellowish below; dark tail.

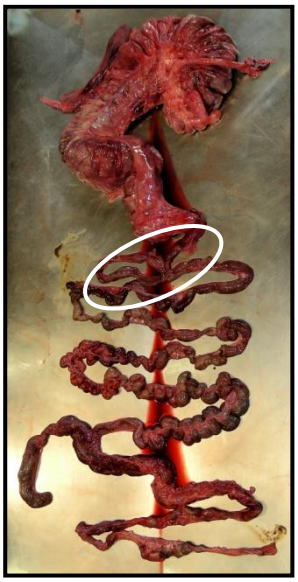
1.2.4 The digestive tract of macropodid marsupials and helminth fauna

The evolutionary changes in the gastrointestinal anatomy of marsupials, followed by a shift in the feeding behaviours, have had a substantial impact on their parasite fauna. Due to their complex digestive system, the subfamily Macropodinae harbours the most diverse range of helminth parasites within the australidelphian radiation (Beveridge & Spratt, 1996).
Species of the subfamily Macropodinae are foregut fermenters similarly to the Ruminantia. However, the macropod stomach is divided into a saccular forestomach and an elongate tubular forestomach, anterior and posterior to the entry point of the oesophagus, respectively, and a small hindstomach, where the enzymatic digestion takes place (Beveridge et al., 1998) (Fig. 5).

Figure 5. Digestive tract of *Macropus giganteus*: 1. oesophagus; 2a. sacciform forestomach; 2b. tubiform forestomach 3. hindstomach; 4. small intestine; 5. caecum; 6a. proximal colon; 6b. distal colon; 7. rectum. An ellipse indicates the predilection-site of *Globocephaloides* adult worms. Scale bar: 5 cm. (Adapted from Hume, 1999)



(Hume, 1999)



(Original)

The forestomach is the primary site of microbial fermentation of coarse plant material, resulting in the production of acetic, propionic and butyric acids. It is lined with glandular epithelium which secretes mucus to maintain the pH between 5 and 8, in addition to the saliva buffering action. In contrast, the fine plant material is deviated directly to the hindstomach, which secrets hydrochloric acid and proteolytic enzymes (Vogelnest & Wood, 2008). Although these anatomical and physiological adaptations of the stomach have allowed kangaroos and wallabies to utilize seasonally low-quality forage (i.e., high fibre and

low protein) in abundant amounts, these adaptations have also provided habitats to a variety of strongyloid nematodes (Beveridge, Spratt, Close, Barker & Sharman, 1989; Beveridge & Spratt, 1996; Hume, 1999).

Beveridge, Spratt and Johnson (2009, *In press*) found that the main predilection-sites of endemic helminths in macropods are the stomach and the elongated small intestine, harbouring 291 and 46 helminth species, respectively, compared with other organs which contain fewer than 8 species (e.g., oesophagus, large intestine, lung, liver, etc.). The oesophagus of *Notamacropus* and *Wallabia* species constitutes an exception due to their stratified, squamous epithelium covered by numerous papilla-like extensions and bacterial plaque, which create an additional fermentative niche for cloacinid nematodes, (Beveridge et al., 1998).

In decreasing order of occurrence in the host, the helminth fauna is composed of, strongyloids, anoplocephalid cestodes, trichostrongyloids, filaroids, and in lower percentage metastrongyloids, spiruroids, oxyuroids, trematodes and other cestodes (Beveridge & Spratt, 1996; Beveridge et al., 1998, 2009 *In press*). This fauna-type is explained by the shift from a simple stomach in phalangeroids and vombatoids to the development of a fermentative forestomach and a grazing diet in macropodoids. In the latter case, the infective larval stage (or third-stage larva) is ingested incidentally with the vegetation.

Usually the effects of this diverse helminth fauna are asymptomatic, even when large burdens occur. For example, *Rugopharynx australis* can reach to numbers of up to 290,000 individuals in the stomach of free-ranging grey kangaroos, without producing any symptoms and/or lesions. However, some parasites may lead to pathological changes, depending on the host species affected.

In *Macropus giganteus*, only five gastrointestinal nematodes from a total of 50 species, reported to date, are pathogenic (unpublished data from Beveridge & Spratt, 2009); *Globocephaloides trifidospicularis* causes duodenal haemorrhage, *Rugopharynx rosemarie* hypertrophic gastritis, *Paramacropostrongylus toraliformis* whitish caecal nodules, and *Strongyloides* spp. associated with *Labiostrongylus* spp. produce erythema and erosive gastritis (Beveridge & Arundel, 1979; Beveridge et al., 1998).

In the case of *Petrogale persephone, G. trifidospicularis* is replaced by *G. macropodis* as the potential causative agent of duodenal haemorrhage, and *Hypodontus macropi* is responsible for caecal haemorrhage and enteritis (Begg, Beveridge, Chilton, Johnson & O'Callaghan, 1995).

By contrast, in *Wallabia bicolor*, *Globocephaloides* spp. and *H. macropi* are encountered in small numbers and do not produce significant lesions. These nematodes are substituted by *Labiostrongylus clelandi* and *Parazoniolaumus collaris*, which both cause gastric nodules containing larvae (Beveridge, Presidente & Speare, 1985).

1.3 Parasite – *Globocephaloides* (Nematoda: Trichostrongyloidea)

The review of the literature pertaining to macropods and their fauna revealed that *Globocephaloides* is one of the main strongyloid nematodes that cause disease in macropodid marsupials. In this section, key aspects of this particular nematode are covered.

1.3.1 Systematics: taxonomy and phylogeny

The systematics of the genus *Globocephaloides* has been controversial and thus it has been difficult to establish its correct taxonomic position. A chronological summary is presented to better understand its taxonomy and phylogeny.

In 1926, Yorke and Maplestone described the genus *Globocephaloides* for the first time from the type-species *G. macropodis* in the host '*Macropus* sp'. The name *Globocephaloides* was suggested by the presence of an enlarged subglobular buccal capsule, similar to that occurring in the genus *Globocephalus* Molin, 1861. However, only a single female was examined and in the absence of a male it was only possible to guess its taxonomic position - "Strongyloidea ?" (Yorke & Maplestone, 1926).

Later, Freitas and Lent (1936) placed the genus *Globocephaloides* as a synonym of *Globocephalus*, considering that species within the former were indistinguishable from *Globocephalus marsupialis*, a nematode of *Metachirus opossum* (Brazilian polyprotodont).

Shortly after, Johnston and Mawson (1939a,b) reported three additional species of *Globocephaloides* in the states of Queensland and New South Wales: *G. wallabiae* and *G. affinis* from *M. dorsalis*, and *G. thetidis* from *Thylogale thetis*. This discovery, caused Johnston and Mawson to disagree with the previous judgement of Freitas and Lent (1936), since the Brazilian species did not occur in Australasia and they differed in a number of important morphological features, such as the structure of the bursa, the position of the vaginae, the jaw-like structures supporting the buccal capsule and the absence of a gubernaculum. For these reasons, Johnston and Mawson (1939a) placed the genus *Globocephaloides* in the Strongylinae.

Subsequently, Kung (1948) reported other new species of *Globocephaloides*, namely *G. trifidospicularis*, from *M. rufogriseus*, which had died in the London Zoological Gardens. This author described both female and male specimens in more detail when compared with the previous descriptions of Yorke and Maplestone (1926) and Johnston and Mawson (1939a,b). Hence, Kung (1948) found it difficult to distinguish the new species from *G. thetidis* or *G. wallabiae*, all closely resembling one another. However, in *G. trifidospicularis* the spicules are trifid and complex, and the author stated that "the spicules are so characteristic that Johnston and Mawson could hardly have failed to comment on them" (Kung, 1948) (see Table 1).

Moreover, this new data gave support for the validity of the genus *Globocephaloides*, in which the spicules are stout, short and branched, compared with *Globocephalus*, with slender and simple ones.

Subsequently, a number of efforts were made to determine the exact taxonomic position of *Globocephaloides*. Popova (1955) referred the genus to the Strongylidae, Chabaud (1965) to the Ancylostomatidae (Ancylostomatoidea), but Inglis in 1968 established the subfamily Globocephaloidinae, placing it in the Amidostomatidae (Trichostrongyloidea) together with other macropodid parasites (Beveridge, 1979).

The next study of the Globocephaloidinae was prepared by Beveridge (1979), who added one more genus, *Amphicephaloides* Beveridge, 1979 along with *Globocephaloides*. This author also supported the amidostomid position based on the dorsal ray and spicules, which were both similar to other amidostomid genera. In his review, Beveridge (1979) did not attempt to classify the subfamily, but rather redefined the species within the Globocephaloidinae. He redescribed *G. trifidospicularis* and *G. macropodis*, and placed *G. wallabiae*, *G. affinis* and *G. thetidis* as synonyms of *G. macropodis*, considering the criteria used to distinguish those three species not to be reliable. On the one hand, *G. affinis* was only erected based on females and a larger buccal capsule, on the other hand *G. wallabiae* and *G. thetidis* were described from a limited number of specimens, using unreliable characters such as worm total length, size of the buccal capsule, length of oesophagus, position of vulva and size of the dorsal tooth. Moreover, the type-specimen of *G. wallabiae* was missing from the collections of the South Australian Museum.

In a later review, Beveridge, Speare and Johnson (1984) agreed that *G. affinis* was indeed a valid species (see Table 1), redescribing both female and male specimens from the host-type *M. dorsalis*.

In 1981, Durette-Desset and Chabaud re-evaluated the taxonomic position of the Globocephaloidinae and transfer it from the Amidostomatidae to the Herpetostrongylidae (Trichostrongyloidea) based on geographical origin (i.e., Australia), host (i.e., Marsupial) and bursal rays (i.e., ray 2 and 3 originating dependently). These authors affirmed that these features were compatible with those from the Herpetostrongylinae (i.e., parasites of reptiles and marsupials in Australia).

Later, Durette-Desset, Beveridge & Spratt (1994) reviewed the systematics of the Strongylida and noticed that *Globocephaloides* species have certain morphological features, which place them unequivocally in the suborder Trichostrongylina. These characters are: teeth at the base of the buccal capsule, amphidelphic female with short ovejector, and male having a dorsal lobe of bursa reduced and migration of ray 4 towards ray 3. Moreover, the monoxeneous life-cycle of *Globocephaloides* is similar to those one occurring in members of the Trichostrongylina.

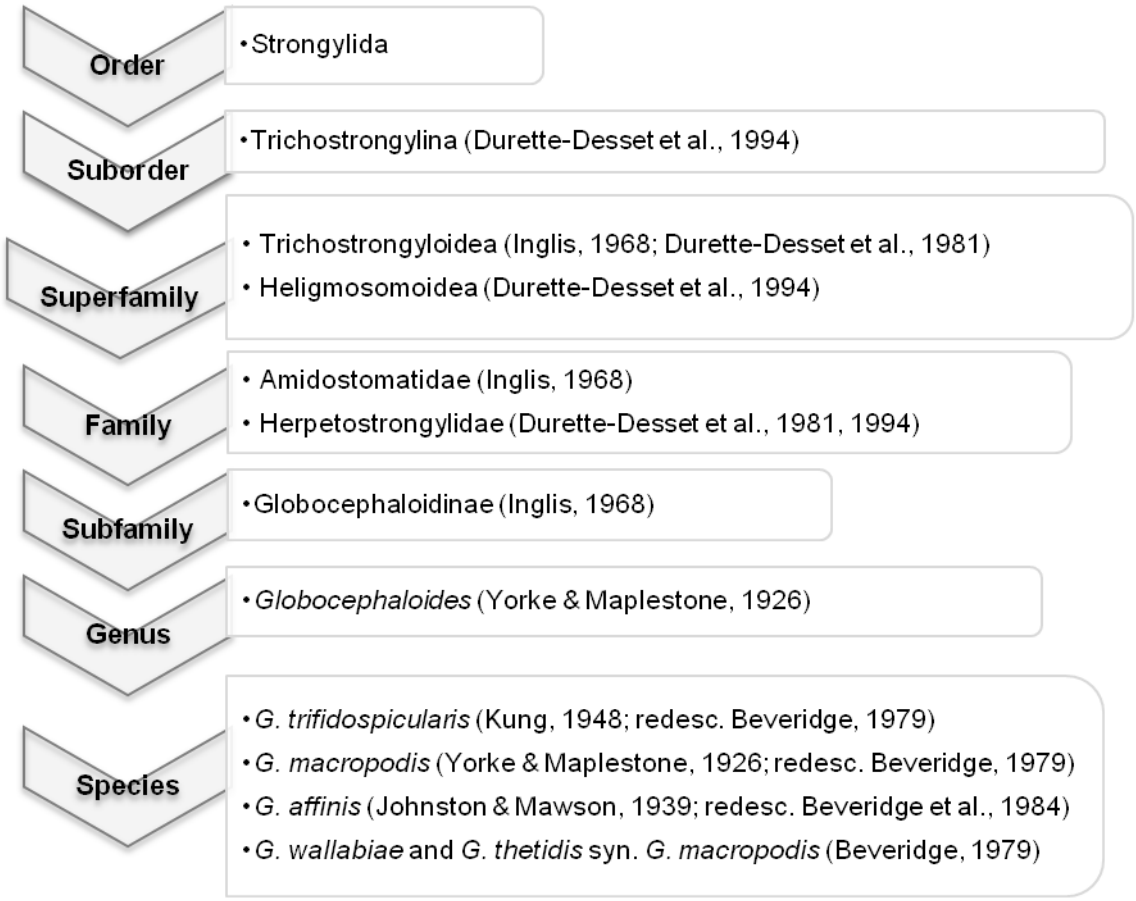
However, three morphological features present in this genus were exceptional for the Trichostrongylina: jaw-like structures, buccal capsule enlarged (not plesiomorphic), absence of cephalic vesicles and lack of synlophe.

Furthermore, Durette-Desset et al. (1994) moved the Herpetostrongylidae from the Trichostrongyloidea (Durette-Desset & Chabaud, 1981) to the Heligmosomoidea, because "each of the major lineages within this superfamily is characteristic of a particular biogeographic region" (Durette-Desset et al., 1994). Nevertheless, the authors still affirmed that "the origin of the Globocephaloidinae, parasitic in macropodid marsupials, is difficult to determine, since species lack synlophes" (Durette-Desset et al., 1994), a major character of the Heligmosomoidea.

Taking into account these classifications were primarily based on morphological features, host, geographical origin, predilection-site and pathological effects, it is clear that subjective interpretations were common.

Figure 6 provides a summary classification accordingly to the present data.

Figure 6. Systematics of *Globocephaloides* spp. according to records to date. Authors associated with first description and subsequent redescriptions (redesc.) are in brackets. (Original)

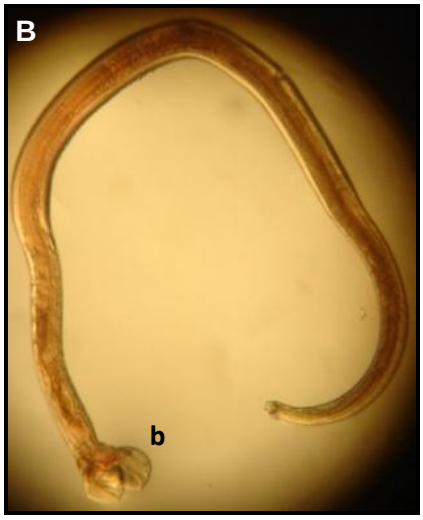


1.3.2 Morphological description

After the first description of the *Globocephaloides* genus by Yorke and Maplestone (1926), there were a number of other descriptions, as mentioned in the previous section. However, it was not until a review by Beveridge (1979), that this genus was described in detail:

a) Conformation - Small red coiled nematode, flat spiral, ventral surface on inside the coil (Fig. 7). Body without longitudinal ridge or alae, covered by numerous transverse striations. Total length ranging from 5.4 to 9.3 mm and maximum width from 0.20 to 0.45 mm, depending on the species, sex and development al stage.

Figure 7. Female (A) and male (B) specimens of *Globocephaloides trifidospicularis* under light microscopy. A “v” indicates the female vulva and a “b” the male copulatory bursa. (Original)



b) Anterior end - Buccal capsule greatly enlarged, thick walled, urceolate, not subdivided, approximately as wide as long; oral opening slit-like, dorsoventrally elongate, without lips, bounded by two lateral jaw-like structures. Large single dorsal tooth and two small subventral teeth arise from oesophagus; dorsal oesophageal gland opens at apex of dorsal tooth. Oesophagus elongate, clavate. Nerve ring encircles oesophagus near anterior extremity. Excretory pore posterior to nerve ring, leading to elongate excretory gland. Deirid small at level of excretory pore.

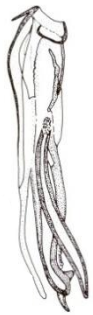
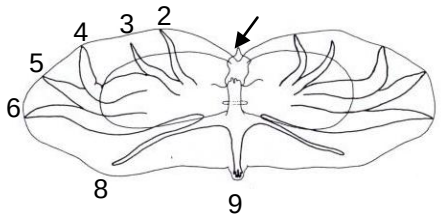
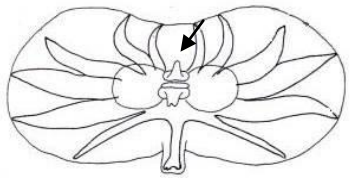
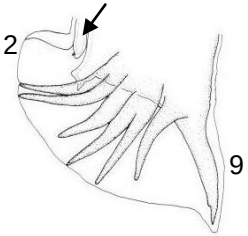
c) Female posterior end - Vulva posterior to mid-body region; amphidelphic; vagina with paired ovejectors, vaginae uterinae short, uteri sac-like, opposed; egg thin-shelled, ellipsoidal; embryo two-celled when egg laid. Tail short, conical.

d) Male posterior end (Fig. 8) - Bursa well developed, lateral lobes elongate, fused with ventral lobes; dorsal lobe greatly reduced; ventroventral and ventrolateral rays separate; lateral rays subequal; externodorsal ray slender; dorsal ray very short, branched at extremity; spicules short, similar, heavily sclerotized, complex, subdivided distally. Genital cone prominent.

Figure 8. Copulatory bursa of *Globocephaloides trifidospicularis* under light microscopy. An arrow points to a bursal ray. (Original)

The morphological features described previously are general for the whole genus, because each *Globocephaloides* morphospecies possesses unique characteristics (Beveridge, 1979; Beveridge et al., 1984) (see appendix I). However, the reliable identification to species can only be based on the posterior end of the male (i.e., spicules and copulatory bursa) (Table 1).

Table 1. Reliable morphological characters to distinguish *Globocephaloides* morphospecies: *G. trifidospicularis*, *G. macropodis* and *G. affinis*. Bursal rays are indicated by numbers: 2. ventroventral, 3. lateroventral, 4. externolateral, 5. mediolateral, 6. posterolateral, 8. externodorsal, 9. dorsal. An arrow indicates the genital cone. (Based on Beveridge 1979; Beveridge et al., 1984)

	G. trifidospicularis	*G. macropodis*	*G. affinis*
Gubernaculum	Absent	Absent	Present
Spicules	• Trifid and heavily scletorized • Main branch with simple, reflexed hook tip; shorter branch sinuous with simple tip; shortest branch with large reflexed hook 	• Bifid and heavily scletorized • Medial branch with dorsally reflexed knob; lateral branch with prominent notch, tips with ventrally flexed knob bearing large beak-like projection 	• Trifid and lightly scletorized • Main branch with serrated lateral margin, and bi-cornuate tip; lateral branch slender with simple tip; other branch simple 
Pattern of bursal rays	• Lateral rays reach margin of bursa • Origin of externodorsal ray independent 	• Externolateral ray does not reach margin of bursa • Origin of externodorsal ray independent 	• Externolateral ray does not reach margin of bursa • Externodorsal ray originates from dorsal ray 

1.3.3 Life cycle

Members of the genus *Globocephaloides* have a direct life cycle (Fig. 9). Eggs are two-celled, with larvae developing within three days and hatching on the third and fourth days. The free-living larval stages occur approximately within ten days, with the first and second ecdysis on the sixth and eighth days, respectively:

a) First-larval stage (L1) – the internal features are obscured by masses of refractile granules filling the intestine and body;

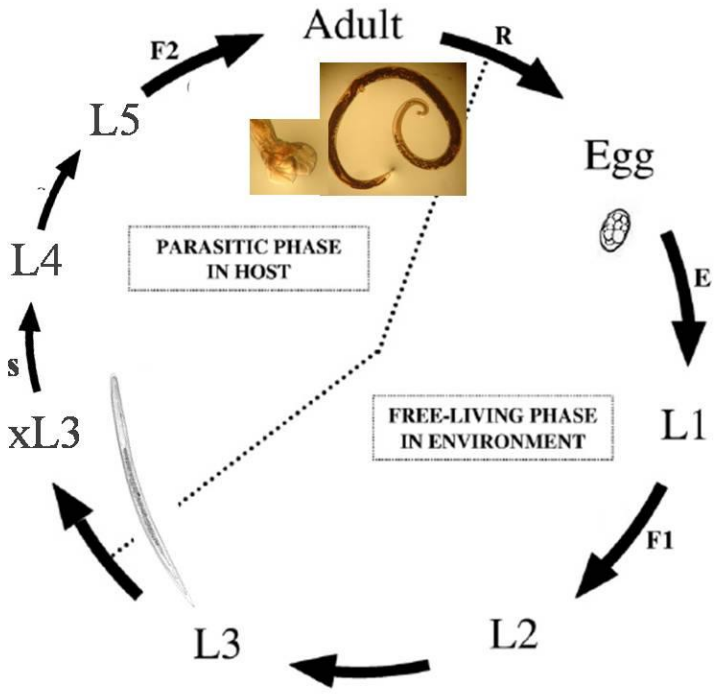
b) Second-larval stage (L2) – retains L1 sheath; fewer granules than L1; oesophagus rhabditiform and visible;

c) Third-larval stage (L3) – retains L1 and L2 sheaths; few granules in both body and intestine.

The infective, L3s are ingested, exsheath in the upper gastrointestinal tract and then migrate, undergoing the fourth-larvae stage (L4), to the duodenum where they establish as dioecious fifth-stage larvae.

The adults are attached to the intestinal mucosa by their buccal capsule and rupture capillaries to feed on blood (Beveridge, 1979).

Figure 9. The life cycle of *Globocephaloides* spp. L1 - first larval stage; L2 - second larval stage; L3 - third larval stage (infective); xL3 - exsheathed L3; L4 - fourth larval stage; L5 – fifth larval stage. Development processes are indicated: embriogenesis (E), sexual differentiation (S), sexual reproduction (R), microbial feeding phase (F1) and host feeding phase (F2). (Adapted from Gasser et al., 2008; Based on Beveridge, 1979)



Beveridge (1979) carried out two experimental infections with *Globocephaloides* larvae. In the first, a juvenile *M. giganteus* (worm-free) was infected by L3s, eggs appeared on faeces after 47 days and remained for more 20 days. In the second experiment, an adult *M. fuliginosus* (*Globocephaloides*-free) was infected with 230 L3s and died on the 28th day. However, only 19 adult worms were found during necropsy and the cause of death was uncertain.

1.3.4 Epidemiology and pathogenicity

A number of epidemiological studies on the helminth fauna of macropodid marsupials have demonstrated that *Globocephaloides* species are an important pathogenic group of parasitic nematodes. Usually, infections are asymptomatic in adult hosts, but juveniles can be seriously affected by *G. trifidospicularis* and to a lesser extent by *G. macropodis*, causing anaemia, hypoproteinaemia and even death, under the conditions of poor nutrition, cold stress and high host density.

Because *Globocephaloides* rupture capillaries to feed on blood, the resultant gastrointestinal lesions are represented by small duodenal hemorrhages in the vicinity of worms (Arundel et al., 1990). Duodenal histopathology is characterized by considerable accumulations of haemosiderin-laden macrophages within the lamina propria of the villi (Arundel et al., 1990).

Depending on the principal mechanism, anaemia due to gastrointestinal parasites can be divided into three groups: a) ingestion of whole blood by the parasite, b) haemorrhage from damaged mucosa and c) nutritional (Symons, 1989). Despite of the occurrence of a small haemorrhage at the attachment site of an individual worm, *Globocephaloides*, as a blood-feeding parasite, causes mainly the first type of anaemia (a), controversially called as 'haemorrhagic anaemia' (Symons, 1989).

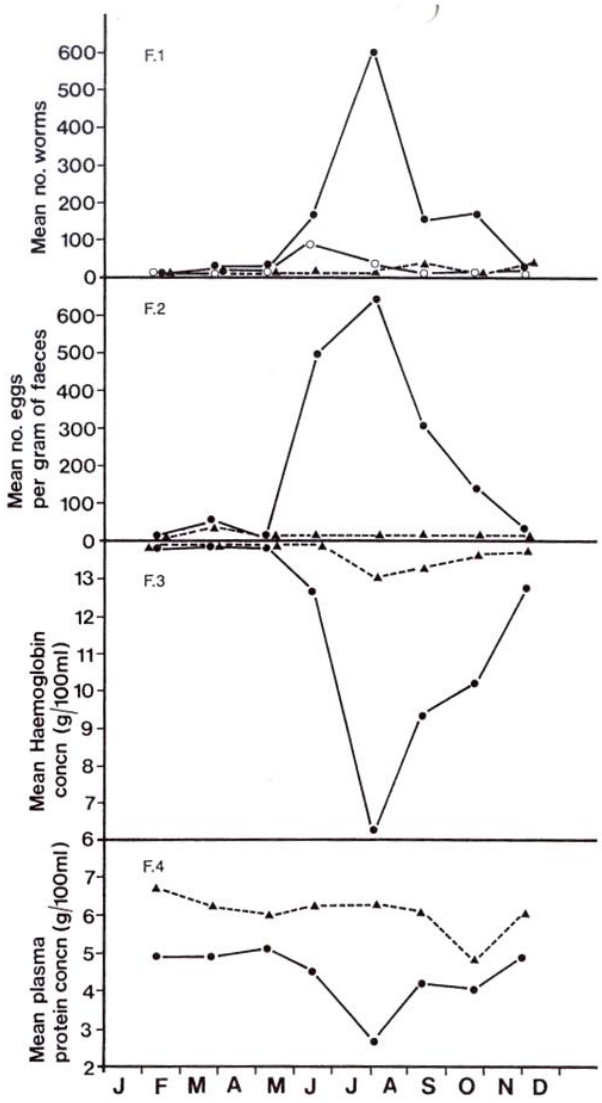
Haemonchus contortus (Nematoda: Trichostrongyloidea), a related parasite in the abomasum, causes the same type of anaemia in small ruminants. Haemonchosis is also characterized by acute anaemia associated with high morbidity or even mortality (Taylor, Coop & Wall, 2007).

In *Globocephaloides* infections, the haematological values have a strong relationship with the number of worms, which often range from 400 to 1500 individuals in weak or moribund hosts, respectively. On average, weak animals have a packed cellular volume (PCV) of 0.20 L/L (reference range 0.47 ± 0.07 L/L), haemoglobin (Hb) of 67 g/L (reference range 154 ± 21 g/L) and total plasma protein (TPP) of 35 g/L (reference range 57 ± 10 g/L) (Arundel et al., 1977; Clark, 2004; Vogelnest & Woods, 2008). Acute anaemia with insufficient erythropoiesis, leading to death, is common in juvenile hosts, when high burdens of worms are associated with winter food depletion, low fat reserve and maximum cold stress. Moribund animals

exhibit a PCV, Hb and TPP as low as 0.10 L/L, 29 g/L and of 23 g/L, respectively (Arundel, Barker & Beveridge, 1977).

Moreover, *Globocephaloides* presents a seasonal pattern of transmission (Fig. 10). The acquisition of worms begins during autumn, reaches a peak in the middle of winter, associated with high rainfall (~ 50 cm), and declines in spring (Beveridge, 1979; Arundel et al., 1990). Similarly, numbers of eggs per gram (EPG) of faeces reflect this seasonal fluctuation and it is likely that juveniles deposit eggs during late winter and spring; the eggs survive to the dry summer and represent a source of infection to the young in the following year.

Figure 10. Seasonal changes in numbers of *Globocephaloides trifidospicularis* and associated haematological values. Months are indicated by their initial letter. In juvenile kangaroos, a closed circle (●) represents adult worms, eggs, haemoglobin or plasma protein concentration, in graphs F.1, F.2, F.3 and F.4, respectively, and an open circle (○) larvae in F.1; a solid triangle indicates (▲) adult kangaroos. (Taken from Arundel et al., 1990)



In addition to seasonal patterns, *Globocephaloides* has differential pathogenicity accordingly to the host age. Arundel et al. (1977) reported that adults of *M. giganteus* were presumably immune, harbouring small numbers of worms (< 50 individuals) at any time of the year, with minor haematological changes and low egg counts (50 EPG is considered as the highest value recorded). In contrast, juveniles had a diminution in both the haemoglobin and plasma concentrations, particularly on the wetter winter months, corresponding to the larvae infection period (> 200 individuals) and high egg counts (as high as 650 EPG).

The reasons for the age resistance are not clear, but as in other parasitic diseases (Symons, 1989) animals become more resistant to primary infections as they reach maturity. Acquired immunity might be related to four mechanisms: a) prevention of migration, b) establishment and development of larvae, c) reduced fecundity of adult worms and/or d) host capability of killing or expelling adults (Symons, 1989; Taylor et al., 2007).

1.3.5 Treatment and control methods

To date, there has been no study regarding treatment or control methods specifically for *Globocephaloides* infections and thus, no drug resistance is recognized.

It is probable that anthelmintics known to kill trichostrongyloid nematodes, such as benzimidazoles (e.g., febendazole, thiabendazole) or avermectins (e.g., ivermectin, moxidectin) are suitable for the treatment of *Globocephaloides* (I. Beveridge, personal communication, March, 2009) (Table 2). For instance, ivermectin binds to glutamate-gated chloride channels (GluCls) leading to paralysis and death of susceptible nematodes, such as *Haemonchus contortus* (Yates, Portillo & Wolstenholme, 2003), because pharyngeal pumping, motility and fecundity ceases. Although there are distinct genetic GluCls characteristics for each group of parasitic nematodes, this mechanism is also reproducible in *C. elegans* (Yates et al., 2003), which is not as closely related to *H. contortus* as *Globocephaloides* species. Thus, it would be expected that ivermectin is able to kill *Globocephaloides* worms.

Regarding the host response, few adverse reactions to medications have been reported in macropods. Speare, Skerratt, Berger and Johnson (2004) reported mebendazole toxicity in captive macropods in Queensland, associated with septicaemia followed by extensive haemorrhage into organs and body cavities, and neutropenia secondary to mebendazole-induced bone marrow depression (i.e., haemorrhagic septicaemic syndrome). The same authors also showed experimentally that mebendazole (50 mg/kg sid for 5 days) is fatal to *Thylogale stigmatica* (red-legged pademelons). So far, other benzimidazoles have not been linked with toxicity in macropods.

To reduce the prevalence of *Globocephaloides* in captive kangaroos and wallabies, general control methods are applicable: a) reduction of population density, b) minimization of cold and nutritional stresses and c) separation of young and adult macropods.

Table 2. Drugs and doses used for helminthiosis in macropods. Single or repeat doses may be required depending on the parasitosis and response. (Based on Vogelnest & Woods, 2008)

Drug	Dose rate
Ivermectin	200-400 µg/kg topical, PO or SC
Moxidectin	500 µg/kg topical, PO or SC
Fenbendazole	25 mg/kg PO sid for 1-5 d
Thiabendazole	44 mg/kg PO

1.3.6 Host affiliation and geographical distribution

Globocephaloides species have different geographical distributions and host specificity (Fig. 11):

a) *G. trifidospicularis* has a wide host range occurring throughout southern Australia (Victoria, New South Wales, South Australia and Tasmania) in free-ranging *M. giganteus*, *M. rufogriseus*, *M. eugenii*, *M. fuliginosus* and *W. bicolor* (Beveridge, 1979; Beveridge & Arundel, 1979; Beveridge et al., 1985; Begg et al., 1995).

b) *G. macropodis* replaces *G. trifidospicularis* in the north (Northern Territory and Queensland), infecting also several host species, particularly *M. agilis*, *M. dorsalis* and *P. persephone* (Beveridge, 1979; Beveridge et al., 1984; Begg et al., 1995; Beveridge et al., 1998).

c) *G. affinis* in contrast is restricted to *M. dorsalis* in north-eastern Australia (Queensland) and, occasionally, to *M. giganteus* where the two hosts occur in sympatry (Beveridge et al., 1984).

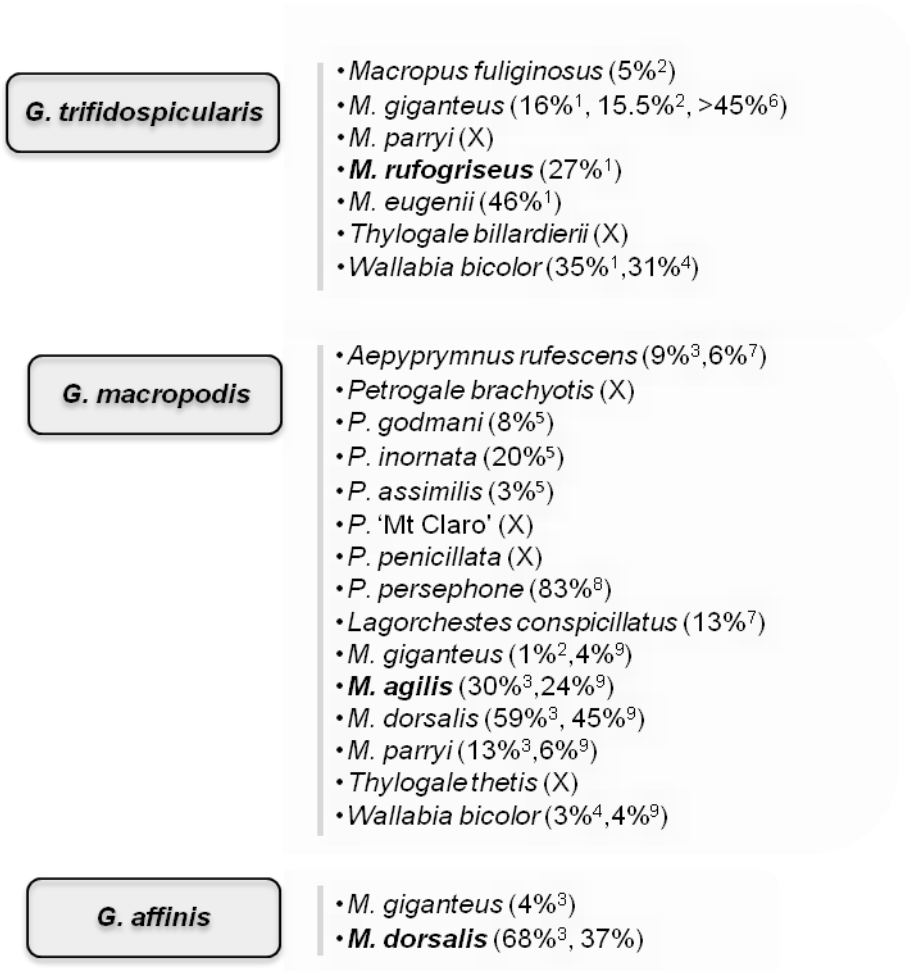
Another interesting characteristic of *Globocephaloides* species is that they present different degrees of pathogenicity accordingly to host species.

In *W. bicolor*, the prevalence (p) of *G. trifidospicularis* is of 31%, but usually worms are present in small numbers (< 50) and do not produce pathological changes (Beveridge et al., 1985). By contrast, in juveniles of *M. giganteus*, *G. trifidospicularis* infection (p = 86%; 207 worms) is accompanied by severe anaemia and can lead to death (Beveridge et al., 1979; Arundel et al., 1990). It was reported that *G. trifidospicularis* was fatal in juvenile eastern grey

kangaroos in a reserve in Victoria (Arundel et al., 1990) and in captive western grey kangaroos housed with eastern grey kangaroos (Vogelnest & Woods, 2008).

Deaths associated with *G. macropodis* have been only reported in a single captive eastern grey kangaroo (Vogelnest & Woods, 2008), but it can be a potential disease agent in *P. persephone* (p = 83%; Begg et al., 1995) which is important in the establishment of captive colonies and conservation of this endangered species of rock-wallaby.

Figure 11. *Globocephaloides* spp. and their respective host records. Prevalence (%) is shown inside brackets and refers to a particular data; an "X" represents unavailable data. The host-type for each *Globocephaloides* species is indicated in bold. Host species are presented in alphabetic order. (Based on Spratt, Beveridge & Walter, 1990)



[1] Beveridge, 1979
[2] Beveridge & Arundel, 1979
[3] Beveridge, Speare & Johnson, 1984
[4] Beveridge, Presidente & Speare, 1985
[5] Beveridge, Spratt, Close, Barker & Sharman, 1989
[6] Arundel, Dempster, Harrigan and Black, 1990
[7] Beveridge, Speare, Johnson & Spratt, 1992
[8] Begg, Beveridge, Chilton, Johnson & O'Callaghan, 1995
[9] Beveridge et al., 1998

Note: Prevalence is only shown for studies with a sample size bigger than 10 animals.

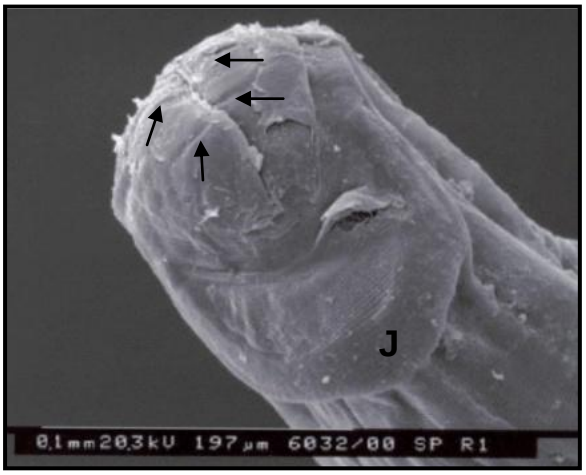
1.4 Current approaches used for the specific identification of nematodes

The accurate identification of parasitic nematodes is central to many areas of research, including epidemiology, ecology and systematics. In the following, relevant morphological, biochemical and molecular approaches for the identification of parasitic nematodes are reviewed.

1.4.1 Morphological approaches

Both light microscopy (LM) and scanning electron microscopy (SEM) are commonly used to identify morphospecies based on phenetic characters. LM has been commonly employed in the morphological characterization of *Globocephaloides*, but SEM provides a much higher magnification and resolution to define objectively the topographical features of the surface of nematodes (Gibbons, 1986). Webley (2004) used SEM to visualize the anterior extremity of a *G. trifidospicularis* specimen (Fig. 12), and detected three depressed canals and a jaw-like structure on each side of the mouth opening.

Figure 12. Scanning electron micrograph of the cephalic extremity of *Globocephaloides trifidospicularis*. An arrow points the neural canal. A 'J' indicates the jaw-like structure. (Taken from Webley, 2004)



Usually, a parasite is first identified to the genus and/or species level using light microscopy, even if subsequent biochemical and/or molecular approaches are to be used. Morphological identification is still considered the mainstay for taxonomic studies of parasitic nematodes. However, it is well recognized that there are limitations with the sole use of morphology to

unequivocally identify species. For example, morphologically similar but genetically distinct (i.e., cryptic) species usually cannot be identified using morphological characters, and subjective interpretation of morphological features can vary considerably among authors (McManus & Bowles, 1996; Chilton, 2004; Gasser, 2006).

A range of biochemical and DNA-based tools can be used to support morphological methods and to provide molecular identification of individual nematodes and genetic data. These tools include multilocus enzyme electrophoresis (MEE), polymerase chain reaction (PCR)-coupled single-strand conformation polymorphism (SSCP) and DNA sequencing.

1.4.2 Biochemical approach: Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MEE), or allozyme electrophoresis, has been employed for many years for systematic studies of a range of parasitic nematodes. This biochemical technique is qualitative and uses allozymes (i.e., different molecular forms of an isozyme derived from different alleles at the same locus) to simultaneously compare two or more samples. The enzymes used in MEE are often inferred to be involved in vital functions of the organism and are more likely to be conserved than other loci. Moreover, these enzymes are representative of the whole genome and can thus be employed as potential genetic markers (Boerlin, 1997).

MEE relies on the principle that the migration of enzymes in an electric field is influenced by the net charge, mass and conformation that they present under non-denatured conditions. After migration, specific histochemical staining procedures can be used to detect the enzyme activity represented by distinct bands in cellulose acetate, polyacrylamide or starch (Andrews & Chilton, 1999).

For instance, Obendorf, Beveridge and Andrews (1991) applied this electrophoretic approach to test the hypothesis that ‘cryptic’ species exist within *G. trifidospicularis* populations. These authors used samples representing pools of worms (rather than individuals) from both *Macropus giganteus* and *M. rufogriseus*, which had been collected in sympatric areas of the state of Tasmania. MEE analysis of 23 enzymes (encoded by 24 loci) yielded reliable markers for *G. trifidospicularis*. For these 24 loci examined, 4 (17%) had fixed allelic differences.

Some studies have shown that the percentage of fixed allelic differences, which identify the presence of distinct biological species, depend on the sample size, geographical origin and speciation events (i.e., allopatric and sympatric speciation). In the case of allopatry, 15% may indicate a lack of gene flow, whereas in sympatry only one fixed allelic difference is considered to be sufficient (Beveridge, 1998; Andrews & Chilton, 1999). However, results must be interpreted cautiously. For example, Obendorf et al. (1991) obtained allelic

differences of 17% in sympatry, but there were only minor and/or unreliable morphological differences between worms, such as the worm total length, spicules length and number of eggs); thus, these authors could not provide clear evidence for the separation of *G. trifidospicularis* into two distinct biological species. Moreover, in this study, Obendorf et al. (1991) used a limited number of host species and geographical areas, which did not allow the authors to make generalizations regarding *Globocepahloides* on the mainland of Australia. Therefore, the hypothesis that *G. trifidospicularis* is a species complex remained to be tested.

1.4.3. DNA-based approaches

DNA-based techniques can be used very effectively to support the morphological and biochemical identification of nematodes. Prior to any DNA-based procedure, it is of major importance to isolate the genomic DNA (gDNA) and to obtain a pure nucleic acid template without host DNA contamination. Protocols have been published (Dawkins & Spencer, 1989; Gasser, Chilton, Hoste & Beveridge, 1993; Gasser et al., 2006b) but, depending on the parasite and/or development stage, the method of gDNA extraction can vary. For example, nematodes (in contrast to cestodes and trematodes) have a tough cuticle and flocculate substances can co-precipitate with nucleic acids during isolation. This 'white precipitate' corresponds to polysaccharides and other unidentifiable compounds (cf. McManus et al., 1985), which may affect subsequent processing or inhibit enzymatic amplification methods (Gasser et al., 1993). Also, free-living larvae are more resistant to physical or chemical disruption, due to an additional, protective sheath.

Therefore, it is important to choose the appropriate extraction method that digests, disrupts or dissolves the cuticular structure, without affecting the final purity of the gDNA. Procedures using DNA-extraction buffer, containing 20 mM Tris-HCl, pH 8, 50 mM EDTA, 1% w/v sodium dodecyl-sulphate (SDS) and proteinase K, followed by direct purification over mini-spin columns (e.g., Wizard DNA Clean-up and Qiagen) have been demonstrated to be highly effective (Gasser et al., 2006b).

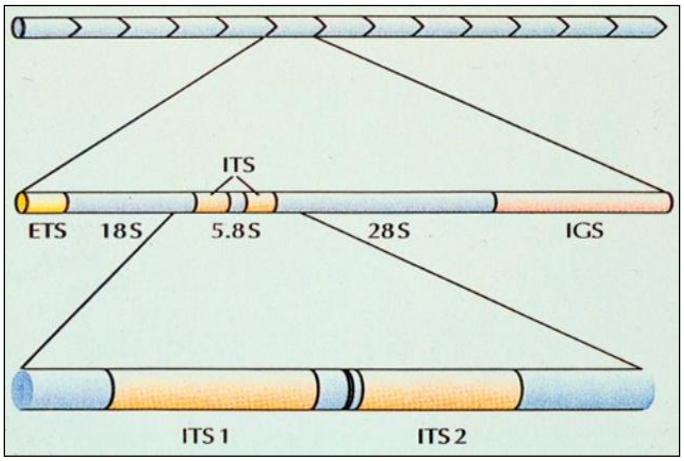
1.4.3.1 DNA target region – genetic marker or locus

It is critical to choose one or more suitable genetic markers for the identification of nematode species. All regions of the nuclear and mitochondrial genomes accumulate mutations over time. Based on the 'concerted evolution' theory, the mutation rate of non-coding regions and

introns is expected to evolve faster than coding regions, which are usually related to a particular vital function in an organism (Gasser & Newton, 2000; Chilton, 2004).

For instance, the nuclear ribosomal DNA (rDNA) exhibits patterns of “concerted evolution”, including both conserved regions for systematic studies and more variable regions for species identification. It contains several tandemly repeated copies of the intergenic non-transcribed spacer (IGS), the external transcribed spacer (ETS) and the transcriptional unit (Fig. 13). This transcriptional unit comprises the 18S (SSU: small subunit), 5.8S and 28S (LSU: large subunit) rRNA genes, separated by the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) located between either 18S and 5.8S genes, or 5.8S and 28S genes, respectively (Chilton, 2004).

Figure 13. Schematic representation of the nuclear ribosomal DNA (rDNA), including the external transcribed spacer (ETS), 18, 5.8 and 28S rRNA genes, the first (ITS-1) and second (ITS-2) internal transcribed spacers, and the intergenic spacer (IGS). (Taken from Schlötterer C. & Tautz D, 1994)



Ribosomal RNA genes display a low rate of heterogeneity and are commonly used for systematic investigations. The divergent (D) domains of the LSU rRNA have been demonstrated to be suitable genetic markers for evolutionary relationships, namely within orders and superfamilies (Chilton, Newton, Beveridge & Gasser, 2001; Chilton, Huby-Chilton & Gasser, 2003; Chilton, Huby-Chilton, Gasser, Beveridge, 2006). For instance, Chilton et al. (2006) used both the SSU and LSU to infer the phylogenetic relationships within the order Strongylida.

By contrast, the ITS region is employed for species delineation, differing sufficiently in sequence among species within a genus but varying little or not at all within a morphospecies (Chilton, Gasser & Beveridge, 1997; Gasser, 1999; Chilton, 2004; Chilton et al., 2006). For example, Chilton, Hoste, Hung, Beveridge and Gasser (1997) described an identical 5.8S

rRNA gene among six *Trichostrongylus* species (i.e., *T. colubriformis*, *T. retortaeformis*, *T. rugatus*, *T. probolorus* and *T vitrinus*), but Hoste, Chilton, Beveridge and Gasser (1998) detected sequence differences in the ITS-1 and ITS-2 between the same species.

A wide range of other studies in parasitic nematodes, including strongylids, have demonstrated the utility and reliability of ITS-1 and ITS-2 as species-specific genetic markers (Stevenson, Chilton & Gasser, 1994; Gasser & Monti, 1997; Hoste et al., 1998; Chilton, Hoste, Newton, Beveridge & Gasser, 1998).

Sequence variation of approximately more than 1.3% in ITS-1 and/or ITS-2 often reflects interspecific variability, whereas differences of less than 1% suggest population heterogeneity or intra-individual variation (i.e., sequence polymorphism between paralogues within an individual nematode) (Stevenson et al., 1995; Hoste et al., 1995, 1998; Chilton, 2004).

In contrast, mitochondrial DNA or microsatellites contain a significant level of intraspecific variability, replacing ITS, if a study is focused in the identification of “strains” or population variation. The mitochondrial gene is maternally inherited and evolves separately from the nuclear genome (Blouin, 2002).

1.4.3.2 The polymerase chain reaction (PCR)

Once a suitable DNA target has been selected, it is necessary to specifically amplify it, so that it can be analysed genetically. The PCR technique (Saiki et al., 1985; Mullis et al., 1986) allows the specific amplification of loci from picogram quantities of genomic DNA (e.g., from individual nematode eggs, larvae or adults).

PCR relies on the enzymatic synthesis *in vitro* of a specific fragment of gDNA, using two oligonucleotide primers which flank the target DNA region to be amplified. In an automated thermal cycler, PCR includes cycles of heat denaturation of the double-stranded DNA, annealing of the primers to their complementary sequences on the opposite strand of the template and extension in both directions from the primer sites, resulting in the production of the double-stranded product (amplicon). In each cycle, the template is replicated by a factor of two, resulting in millions of copies of DNA for subsequent analysis using one or more methods (such as agarose gel electrophoresis, mutation scanning analysis and/or sequencing) (Nelson & Cox, 2008).

During PCR procedures, it is important to be aware of potential problems with contamination of the target DNA with erroneous DNA, particularly “carry-over” contamination from previous PCRs, and/or the contamination of the reagents with amplicons or genomic DNA, particularly if conserved primers are used for amplification. Therefore, a negative (i.e., no template) and positive (i.e., genomic DNA known to amplify) controls should be included in each PCR run.

The optimization of cycling conditions is critical to achieve maximum specificity and fidelity (Gasser, 1999). No PCR protocol is generally applicable; thus, each PCR requires optimization to avoid misincorporations, nonspecific products and primer-dimer formation (Gasser et al., 2006b).

The following aspects are to be taken into account in the optimization of the polymerase chain reaction:

a) Enzyme (DNA polymerase): the common enzyme used for the amplification of gDNA is *Taq* polymerase, a heat-stable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* (Nelson & Cox, 2008).

Taq DNA polymerase concentrations should range between 0.5 and 1.25 units per 50 µl amplification reaction. Extra enzyme concentrations are normally neutral in the results, but when associated with long extension times it may produce nucleotide misincorporations because of the 5'→3' exonuclease activity of *Taq*. In contrast, if concentrations are too low, insufficient amount of the target product is generated (Innis & Gelfand, 1990).

Taq polymerase is crucial for PCR fidelity, since it can introduce nucleotide errors at a rate of ~1-2 x 10^{-5} per nucleotide (Grunenwald, 2003). Moreover, there is a virtual failure of *Taq*, termed 'jumping PCR', that is in the reality due to the synthesis of a sequence with one or more polymorphic sites, producing four double-stranded (ds) DNAs instead of two (Gasser, 1999).

This critical cycling reagent has different commercial suppliers, therefore to achieve the maximum fidelity, a serial titration is recommended for *Taq* DNA polymerase.

b) Magnesium: the decision on the Mg^{2+} concentration is also essential for PCR fidelity and specificity, because it affects primer annealing, denaturation temperatures, product specificity, formation of 'primer-dimer' and *Taq* performance (Innis & Gelfand, 1990). The use of magnesium can vary between kits; for example, Gasser et al. (2006b) reported that 3-3.5 mM is optimal for Promega PCR kit using *Taq*™ pol. However, even under optimal $MgCl_2$ conditions, is important to ensure that dNTPs concentration is not excessive, because they compete with *Taq* for magnesium (e.g., a dNTP increase of 4-6 mM would reduce *Taq* polymerase performance by 30%) (Griffin & Griffin, 1994).

Taking into account all these factors, an initial serial titration is always advised.

c) Deoxyribonucleotide triphosphate (dNTP): dATP, dCTP, dGTP and dTTP must be incorporated in equal concentrations to minimize misincorporation errors (Innis & Gelfand, 1990). dNTPs should also be used in the lowest concentration possible that still amplifies the target DNA, without producing mispriming at undesirable positions and misincorporated nucleotides. Normally, 200 µM of each dNTP per 50 µl reaction is sufficient.

d) Primers: primer concentrations should range from 100 to 500 pmol. Higher concentrations increase the chance of 'primer-dimers', which turn out to be products that compete with the target DNA for enzyme, dNTPs and even primers (Innis & Gelfand, 1990).

Furthermore, the choice of primers should be based on the target DNA and type-organism under investigation. In the case of closely-related parasites, target sequences are reasonably conserved (i.e., similar) and thus, oligonucleotide primers that are known to hybridize with the gDNA of correlated parasites can be used in a preliminary PCR coupled targeted sequencing to verify the identity of the amplicon under study (Gasser, 1999).

e) Buffer: The appropriate buffer is 10 to 50 mM Tris-HCl to obtain a pH of 8.3-8.8 at 20ºC (Innis & Gelfand, 1990). Also, it is recommended to use a coloured buffer for amplifications that will be detected on agarose gels, whereas a colourless one is advised when using dyes or other subsequent fluorescent methods (Griffin & Griffin, 1994).

f) Cycling parameters (denaturation, annealing and extension): Time and temperature of the cycling parameters depend on the length and base composition of the target sequence and melting temperature of the oligonucleotide primers.

In the sole case of denaturation, *Taq* polymerase is also critical for the time/temperature binomial, since the enzyme activity decreases above 85ºC; denaturation conditions of 94-95ºC for 30 seconds are enough to denature the DNA, without losing substantial *Taq* activity (half-life of 40 minutes at 95ºC) (Innis & Gelfand, 1990). However, in the case of a G+C rich target, higher temperatures (95-97ºC) are necessary to break all hydrogen bonds.

For the annealing parameter, temperatures can range between 50 and 72ºC (Innis & Gelfand, 1990), but depending on the GC content of the target sequence it should be around 60ºC for GC greater than 50% and 55ºC for GC equal or less than 50% (Kramer & Coen, 2001).

Finally the extension conditions should be at least 60 sec/kb product sequence at 72ºC.

g) Number of cycles: after the optimization of the previous parameters, the cycle numbers are only dependent on the initial gDNA concentration. Usually, 25-40 cycles are enough for amplification, but “if you have to go more than 40 cycles to amplify a single-copy gene, there is something seriously wrong with your PCR” (Kary Mullis, 1986). More than 40 cycles will increase the chance of nonspecific backgrounds (Innis & Gelfand, 1990).

1.4.3.3 Methods for the analysis and characterization of PCR products

PCR products can be subjected to further analysis using methods, such as agarose gel electrophoresis, mutation scanning and sequencing.

1.4.3.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis is used for the separation of PCR products of up to ~23 kb. Regarding DNA analyses, horizontal agarose gels are the gold standard method, because they are submerged in buffer to prevent from drying out (Westermeier, 2005).

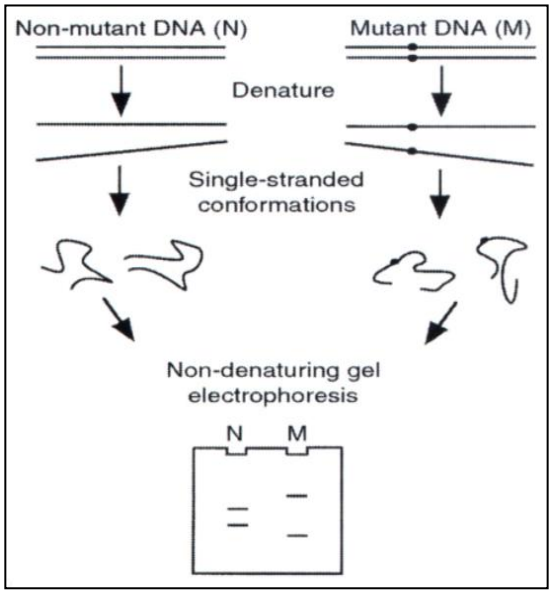
During electrophoresis, amplicons migrate through the electric field and are separated only by size. The charge/mass ratio is usually equal for DNA molecules (Voytas, 2003) and does not influence the differential migration.
The resulting gels are then stained with fluorescent dyes (e.g., ethidium bromide or SYBR Green) and bands are visible under UV light.
This technique can be a preparative step for subsequent analysis, particularly when similar bands in an agarose gel represent amplicons that contain high levels of heterogeneity.

1.4.3.3.2 Mutation-scanning techniques

Agarose gel electrophoresis does not allow amplicons of differing sequences to be differentiated. A range of other approaches, called 'mutation scanning', are available to accurately identify distinct sequences-type between and within amplicons, which has important implications for the study of populations genetic variation.
These techniques separate DNA molecules not only based on size but also considering chemical or physical characteristics. Some examples are heteroduplex analysis, single-strand conformation polymorphism (SSCP), denaturating gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and single nucleotide polymorphism (SNP) (Gasser, 1998; Westermeier, 2005). For instance, DGGE can detect point mutations in fragments up to 500 bp (mutation rate = 100%), but it is still reliable for fragments of between 500 bp to 1 kb (Gasser, 1998, 1999). However, if it is known a *priori* that the target sequence is relatively short (100 bp to 400 bp) SSCP is a good alternative, having a similar level of resolution, without needing to be extensively optimized for each sequence-type and organism (Gasser, 1998, 1999; Gasser et al., 2006b).
The SSCP technique was developed by Orita et al. (1989) for the detection of polymorphisms in human DNA, but applications have extended to the detection of mutations, allele separation, species identification and exclusion of PCR contamination (Griffin & Griffin, 1994). Several studies (Gasser & Monti, 1997; Gasser & Chilton, 2001) have successfully employed SSCP to visually detect sequence variation in PCR products within and among specimens or populations for a variety of parasites.
SSCP analysis is based on the principle that the electrophoretic mobility of a single-stranded (ss) DNA molecule in a non-denaturing gel depends on its length, molecular weight and conformation (Fig. 14). In solution, ssDNA fragments acquire secondary and tertiary conformations due to intra-strand base pairing; thus, if a single nucleotide substitution exists in the primary sequence of a particular ssDNA, the migration through the gel is different from a non-mutant ssDNA. Under optimal conditions, a point mutation would cause a difference in mobility, and would thus be detected based on a distinct banding pattern (i.e., shift) in a non-denaturating gel (Gasser et al., 2006b). Usually, 50-100% of mutations can be detected for

fragments between 100 and 200 bp in length, but recent studies have demonstrated that point mutations can be resolved for amplicons up to 450-500 bp (Gasser et al, 2007). Also, SSCP can be more sensitive at detecting sequence polymorphisms than sequencing especially for screening proposes (R. Gasser, personal communication, April 2009). However, the mutation detection rate of SSCP can be altered according to the sequence length (i.e., >500 bp) and nucleotide composition (i.e., A+T or G+C richness), electrophoresis temperature and voltage, gel composition and operator errors (Gasser et al., 2006b).

Figure 14. The principle of PCR-based SSCP analysis. A single mutation (represented by a dot on a DNA strand) leads to the formation of different single-strand conformations of the mutant DNA (M) compared with the non-mutant molecule (N), resulting in differential mobilities in a non-denaturating gel matrix. (Taken from Gasser et al. 2006b)



Another characteristic of SSCP is that it can be performed under isotopic (using radioactively labeled amplicons) or non-isotopic ('cold') conditions. The former has the advantage of being able to detect mutant sequences within an amplicon, which may represent a small percentage of the overall copies, but is potentially hazardous for the operator. In the absence of facilities to handle radio-isotopes, the non-isotopic SSCP is a valuable approach, since after electrophoresis, the gel can be stained with SYBR Gold (Invitrogen), silver or ethidium bromide (Bio-Rad) and photographed (Gasser et al., 2006b).

The SSCP technique allows the accurate display of sequence variation in ITS within and among a range of species of parasitic nematodes of livestock (reviewed by Gasser & Chilton, 2001; Gasser, 2006; Gasser, Bott, Chilton, Hunt & Beveridge, 2008), and of macropods,

such as *Labiostrongylus longispicularis* (see Huby-Chilton, Beveridge, Gasser & Chilton, 2001), *Hypodontus macropi* (see Gasser, Zhu, Beveridge & Chilton, 2001) and *Zoniolaimus mawsonae* (see Huby-Chilton, Beveridge, Gasser & Chilton, 2002), collected from different host species and geographical localities.

For instance, Huby-Chilton et al. (2002) visualized two distinct SSCP banding patterns for the ITS-2 region of individuals representing *Z. mawsonae* from *M. rufus*. This result was further supported by the sequencing of the same region (*D* = 4.8%) and morphological examination (different lengths of the spicules, bursa conformation and genital cone), which ultimately led to the erection of a new species, *Z. latebrosus*.

Also, in a limited study (Webley, Beveridge & Coulson, 2004), seven individual worms of *G. trifidospicularis* from *M. fuliginosus* and *M. eugenii* (from Kangaroo Island, South Australia) were analyzed by SSCP to explore intraspecific variation in the ITS-2; however, no heterogeneity was detected among samples.

1.4.3.3.3 DNA sequencing

Usually, DNA sequencing follows mutation detection methods, when large numbers of samples need to be screening, because it is more costly and time-consuming. Thus, DNA sequencing is often used to obtain the quantitative data of a target mutant nucleotide sequence detected by a mutation-scanning approach (cf. Gasser et al., 2006b). Sequencing allows the determination of the nucleotide sequence and thus the definition of nucleotide variation (i.e., substitutions, insertions, deletions and/or polymorphism).

One of the commonly used techniques for the DNA sequencing is the ‘dideoxy method’, first developed by Fred Sanger in 1975. It uses dideoxyribonucleotide triphosphates (ddNTPs) which are specific chain-terminating analogues of the normal deoxyribonucleotide triphosphates (dNTPs) lacking the 3’-hydroxyl group. These ddNTPs act as terminators when incorporated into a “growing” DNA chain by DNA polymerase, since the chain does not contain the 3’-hydroxyl group to form a phosphodiester bond with the next nucleotide (Sanger, 1980; Lodish et al., 2004). In this technique, four reactions are performed simultaneously and within each tube there is a mix of: 4 dideoxyribonucleotide triphosphates (ddATP, ddTTP, ddCTP or ddGTP); 4 deoxyribonucleotide triphosphates (dATP, dTTP, dCTP or dGTP); and a primer (with a dye marker) to initiate the DNA synthesis.

The resultant fragment is usually detected using dye fluorescence in an automated machine; hence, a different colour is emitted, depending on the nucleotide composition, allowing the direct reading of the nucleotide sequence (Griffiths, Miller, Suzuki, Lewontin & Gelbart, 1996; Lodish et al., 2004). For instance, using the program Sequence Scanner (Applied Biosystems), a fragments ending in G, T, A or C shows a black, red, green or blue colour, respectively.

Dideoxy sequencing has been employed for the quantification of sequence variation in ITS within and among a range of species of parasitic nematodes (reviewed by Gasser, 2006), including those of macropodid marsupials. For example, DNA sequencing was used in the detection of heterogeneity within *Hypodontus macropi* (see Chilton, Gasser & Beveridge, 1995), *Papillostrongylus labiatus* (see Chilton, Huby-Chilton, Gasser & Beveridge, 2002), *Labiosimplex australis* (see Chilton, Huby-Chilton, Smales, Gasser & Beveridge, 2009) and *Cloacina* (see Chilton, Huby-Chilton, Johnson, Beveridge & Gasser, 2009).
SSCP-coupled sequencing was applied to *Labiostrongylus longispicularis* (Huby-Chilton et al., 2001) and *Zoniolaimus mawsonae* (see Huby-Chilton et al., 2002).

1.4.4 Phylogenetic analyses

DNA sequence data can be further used to establish the relationships of organisms, including nematodes. Phylogenetic trees, for example, display visually the genetic or evolutionary relationships of the taxa (i.e., sequences) under study.
A tree is composed of branches (i.e., line that connects two nodes) and nodes, which can be either terminal, representing taxa, or internal, representing ancestral relationships. A phylogenetic analysis includes an ingroup (i.e., taxa being compared) and an outgroup (i.e., the taxon with which the ingroup is being compared) (Hall, 2004).
There are four tree building methods: neighbour joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). None of these methods is perfect for all circumstances; the choice depends on the complexity of the data set and algorithm used.
Bayesian Inference is gaining acceptance, being cost and time efficient and readily accessible (e.g., MrBayes program can be obtained *via* internet). BI analysis is a character-based method, like ML and MP, but instead of seeking a single tree that maximizes the likelihood of the data set, it seeks the best set of trees (i.e., those with greatest likelihoods) (Hall, 2004).
The BI relies on a ‘generation’ process, by creating trees and evaluating each new tree, accepting the new if the posterior probability (pp) is greater than the previous one(s); otherwise, the state remains the same. As the number of generations increases, the acceptance of a new tree is random - this point is called the ‘stable likelihood’. Finally, a consensus tree is displayed for the taxa, accordingly to the relationships of the variations present in the primary sequence data.
Although methods for constructing phylogenies attempt to display the ‘correct’ tree, it is important to understand that these analyses are only deductions of historical events. The topology of the tree can never be assumed to reflect accurately the similarity among the taxa (Hall, 2004).

1.5 Conclusions from the literature review and research aims

This literature review has shown that *Globocephaloides* species are important parasites of macropodid marsupials in Australia. Although *Globocephaloides* infections are usually asymptomatic in adult macropods, some members of this genus can be highly pathogenic in juvenile animals, causing anaemia, hypoproteinaemia and even death, particularly under the conditions of poor nutrition, cold stress and high host density. Particular attention needs to be paid to these parasites, because some of their hosts are listed as endangered native fauna (e.g., *Petrogale persephone*) and also because they might become potential pathogens (for example due to host-switching events) in a range of other host species with the same or similar physiological, gastrointestinal and nutrition features.

Given that the identification and classification of *Globocephaloides* species have been based mainly on traditional approaches (i.e., morphology, host and geographical origin), there are significant controversies regarding their systematics and population structures. Previous studies have proposed that both *G. trifidospicularis* and *G. macropodis* represent complexes of species, since they have wide host and geographical ranges compared with *G. affinis* which is relatively limited. However, there has been no major attempt to test this proposal. Together with morphological approaches, PCR-based mutation scanning and sequencing, employing suitable genetic markers in ITS rDNA, are available for the specific identification of strongylid nematodes. The combined application of these tools can now explore the species composition of *Globocephaloides* from different host species and assist in improving our knowledge of the systematics, speciation events, geographical distribution and pathogenicity of members of this genus.

Therefore, to test the hypothesis that *G. trifidospicularis* and *G. macropodis* each represent a species complex, the following aims were set:

1. To assess the genetic variation within and among *Globocephaloides* individuals and populations from different macropodid hosts and geographical distributions in Australia, using PCR-coupled SSCP, followed by targeted sequencing.
2. To use the molecular data to establish the genetic relationships within *Globocephaloides* species and among other related trichostrongyloid nematodes.
3. In the event of the discovery of new and/or cryptic species, to morphologically describe them in detail using light and scanning electron microscopy.
4. To gain improved insights into the host affiliation and geographical range of each *Globocephaloides* species.
5. To appraise the findings from the present study and compare them with previous investigations, in order to draw final conclusions.

Chapter 2
Material and Methods

2.1 Collection of parasites

During necropsy, adult specimens of *Globocephaloides* (*n* = 156) were collected from the small intestines of a range of infected macropodid marsupials from different states in Australia (Figs. 15 and 16); the hosts were mainly “road-kills” (Fig. 17). In light infections, worms were individually collected and washed in physiological saline. In moderate to heavy infections the duodenal content was washed in water and allowed to sediment. The residue was examined using a dissection microscope (Fig. 18). A pool of *Globocephaloides* worms (≈ 3 to 20 individuals) from each host was transferred to an Eppendorf tube (1.5 ml), washed in physiological saline and frozen at -70ºC. Information of specimens is shown in Table 3.

Table 3. Host and geographical origins of *Globocephaloides* specimens subjected to morphological and molecular analysis.

Host species	Geographical origin	Morphospecies	No. of individual worms analyzed
Macropus eugenii	Melbourne (captive), Victoria	*G. trifidospicularis*	7
	Kangaroo Island, South Australia		15
Wallabia bicolor	Halls Gap, Victoria		2
	Healesville, Victoria		4
	Buangor, Victoria		4
	Brimpaen, Victoria		2
	Werribee, Victoria		10
	Mount Zero, Victoria		3
M. giganteus	Evandale, Tasmania		9
	Portland, Victoria		20
	Bellbrae, Victoria		10
	Mount Zero, Victoria		2
M. fuliginosus	Dadswell’s Bridge, Victoria		6
	Brimpaen, Victoria		1
M. rufogriseus	Sasafrass, Tasmania		18
	Brimpaen, Victoria		3
	Laharum, Victoria		4
M. dorsalis	Yeppoon, Queensland	*G. macropodis*	8
	Rockhampton, Queensland		5
	Kawonga, Queensland		2
	Bowen, Queensland		1
M. agilis	Townsville, Queensland		9
M. dorsalis	Kawonga, Queensland	*G. affinis*	10

Figure. 15. Australian map showing the geographical origin of *Globocephaloides* specimens. A blue star indicates *G. macropodis*, a red star *G. affinis* and a green star *G. trifidospicularis*.

Figure 16. Map of the state of Victoria showing the localities from where *Globocephaloides trifidospicularis* specimens (green star) were collected: 1. Portland; 2. Brimpaen; 3. Laharum; 4. Halls Gap; 5. Mount Zero; 6. Dadswell's Bridge; 7. Buangor; 8. Bellbrae; 9. Werribee; 10. Melbourne; 11. Healesville.

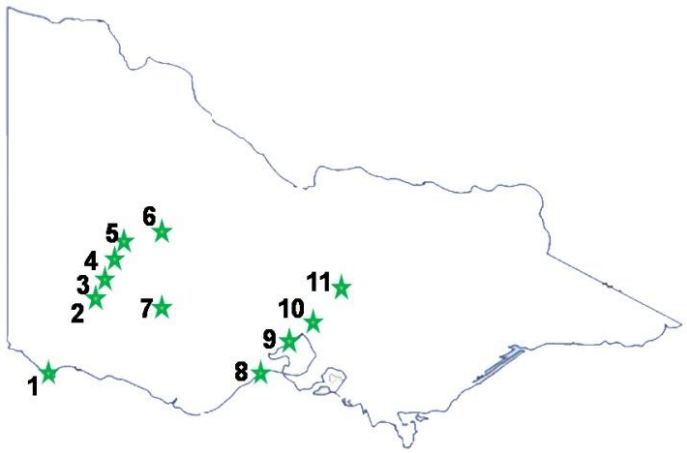
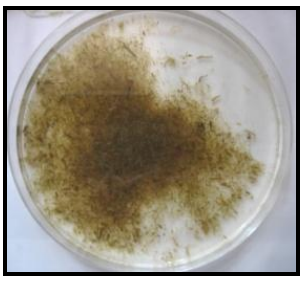


Figure 17. Necropsy *in situ* of a road-killed *Wallabia bicolor*; Point Addis, Victoria. (Original)

Figure 18. A. *Globocephaloides* worms inside the duodenum of *Macropus giganteus.* B. Sediment of the duodenum content, containing *Globocephaloides* worms (not visible). C. Macroscopic view of *Globocephaloides* worms. (Original)

A



B

C

2.2 Preparation of samples and morphological identification

The frozen worms were thawed and again washed extensively in physiological saline. The anterior and posterior ends (1 mm) of individual worms were excised under a dissection microscope and cleared in a drop of lactophenol prior to morphological examination. The mid-body section (2-5 mm) was cut into two parts. One part was then used for the isolation of genomic DNA and the other remained frozen (for any further analyses).

All samples were morphologically examined by light microscopy and indentified to species according to the available descriptions (Beveridge et al, 1984; Beveridge, 1979). Samples were also maintained as voucher specimens.

2.3 Molecular study: genetic characterization of *Globocephaloides* species

2.3.1 Isolation and purification of genomic DNA

Genomic DNA was isolated from individual worms using a standard small-scale sodium dodecyl-sulphate (SDS)/proteinase K treatment (Gasser, Chilton, Hoste & Beveridge, 1993). To each Eppendorf tube was added 155 µl of extraction solution (containing 5 µl Proteinase K in 150 µl of extraction buffer - 20 mM Tris-HCl, pH 8.0, 100 mM EDTA and 1% SDS).
The tubes were incubated at 37ºC overnight (usually 14 h). After incubation, genomic DNA was purified directly using a mini-spin column (Wizard® DNA Clean-Up, Promega) employing a vacuum manifold, according to manufacturer's recommendations.
Prior to storage at -20ºC, the concentration of the gDNA in each Eppendorf tube was measured by spectrophotometry (NanoDrop® 1000).

2.3.2 Analysis of the internal transcribed spacers (ITS) of nuclear ribosomal DNA

The first (ITS-1) and second (ITS-2) internal transcribed spacers of nuclear ribosomal DNA (rDNA) were selected for use in this study, as they provide a species-specific marker for a wide range of strongylid nematodes (reviewed by Chilton, 2004; Gasser, 2006, Gasser et al., 2008).

2.3.2.1 Amplification of the second internal transcribed spacer (ITS-2) by polymerase chain reaction (PCR)

The ITS-2^{+} region (i.e., ITS-2 plus ~90 bp flanking sequences, comprising ~20 bp at the 3' end of the 5.8S rRNA gene and ~70 bp at the 5' end of the 28S rRNA gene) was amplified by polymerase chain reaction (PCR) from ~5-50 ng of genomic DNA using oligonucleotide primers NC1 (forward: 5´-ACGTCTGGTTCAGGGTTGTT-3) and NC2 (reverse: 5´-

TTAGTTTCTTTTCCTCCGCT -3´), designed to regions of 5.8S and 28S rRNA genes of the free-living nematode, *Caenorhabditis elegans* (Gasser et al, 1993), respectively.

PCR was performed in 50 µl volumes using 100 pmol of each primer, 200 µM of each dNTP, 3.0 mM of $MgCl_2$ and 1.25 U of *Taq* polymerase (GoTaq, Promega); a no-template ("negative") control was included with each run. The cycling conditions used for PCR were: 94 °C for 5 min (initial denaturation), then 30 cycles of 94 °C for 30 sec (denaturing), 55 °C for 30 sec (annealing), 72 °C for 30 sec (extension), followed by 72 °C for 10 min (final extension), in a thermal cycler (2720, Applied Biosystems).

Aliquots (5 µl) of individual PCR products were run in 1.5% agarose gels at 100 V in Tris/Boric Acid/EDTA Buffer (TBE: 65 mM Tris-HCL, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad) for 45 min, stained with ethidium bromide for 20 min and then photographed, following destaining in H_2O for 15 min.

Each PCR product amplified represented a single band on an agarose gel.

2.3.2.2 Single-strand conformation polymorphism (SSCP) analysis of ITS-2$^+$ amplicons

This method follows protocol B, described by Gasser, Hu, Chilton, Campbell, Jex, Otranto, Cafarchia, Beveridge & Zhu (2006).

In brief, 2 µl of individual amplicon were mixed with 4 µl of DNA sequence stop solution (cat. no. Q408A, Promega) and 4 µl of H_2O. After denaturation at 94ºC for 30 min and subsequent snap-cooling on a freeze block (-20ºC) for 1-2 min, 6 µl of individual samples were loaded into the wells of a prefabricated GMA gel (S-2x25 or S-2x13, Elchrom Scientific AG) that had been pre-run in an SEA2000 apparatus (Elchrom Scientific AG) for 30 min.

Electrophoresis was performed at 74 V and 7.4ºC (constant) for 18 h. Following electrophoresis, the gel was separated from the back sheet with a nylon line and subsequently stained in SYBR Gold (Invitrogen; according to the manufacturer's instructions) for 20 min, destained in H_20 for 15 min and then photographed digitally (GelDoc, BioRad).

2.3.2.3 Amplification of the internal transcribed spacers by PCR

Based on the banding patterns detected in the SSCP analysis of the ITS-2$^+$ region, samples were selected for the amplification of the entire ITS-1 and/or ITS-2 region (Table 4).

The rDNA region comprising the ITS-1, ITS-2 and the 5.8S gene (designated ITS$^+$), was amplified by the polymerase chain reaction (PCR) from 5-50 ng of genomic DNA using oligonucleotide primers NC16 (forward: 5´- AGTTCAATCGCAATGGCTT-3´) and NC2

(reverse: 5´-TTAGTTTCTTTTCCTCCGCT-3´), designed to conserved regions of 18S rRNA gene of strongylid nematodes (Chilton, Huby-Chilton & Gasser, 2003) and 28S rRNA gene of the free-living nematode *Caenorhabditis elegans* (Gasser et al, 1993), respectively.
PCR was performed as for ITS-2^+, but employing 3.5 mM of $MgCl_2$. The cycling conditions used were: 94 °C for 5 min (initial denaturation), then 35 cycles of 94 °C for 40 sec (denaturing), 52 °C for 60 sec (annealing), 72 °C for 75 sec (extension), followed by 72 °C for 10 min (final extension).
PCR products were analyzed by electrophoresis agarose gel, as for ITS-2^+.

2.3.2.4 Sequencing of the ITS-2 and ITS-1 regions

ITS^+ amplicons were column-purified (Wizard PCR-Preps, Promega), according to the manufacturer's instructions, and then subjected to automated, bi-directional sequencing using the same primer set as used for amplification as well as primer NC1 and its reverse complement NC1R (5´-AACAACCCTGAACCAGACGT-3´; Chilton, Hoste, Hung, Beveridge & Gasser, 1997).
Forward and reverse sequences were analyzed for base purity using the computer program Sequence Scanner (Applied Biosystems®). Reverse sequences were also converted into their complement sequences (http://www.bioinformatics.org/sms2/rev_comp.html) and then clustered with the respective forward sequence using the computer program BioEdit® (www.mbio.ncsu.edu/BioEdit/page2.html) to confirm the sequence strength (i.e., similarity).
All sequences (representing each individual worm) were then aligned using the program BioEdit® and adjusted manually. The pairwise differences between sequences (*D*) were calculated using the formula *D* (%) = [1 - (*M*/*L*)] x 100, where *M* is the number of alignment positions at which the two sequences have a base in common and *L* is the total number of alignment positions over which the two sequences are compared Chilton, Gasser & Beveridge (1995).
Nucleotide sequence data have been deposited in the GenBank database under accession numbers GQ131400 - GQ131409.

2.3.2.5 Secondary structure prediction for the ITS-2 precursor rRNA

A secondary structure model for the ITS-2 precursor rRNA of *Globocephaloides* species was manually constructed using reference structures for other trichostrongyloid and strongyloid nematodes (Chilton, Hoste, Newton, Beveridge & Gasser, 1998; Hung, Chilton, Beveridge &

Gasser, 1999). This structure was used to examine the positions of nucleotide alterations and to optimize the sequence alignment for the subsequent phylogenetic analysis.

2.3.3 Phylogenetic analyses of *Globocephaloides* species

Phylogenetic analysis of ITS-1 and ITS-2 sequence data was conducted using Bayesian Inference (BI), employing the software package MrBayes v.3.1.2 (http://mrbayes.csit.fsu.edu/index.php).

Posterior probabilities (pp) were calculated *via* 2,000,000 generations (ngen = 2,000,000; burnin = 2,000) using the Monte Carlo Markov Chain method and four simultaneous tree building chains (nchains = 4), with every 10-th tree being saved (samplefreq = 10).

Evolutionary distance was calculated using the General Time Reversible (GTR) evolutionary model (nset = 6), allowing for a gamma-shaped variation in mutation rates between codons (rates = gamma).

Nematodirus battus (accession nos. AJ251569.1 and Y14010; ref. [1]) was selected as the outgroup. Upon completion of the analysis, a consensus tree was constructed in FigTree v1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4 Morphological study: morphological characterization of *Globocephaloides macropodis* genotypes

2.4.1 Light microscopy

Specimens identified as *G. macropodis* in the molecular study and also all specimens identified as *G. macropodis* or *G. wallabiae* in the collection of the South Australian Museum (SAM), Adelaide, were examined (registration numbers of vouchers are cited in the text) (see appendix II).

Individuals were cleared in lactophenol and then examined by light microscopy. Only collections containing at least one male specimen were identified to species.

Measurements were made with the aid of an ocular micrometer, and drawings were made with a drawing tube attached to an Olympus BH2 microscope. Measurements in adult worms included the total length, maximum width, buccal capsule wide and length, tooth length, oesophagus length, and nerve-ring, excretory pore and deirid from anterior end. The spicules length was also measured for male specimens and the tail length, vulva from posterior end and eggs length for female specimens. In the descriptions, measurements are given in

millimetres as the range, followed by the mean of ten measurements, in parentheses. Buccal capsule measurements were made on lateral views, including the jaw-like structures or battlements (width) and from the base of dorsal tooth to the anterior extremity of the buccal capsule (length).

2.4.2 Scanning electron microscopy

Selected specimens were examined further by scanning electron microscopy (SEM). Specimens were dehydrated in ethanol, dried in hexamethyldisilasane (ProSciTech, Townsville), mounted on stubs with double-sided tape, sputter-coated with gold and viewed in a Siemens Autoscan microscope at an accelerating voltage of 5 kV.

2.5 Phylogenetic analysis: relationships between the genus *Globocephaloides* and selected trichostrongyloid genera

The partial sequences of the first (D1) and second (D2) divergent domains of the 28S rDNA sequence data (available upon request from N. Chilton, 2009) of 31 species of trichostrongyloid nematode, including *G. trifidospicularis*, were aligned using the ClustalX 2.0.11 program (http://www.clustal.org).

Phylogenetic analysis of the partial LSU rDNA sequence data was conducted using Bayesian Inference (BI), employing the software package MrBayes v.3.1.2 (http://mrbayes.csit.fsu.edu/index.php).

Posterior probabilities (pp) were calculated *via* 2,000,000 generations (ngen = 2,000,000; burnin = 2,000) using the Monte Carlo Markov Chain method and four simultaneous tree building chains (nchains = 4), with every 10-th tree being saved (samplefreq = 10).

Evolutionary distance was calculated using General Time Reversible (GTR) evolutionary model (nset = 6), allowing for a gamma-shaped variation in mutation rates between codons (rates = gamma).

C. elegans (accession number X03680) was selected as the outgroup. Upon completion of the analysis, a consensus tree was constructed in FigTree v1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Chapter 3
Results and Discussion

3.1 Molecular study: genetic characterization of *Globocephaloides* species

In a first step, ITS-2$^+$ amplicons (~350 bp) were produced from genomic DNA samples from 156 individuals of *G. trifidospicularis* (*n* = 121), *G. macropodis* (*n* = 25) and *G. affinis* (*n* = 10) (Table 4).

Although no variation in size was detected among amplicons on agarose gels (representative gels shown in Fig. 19), SSCP analysis allowed the display of significant sequence heterogeneity in ITS-2$^+$ both within and among samples (representative profiles shown in Fig. 20).

Figure 19. Representative agarose gels of the ITS-2$^+$ amplicons (~350 bp) derived from selected individual worms: **A.** *Globocephaloides trifidospicularis* from *M. giganteus* (Victoria); **B.** *G. macropodis* from *M. dorsalis* (Queensland); **C.** *G. affinis* from *M. dorsalis* (Queensland). Φ = 100 bp ladder.

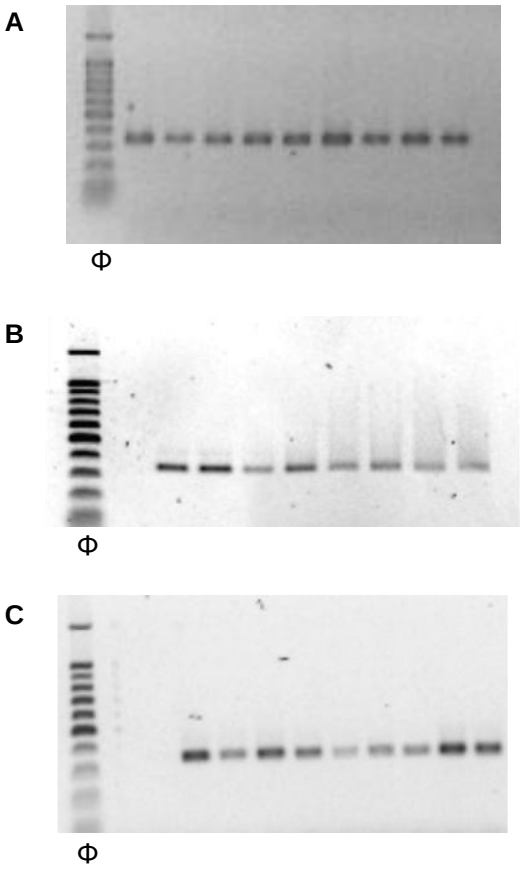
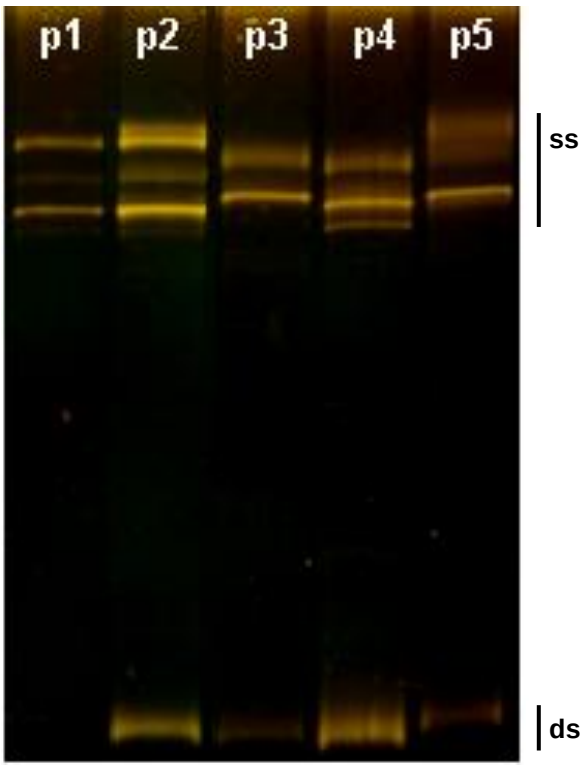


Table 4. Host and geographical origins of *Globocephaloides* specimens subjected to molecular analysis of the first (ITS-1) and second (ITS-2) internal transcribed spacers plus flanking sequence. Single-strand conformation polymorphism (SSCP) profiles for ITS-2 are listed. Accession numbers of representative ITS-1 and ITS-2 sequences derived from individual nematodes selected based on SSCP profiles are also listed.

Host species	Geographical origin	Morphospecies	No. of individual worms subjected to SSCP analysis	SSCP profile (ITS-2^{+})	Accession no. (ITS-2)	Accession no. (ITS-1)
Macropus eugenii	Melbourne (captive), Victoria	*G. trifidospicularis*	7	p1	GQ131400	
	Kangaroo Island, South Australia		15	p1	GQ131400	GQ131404
Wallabia bicolor	Halls Gap, Victoria		2	p1	GQ131400	
	Healesville, Victoria		4	p1	GQ131400	
	Buangor, Victoria		4	p1		
	Brimpaen, Victoria		2	p1		
	Werribee, Victoria		10	p1		
	Mt Zero, Victoria		3	p1		
M. giganteus	Evandale, Tasmania		9	p1	GQ131400	GQ131404
	Portland, Victoria		20	p1	GQ131400	
	Bellbrae, Victoria		10	p2	GQ131400	
	Mt Zero, Victoria		2	p1		
M. fuliginosus	Dadswell's Bridge, Victoria		6	p1	GQ131400	GQ131405
	Brimpaen, Victoria		1	p1		
M. rufogriseus	Sasafrass, Tasmania		18	p1	GQ131400	GQ131406
	Brimpaen, Victoria		3	p1		
	Laharum, Victoria		4	p1	GQ131400	GQ131404
M. dorsalis	Yeppoon, Queensland	*G. macropodis*	8	p3	GQ131401	GQ131407
	Rockhampton, Queensland		5	p3		
	Kawonga, Queensland		2	p3		
	Bowen, Queensland		1	p3		
M. agilis	Townsville, Queensland		9	p4	GQ131402	GQ131408
M. dorsalis	Kawonga, Queensland	*G. affinis*	10	p5	GQ131403	GQ131409

A total of five different SSCP profiles were displayed for all 156 amplicons: profiles p1 and p2 were displayed for *G. trifidospicularis* from five different macropodid hosts, p3 for *G. macropodis* from *M. dorsalis,* p4 for *G. macropodis* from *M. agilis*, and p5 for *G. affinis* (Table 4).

Figure 20. Representative gel displaying single-strand conformation polymorphism (SSCP) analysis of ITS-2 amplicons derived from selected individual worms of *Globocephaloides trifidospicularis* (profiles p1 and p2), *G. macropodis* (profiles p3 and p4) and *G. affinis* (profile p5). Single-stranded (ss) and double-stranded (ds) DNAs are indicated.



Thirteen samples representing all five SSCP profiles (p1-p5) and different host and geographical origins (Table 4) were selected for the sequencing of ITS-2^{+}. The ITS-2 sequences determined varied in length from 243 to 254 bp (Fig. 21) and in G+C content from 34 to 38%.

For *G. trifidospicularis*, the different SSCP banding patterns detected between profiles p1 and p2 corresponded to one deletion at the 5' end of the 28S rDNA sequence flanking the ITS-2^{+}.

For *G. macropodis*, the variation in ITS-2 sequence between profiles p3 (*M. dorsalis*) and p4 (*M. agilis*) was 7.1%, which related to 18 mutations over an alignment length of 254 positions, represented by 7 indels (insertions/deletions), 10 transitions (A ↔ G, *n* = 7; C ↔ T, *n* = 3) and 1 transversion (A ↔ T) (see Fig. 21). Based on this latter finding, *G. macropodis* from *M. agilis* was a distinct genotype from *G. macropodis* from *M. dorsalis*. Sequence differences in ITS-2 among species/genotypes ranged from 9% to 13% (Table 5).

The nucleotide alterations in ITS-2 within and among *Globocephaloides* species/genotypes were linked to the proposed secondary structures for the ITS-2 precursor rRNA (Chilton et al., 1998; Hung et al., 1999) (Fig. 22).

The majority of the substitutions, insertions and/or deletions occurred in loops and/or bulges (i.e., unpaired regions) on stems II, VI and VII, altering, in some cases, the base-pairing arrangements and conformation. For instance, the end loop of the stem VI varied in length and shape among all *Globocephaloides* species or genotypes, and the bulge in stem VII was adjusted for *G. affinis* relative to the other species/genotypes. In spite of significant nucleotide variation in ITS-2 among species/genotypes of *Globocephaloides*, the complete or partial compensatory nucleotide changes did not appear to alter the overall stability of the secondary structure model, and the conserved core regions were essentially maintained.

To complement the SSCP-based analysis of sequence variation in ITS-2, ITS-1 sequences were determined for selected samples (*n* = 8) linked to particular hosts and geographical locations in Australia (see Table 4). The ITS-1 sequences determined were 465-472 bp in length (Fig. 21) and had a G+C content of 40-41%.

For *G. trifidospicularis*, the sequence variation in ITS-1 was ≤ 0.2% (Fig. 21); this variation was associated with two substitutions (G ↔ A and T ↔ C at alignment positions 228 and 321, respectively) and four polymorphic positions (alignment positions 193, *n* = 1; 321, *n* = 2; 421, *n* = 1) (Fig. 21).

For *G. macropodis*, the sequence variation in ITS-1 between specimens from *M. agilis* and *M. dorsalis* was 5.2%, which related to 4 indels (insertions/deletions), 14 transitions (A ↔ G, *n* = 5 C ↔ T, *n* = 9) and 7 transversion (A ↔ T, *n* = 3; C ↔ A, *n* = 3; G ↔ T, *n* = 1). This sequence variation between the genotypes of *G. macropodis* (from *M. agilis* and *M. dorsalis*) was in agreement with that recorded in ITS-2.

Sequence differences in ITS-1 among species and genotypes of *Globocephaloides* ranged from 7 % to 19% (Table 5).

Figure 21. Alignment of the ITS-1 and ITS-2 sequences representing *Globocephaloides* species/genotypes: *G. trifidospicularis* (accession nos. GQ131400 and GQ131404-GQ131406); *G. macropodis* (GQ131401, GQ131402, GQ131407 and GQ131408); and *G. affinis* (GQ131403 and GQ131409). An alignment position identical to that of the top sequence is indicated by a dot. An alignment gap is represented by a dash. Based on International Union of Pure and Applied Chemistry (IUPAC) codes, Y = C and/or T; R = A and/or G. Numbers in round brackets indicate the length (bp) of each sequence. Sequence elements corresponding to stems I-VII of the ITS-2 precursor rRNA are underlined. Only samples with sequence variation are shown. Published sequence data (Chilton, Huby-Chilton, Gasser, Beveridge, 2006) infer rRNA gene boundaries.

ITS-1

```
                10        20        30        40        50        60        70        80        90        100       110       120       130       140       150
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GQ131404    TCGAAA-CCAAAACATGGTTCACACGCTCTGATCGAATGAGAAACCAACATGCTAGCTGGATTTGACGACCTTGTCGTTAAACGTTGGGAGTATCGACCACCGTTAGAGCTCTATTTGAGGTGTCTACGTGCGAGGCATGAGTCGTTCAA
GQ131405    ......-.....................................................................................................................................................
GQ131404    ......-.....................................................................................................................................................
GQ131404    ......-.....................................................................................................................................................
GQ131406    ......-.....................................................................................................................................................
GQ131407    ......-......T......T.....................................A.....................................T.....................T.....................T..
GQ131408    ......-......T......T.............A..C.................G....T....A.....T.................A.....................................T.....................
GQ131409    ......A........C.........A..T.....C..A..........T.....G.TG......T.....T.GT....G...T.....A.............TT...A........C..........GT...T.....................

                160       170       180       190       200       210       220       230       240       250       260       270       280       290       300
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GQ131404    GAGTGGCGGCTGTGATTGTACGCACAAAGTTCCCATTTTCTACGTTCA-CGTTGTAAATGGTTGAGCTTAA-GACTTGATGAGCATTGCTGGAATGCCGCCTCTATTA-TTAT--TTG-ATT---GTTGTATTGGCGGTTATGTACATAG
GQ131405    ..................................................-.....................-.....A.............................-....--...-...---.....................
GQ131404    ..................................................-.....................-.....................................-....--...-...---.....................
GQ131404    ..................................................-.....................-.....................................-....--...-...---.....................
GQ131406    .........................................Y.....-.....................-.....................................-....--...-...---.....................
GQ131407    ..........C.........................C.....T.....-...G...G............G.T.....................................---...--...-...---.....................
GQ131408    ........................T.............C............T...G...G............G.T.....................................---...TT...-...---.....................
GQ131409    ........A..A............TG..........C.....T.....---.A...G.....A........-.............................G..GTT...-G...T...GTT..........T.............TA

                310       320       330       340       350       360       370       380       390       400       410       420       430       440       450
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GQ131404    ACGAATCATGATGCTAAATTTGAGCTTGT-TTATGTACAATACCTG-AACTAAATCAGGAAACCTTAATGACCTGTCATAACGAAATCTTTCGTGATTTCGACGGGTGCCAAGACAACAGATAACACTACTAGATTTGAATTTT-GCAGA
GQ131405    ...........................-.............-.....................................................................-.....
GQ131404    .....................Y........-.............-.....................................................................-.....
GQ131404    .....................Y........-.............-.....................................................................-.....
GQ131406    .....................C........-.............-.....................................................R.....................-.....
GQ131407    T.....A...T..G.G....C........-.............-..A.....................................G.....-A...................T...G........CA............T....T.....
GQ131408    TT....A...T..G.G....C.......AGC.............-.....................................G......-A...................T...G.....................T.....
GQ131409    TT..T.AC.A.......T..CA.......-A.............G...C.....................A..G..AA..G....C-........AT.................C....T....T..T.A..TG.....T.....

                460       470
            ....|....|....|....|....|....
GQ131404    AATGTGATTGTTTATAATCACGAAATGAA  (466)
GQ131405    ............................  (466)
GQ131404    ............................  (466)
GQ131404    ............................  (466)
GQ131406    ............................  (466)
GQ131407    ........A......T.....A.C.....  (465)
GQ131408    ........A............A.C.....  (469)
GQ131409    ........C.....CG.A...A.T.CAG.  (472)
```

Figure 21. (Continuation)

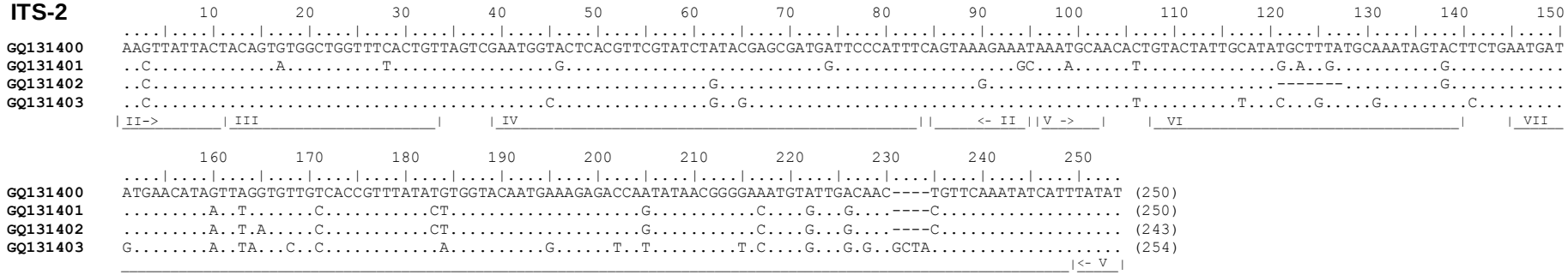
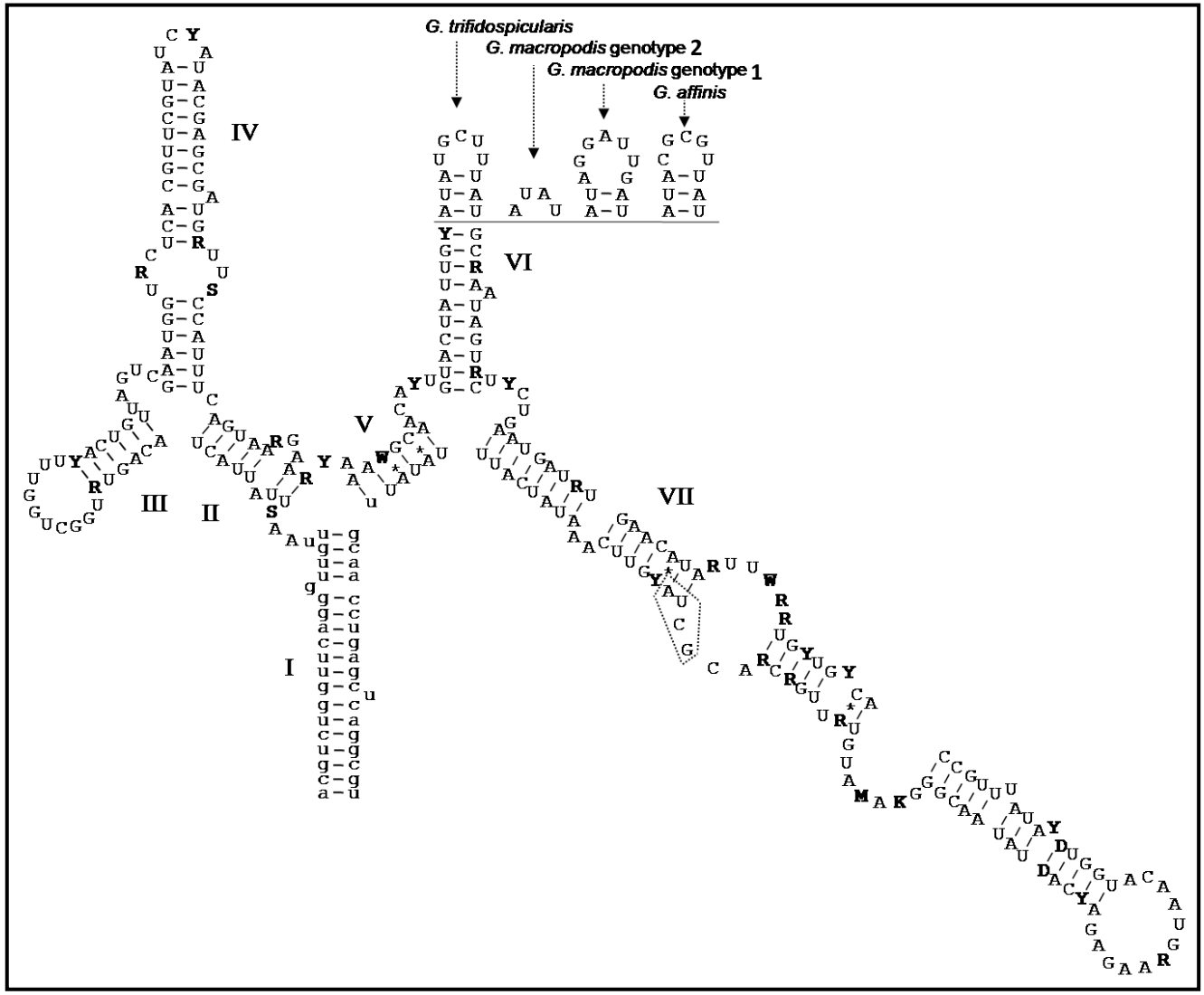


Table 5. Pairwise comparison of sequence differences (%) in ITS-1 (upper diagonal) or ITS-2 (lower diagonal) among *Globocephaloides* species and within *G. macropodis*. GenBank accession numbers for selected sequences representing each species and/or genotype are: GQ131400-GQ131403 for ITS-2, and GQ131404 and GQ131407-GQ131409 for ITS-1.

***Globocephaloides* species/genotype**	(1)	(2)	(3)	(4)
(1) *G. trifidospicularis*	-	7.3	8.4	17.7
(2) *G. macropodis* genotype 1	9.1	-	5.2	19
(3) *G. macropodis* genotype 2	8.7	7.1	-	18.6
(4) *G. affinis*	11.4	13	12.2	-

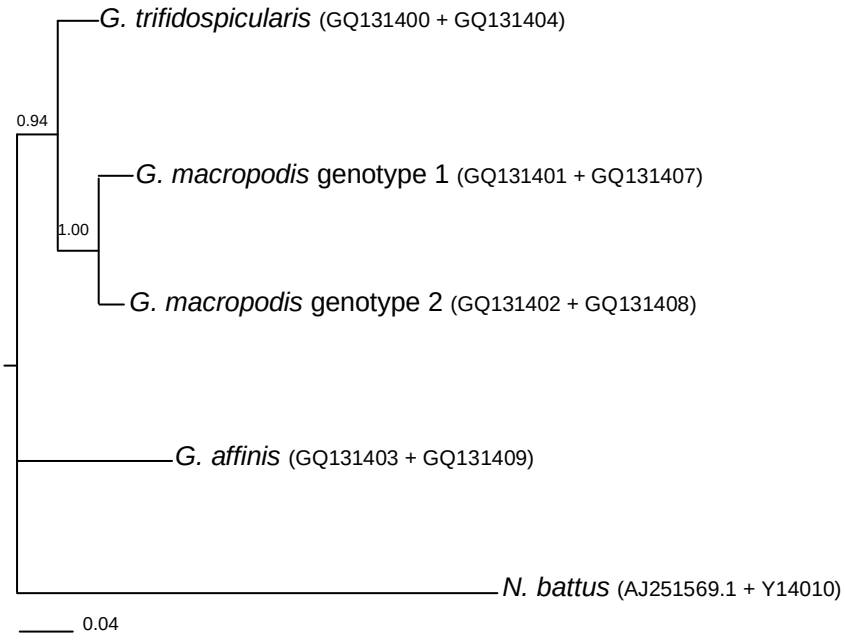
Figure 22. Predicted consensus secondary structure of the ITS-2 precursor ribosomal RNA (rRNA) and flanking regions (i.e., 3'-end of the 5.8S rRNA gene and the 5'-end of the 28S gene) for *Globocephaloides trifidospicularis* (accession number GQ131400), *G. macropodis* genotype 1 (GQ131401), *G. macropodis* genotype 2 (GQ131402) and *G. affinis* (GQ131403). Helices are indicated by roman numerals (cf. Fig. 21). Nucleotides in lower-case letters represent part of the 5.8S or 28S rRNA genes (stem I). Based on International Union of Pure and Applied Chemistry (IUPAC) codes, Y = C and/or T; R = A and/or G; S = C and/or G; M = A and/or C; W = A and/or T; D = not C. Partial compensatory nucleotide changes are marked with asterisks. The region demarked by a dotted line in stem VII represents an insertion in the sequence for *G. affinis* compared with the other species studied (cf. Fig. 21).



Having assessed genetic variation within and among populations of *Globocephaloides* from different geographical locations, a phylogenetic analysis of the concatenated ITS-1+ITS-2 sequence dataset was conducted using BI to establish the relationships among *G. affinis*, *G. trifidospicularis* and the two genotypes of *G. macropodis*. The ITS-1 and ITS-2 sequences were both aligned manually; the alignment of ITS-2 sequences (to achieve maximum positional homology) was guided by the precursor rRNA secondary structure model (*cf*. Fig. 22).

The phylogram (Fig. 23) shows that *G. affinis* was the most genetically distinct species relative to *G. trifidospicularis* and the two genotypes of *G. macropodis* (which both formed a clade that was strongly supported; pp = 0.94-1.00).

Figure 23. Phylogram depicting the genetic differences between selected individuals of *Globocephaloides*, based on an analysis by Bayesian inference of concatenated ITS-1 and ITS-2 sequence data. Concatenation is indicated by a plus sign. *Nematodirus battus* represents the outgroup. The posterior probability (pp) for each clade is given at each respective node.



The present study elucidated, using a mutation scanning approach, the genetic make-up of *Globocephaloides* populations from seven different macropodid hosts from disparate geographical regions in Australia using genetic markers (i.e., ITS-1 and ITS-2) known to allow the specific identification of strongylid nematodes of macropodids and other vertebrates

(Gasser & Chilton, 2001; Blouin, 2002; Chilton, 2004; Gasser, Gruijter & Polderman, 2006; Gasser, 2006; Gasser et al., 2008).

The low degree of nucleotide variation (≤ 0.2%) detected in the ITS-1 region within *G. trifidospicularis* from five host species and thirteen geographical locations was less than the levels of intraspecific variation (usually ≤ 1%) detected for a wide range of strongylid nematodes (Hoste et al., 1993, 1995, 1998; Stevenson et al., 1995; Huby-Chilton et al., 2001; Chilton, 2004; Gasser, 2006), including those of the suborder Trichostrongylina, to which the genus *Globocephaloides* belongs. The sequence homogeneity in ITS rDNA within *G. trifidospicularis* contrasts with previous MEE results, showing a fixed allelic difference of 17% between (pooled) samples of *G. trifidospicularis* from sympatric hosts in Tasmania (Obendorf et al., 1991), and does not support a previous proposal that *G. trifidospicularis* represents a complex of at least two species. In spite of the discordance between the present and MEE results (Obendorf et al., 1991), multiple genetic loci that display more sequence variation within species (such as mitochondrial or microsatellite markers) could be used to retest this previous hypothesis.

There is a precedent for cryptic species in strongylid nematodes of macropodid marsupials. A number of previous studies have discovered cryptic species and/or proposed adaptive speciation (Gasser et al., 2001; Huby-Chilton et al., 2001, 2002; Chilton et al., 1995, 2002, 2009a,b). For example, Huby-Chilton et al. (2002) used a mutation scanning-sequencing approach to detect 5.2% sequence variation in ITS-2 between two forms of *Zoniolaimus mawsonae* (Nematoda: Strongyloidea) from *M. rufus* and concluded that they represented sibling species. This conclusion was supported by a detailed morphological examination showing delineation based on spicule length as well as genital cone and bursal conformations.

The current molecular findings revealed genetic homogeneity within *G. macropodis* from *M. agilis* and *M. dorsalis*, based on both SSCP analysis and sequencing. There was a consistent sequence difference of 7.1 and 5.2% in ITS-2 and ITS-1, respectively, between the two genotypes of *G. macropodis* representing each host species. These percentages were more than five times greater than the nucleotide variation usually detected in either ITS-1 or ITS-2 within species (<1%) and similar to the variation in these rDNA regions detected among species (1.3-7.6%) of trichostrongyloid nematodes (Stevenson et al., 1995; Hoste et al., 1995, 1998; Chilton, 2004).

This information indicates that the two genotypes of *G. macropodis* from *M. dorsalis* (= genotype 1) and *M. agilis* (= genotype 2) are probably sibling species.

3.2 Morphological study: morphological characterization of *Globocephaloides macropodis* genotypes

To independently test the proposal that *G. macropodis* is a composite of two distinct species, a detailed morphological study was required. Therefore, the posterior and anterior ends of male and female specimens (AHC 45367) from *G. macropodis* used for the molecular study were examined in detail by light microscopy.

The morphological data indicated that the specimens from *M. dorsalis* (genotype 1) were different to those found in *M. agilis* (genotype 2). *Globocephaloides* worms from *M. dorsalis* (Fig. 24) possess longer spicules (0.48 mm), "foot-like" spicule tips, the externolateral ray invariably reaches the margin of the bursa and the externodorsal ray originates from the posterolateral ray. By contrast, in *Globocephaloides* worms from *M. agilis (*appendix I), the spicules (0.41 mm) have a prominent notch on one branch and the tips are ventriflexed, bearing a large rostrate projection; also the externodorsal ray originates independently from lateral and dorsal rays.

In the present study, the distinct molecular and morphologic characteristics between *G. macropodis* genotypes instigated the erection of a new species. Since *G. wallabiae* was first described from *M. dorsalis* in Queensland by Johnston and Mawson (1939a), this species was here resurrected and is not synonymous with *G. macropodis*, as Beveridge (1979) considered in the absence of the type-specimens in SAM (presumably lost) and based on the description (Johnston and Mawson, 1939a) of the spicules tips ("spoon-like").

Furthermore, *G. wallabiae* is redescribed in detail (Fig. 24) and all specimens in the SAM collection, identified as *G. macropodis* or *Globocephaloides* sp., were re-examined to rectify the species name, and to gain insights into the host specificity and geographical distributions.

3.2.1 *Globocephaloides wallabiae*: redescripiton and associated host species

***Globocephaloides wallabiae* Johnston & Mawson, 1939a**

✶ Material examined from:

- *Macropus dorsalis* (type-host) (Queensland): 1 ♂ , Clement's Ck, Marlborough (AHC 12119); 2 ♂ , Mt. Surprise (AHC 11141); 1 ♂ , 4 ♀ , Milman (AHC 12253); 1 ♂ , 1 ♀ , Palamana Stn via Charters Towers (AHC 12745); 2 ♂ , Rockhampton (AHC 32457, 41481); 3 ♂ , Harvest Home Stn via Charters Towers (AHC 45368); 10 ♂ , 9 ♀ , Milman (AHC 12023); 3 ♂ , 5 ♀ , Yeppoon (AHC 45367);

- *Petrogale mareeba* (Queensland): 1 ♂ , Banggarra Stn via Ravenshoe (AHC 33472);
- *Petrogale assimilis* (Queensland): 1 ♂ , 2 ♀, Valley of Lagoons Stn via Greenvale (AHC 13169).

✶ Redescription (Fig. 24):

Small nematodes, coiled in loose, flat coil; ventral surface on inside of coil. Body without longitudinal ridges or alae, covered by numerous transverse annulations. Annulations extremely fine, close together posterior to level of buccal capsule, slightly wider apart in region from excretory pore to posterior end.

Mouth opening oval, slit-like when closed, dorsoventrally elongate. Lips absent; mouth surrounded by 2 lateral, jaw-like structures, joined at dorsal and ventral margins. Each jaw bears 3 finger-like extensions of neural pulp which reach mouth opening. Cephalic papillae seen only in apical views: 2 amphids, 4 tiny conical cephalic papillae; labial papillae not seen. Jaws supported posteriorly by U-shaped buttress-like elevations of cuticle and subcuticular tissues on dorsal and ventral aspects, extending to level of anterior extremity of oesophagus, running anteriorly and laterally. Buccal capsule large, urceolate, with thickened rim at base, thinning markedly anteriorly; walls also thin dorsally and ventrally. Single pointed, elongate dorsal tooth arises from oesophagus; 2 tiny subventral teeth present, not projecting into buccal capsule.

Dorsal oesophageal gland opens through apex of dorsal tooth. Oesophagus elongate, clavate, slightly wider at posterior end.

Nerve ring encircles oesophagus near anterior extremity. Excretory pore posterior to nerve ring, leading to elongate excretory gland. Deirid small, at level of excretory pore.

Male. Measurements of 10 specimens from *Macropus dorsalis*.

Total length 4.40-5.54 (4.94); maximum width 0.11-0.29 (0.27); buccal capsule 0.090-0.110 (0.095) wide × 0.070-0.080 (0.077) long; tooth 0.030-0.045 (0.037) long; oesophagus 0.56-0.75 (0.62) long; nerve-ring 0.14-0.22 (0.19), excretory pore 0.19-0.27 (0.23) and deirid 0.19-0.30 (0.23) from anterior end; spicules 0.40-0.56 (0.48) long.

Lateral lobes of bursa enlarged, fused with ventral lobes. Dorsal lobe very small, separated from lateral lobes. Ventroventral and ventrolateral rays separated, slender, reaching margin of bursa. Lateral rays broad at origin, subequal, externolateral ray directed ventrally, variable, reaching or not quite reaching margin of bursa; other lateral rays directed dorsally, reaching

margin of bursa. Externodorsal ray originating from posterolateral ray, independently from dorsal ray, not quite reaching margin of bursa. Dorsal ray short, divided into 4 very short branches at distal extremity. Two dome-shaped bullae overlie origins of lateral and ventral rays.

Genital cone prominent; ventral lip of cone forms elongate, conical projection between bullae. Dorsal lip gives rise to elongate, distally bilobed projection.

Spicules short, complex, heavily sclerotized, yellow with numerous longitudinal ridges on spicules bodies. Spicules bifid distally. Main branch of spicule terminates in foot-like projection. Medial branches of spicules slender, simple, shorter than lateral branches, tip simple. Gubernaculum absent.

Female. Measurements of 10 specimens from *Macropus dorsalis*.

Length 4.79-11.40 (7.54); maximum width 0.23-0.42 (0.31); buccal capsule 0.090-0.110 (0.103) wide, 0.070-0.100 (0.083) long; tooth 0.030-0.045 (0.037); oesophagus 0.65-1.02 (0.79) long; nerve ring 0.15-0.28 (0.19), excretory pore 0.19-0.34 (0.27), deirid 0.22-0.345 (0.27) from anterior end.

Tail 0.13-0.21 (0.18) long; vulva 1.92-3.70 (2.62) from posterior end; 0.06-0.11 (0.96) egg. Tail short, simple, conical.

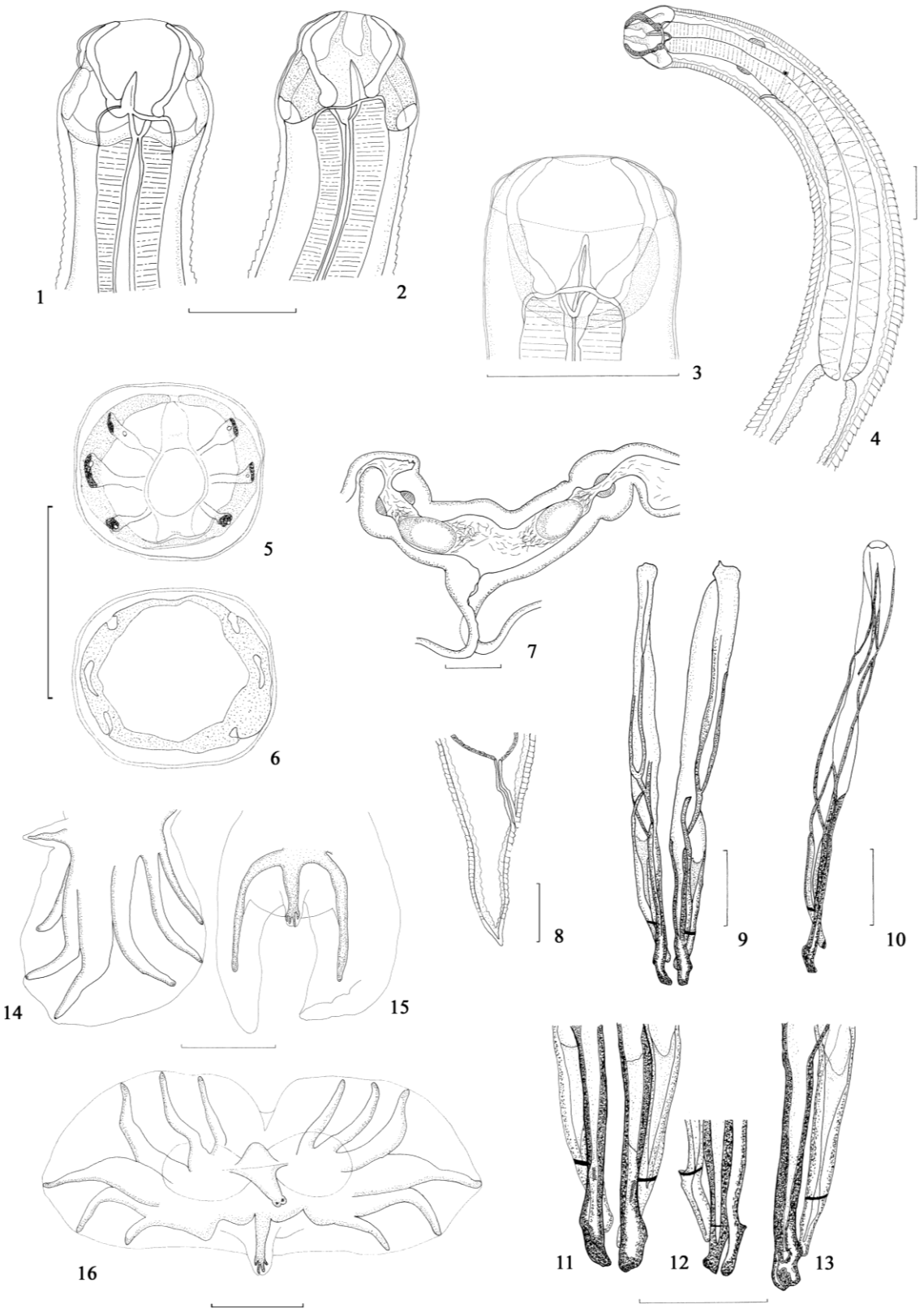
Vulval lips prominent, lips approximately equal. Vagina short; ovejectors paired. Uteri large, sac-like, opposed. Egg thin-shelled, ellipsoidal.

✶ SEM (Fig. 25):

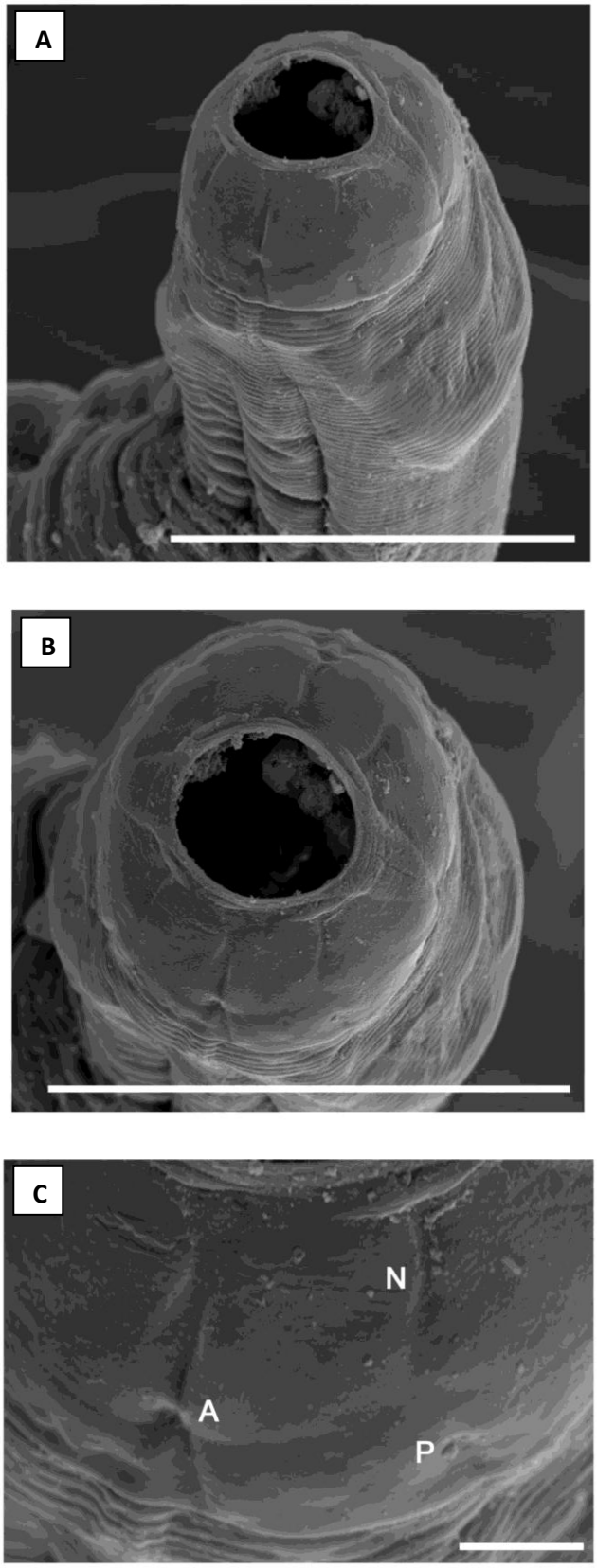
Scanning electron micrographs of *G. wallabiae* clearly revealed paired amphids and four tiny, conical cephalic papillae situated mid-way along the neural channels, which are clearly visible in lateral views of the head. The presence of these papillae has not been reported in previous studies of the genus (Beveridge, 1979; Beveridge et al., 1984).

The neural canals extend anteriorly beyond the papillae to the vicinity of the stoma. However, no labial papillae were detectable using either light or SE microscopy.

Figure 24. *Globocephaloides wallabiae*. 1. Cephalic end, sub-dorsal view. 2. Cephalic end, lateral view. 3. Cephalic end, ventral view. 4. Anterior end, lateral view. 5. Cephalic end, apical view. 6. Optical transverse section through buccal capsule showing indentations for neural canals. 7. Vulva and ovejector, lateral view. 8. Female tail, lateral view. 9. Spicules, ventral view. 10. Spicule, lateral view. 11. Distal tips of spicules, lateral view. 12. Distal tips of spicules, sublateral view. 13. Distal tip of spicule, ventral view. 14. Bursa, lateral view. 15. Dorsal lobe of bursa, dorsal view. 16. Bursa, apical view of flattened bursa. Scale-bars = 0.1 mm. (Original)



Figures 25. Scanning electron micrographs of the cephalic extremity (A, B and C) of *Globocephaloides wallabiae*. Figure C shows a close-up of the cephalic papillae (P), amphid (A) and neural canal (N). Scale-bars = 0.1 mm (figures A and B) and 10 µm (figure C). (Original)



3.2.2 *Globocephaloides macropodis*: associated host species

***Globocephaloides macropodis* Yorke & Maplestone, 1926**

✶ Material examined from:

- *Macropus agilis* (type-host) (Queensland): 3 ♂ , 6 ♀ , Hervey's Range, Townsville (AHC 7165, 7517); 3 ♂ , 6 ♀ , Townsville (AHC 7565, 41483-41487); Northern Territory: 1 ♂ , 3 ♀ , Tipperary Stn (AHC 9951); (Papa New Guinea): 2 ♂ , Bula Plain, Bensbach Province (AHC 33878);
- *Petrogale persephone* (Queensland): 3 ♂ , 3 ♀, Proserpine (AHC 23715); 9 ♂ , 10 ♀ , Flame Tree Hill, Proserpine (AHC 24206, 30758); 1 ♂ , 1 ♀ , Mandalay Rd., Proserpine (AHC 24207); 5 ♂ , 2 ♀ , O'Haras Hill, Dingo Beach (AHC 24209, 30759); 1 ♂ , Strathdickie (AHC 24211); 2 ♂ , 1 ♀ , Mt. Lucas, Proserpine (AHC 30755, 30762);
- *Petrogale brachyotis* (Northern Territory): 1 ♂ , Narbarlek (AHC 10835);
- *Petrogale inornata* (Queensland): 2 ♂ , 1 ♀ , Apis Ck Stn via Marlborough (AHC 11105);
- *Aepyprymnus rufescens* (Queensland): 2 ♂ , 2 ♀ , Ayersville (AHC 8841)
- *Macropus parryi* (Queensland): 1 ♂ , 1 ♀ , Inkerman Stn via Home Hill (AHC 12364);
- *Macropus giganteus* (Queensland): 3 ♂ , 1 ♀ , Balgal (AHC 13422);
- *Macropus dorsalis* (Queensland): 3 ♂, Harvest Home Stn via Charters Towers (AHC 12744);
- *Largochestes conspicillatus* (Queensland): 3 ♂ , Bohle (AHC 12837).

3.2.3 *Globocephaloides macropodis* and *G. wallabiae*: comparison of host specificity and geographical distribution

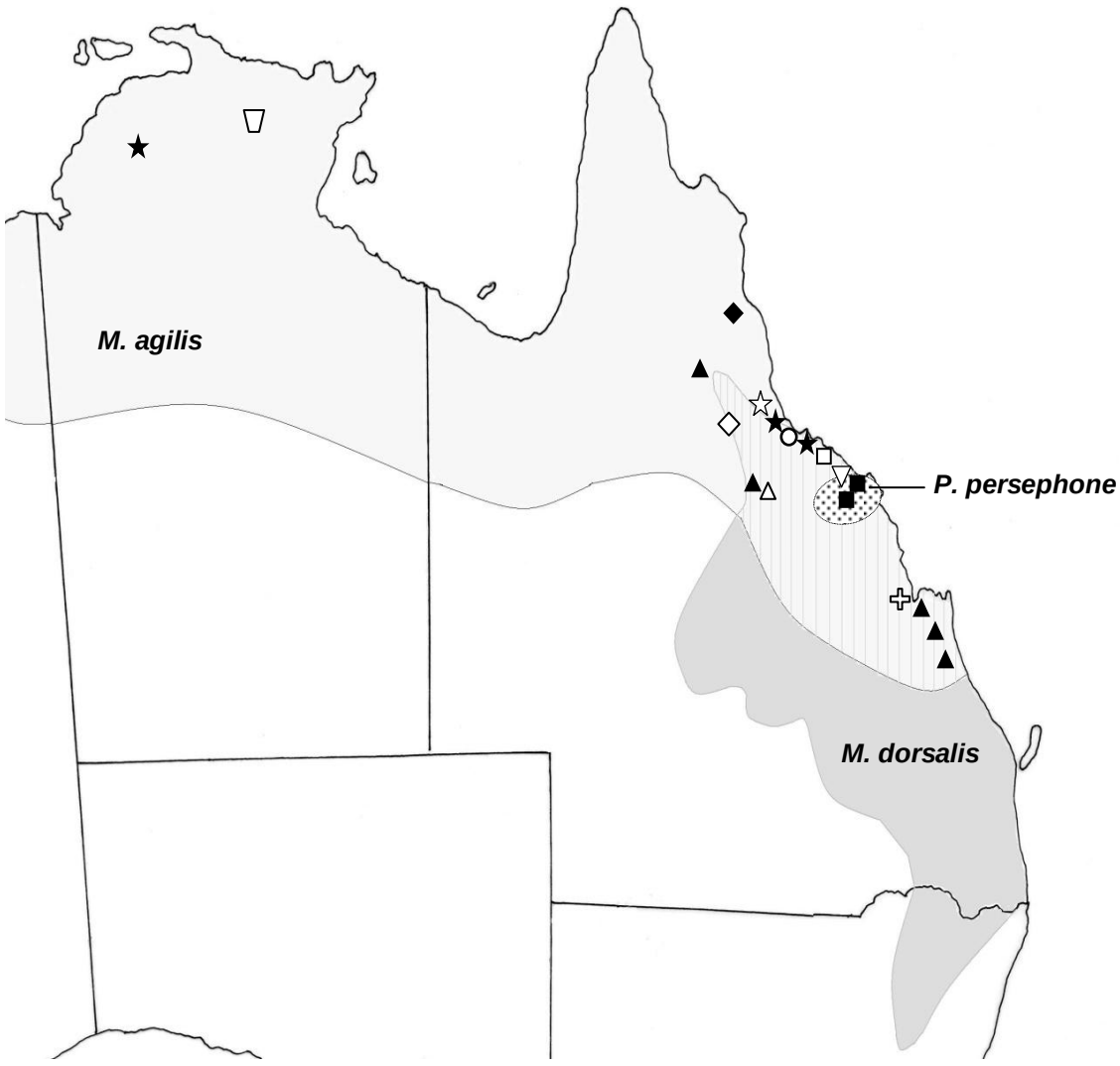
In the present study, the morphological data clearly confirmed the validity of *G. wallabiae* and additional host and geographical records are provided.

G. wallabiae occurs commonly in *M. dorsalis* (prevalences of 45-59% being reported as *G. macropodis*; Beveridge at al., 1984, 1998), but is also present in *Petrogale mareeba* and *P. assimilis* based on the re-examination of specimens formerly identified as *G. macropodis*. Its geographical range appears to be restricted to north-eastern Queensland (Fig. 26).

In contrast, *G. macropodis* generally infects *M. agilis* and *P. persephone* in both the Northern Territory and northern areas of Queensland at prevalences of 24-30% (Beveridge at al., 1984; Beveridge at al., 1998) and 83% (Begg et al., 1995), respectively (Figure 26). Additionally, *G. macropodis* has been reported at lower prevalences (4-20%) from

Aepyprymnus rufescens, P. brachyotis, *P. inornata*, *M. parryi, M. giganteus* and *Lagochestes conspicillatus* (Beveridge at al., 1984, 1989; 1992, 1998), which are probably incidental hosts, due to switching in areas of host sympatry.

Figure 26. Distribution of *Globocephaloides wallabiae* Johnston & Mawson, 1939 and *G. macropodis* Yorke & Maplestone, 1926 in northern and eastern Australia. Records of *G. wallabiae* are indicated by solid triangles in *Macropus dorsalis* (*n*=8, indicates that in total 8 hosts were examined), solid diamond in *Petrogale mareeba* (*n*=1) and open diamond in *P. assimilis* (*n*=1). Records of *G. macropodis* from *M. agilis* (solid stars, *n*=9) and from *P. persephone* (solid squares, *n*=9). Other incidental findings of *G. macropodis* are indicated by open symbols: *P. brachyotis* (open trapezium, *n*=1), *P. inornata* (open cross, *n*=1), *Aepyprymnus rufescens* (open square, *n*=1), *Largochestes conspicillatus* (open circle, *n*=1), *M. dorsalis* (open triangle, *n*=1), *M. parryi* (open inverted triangle, *n*=1) and *M. giganteus* (open star, *n*=1). Geographic ranges of *M. dorsalis* (dark grey), *M. agilis* (light grey) and *P. persephone* (black dots) are shown. Sympatric ranges of *M. dorsalis* and *M. agilis* are shown with grey stripes.



Beveridge et al. (1989) also reported *G. macropodis* from *P. godmani* and *P. sharmani*, but only one or two female nematodes were present in collections and therefore it is not possible to determine which species of *Globocephaloides* was involved in those infections.
Moreover, in the present study, a mixed infection by *G. wallabiae*, *G. macropodis* and *G. affinis* was found in a single *M. dorsalis* from the Charters Towers area of Queensland. This finding is in accordance with Beveridge (1984), who also reported mixed infections of *G. wallabiae* (as *G. macropodis*) and *G. affinis* in the same host and geographical region.
In conclusion, the primary host of *G. wallabiae* is *M. dorsalis* in north-eastern Queensland and the re-examination of all available specimens of *G. macropodis* indicate that it occurs mainly in *M. agilis* in the Northern Territory, northern Queensland and Papua New Guinea as well as in *P. persephone* in north-eastern Queensland. Therefore, the geographical distribution of *G. wallabiae* and *G. macropodis* overlaps (Fig. 26).

3.2.4 *Globocephaloides thetidis*: a synonym of *G. wallabiae*

***Globocephaloides thetidis* Johnston & Mawson, 1939b**

✶ Material examined from:

- *Thylogale thetis* (New South Wales): 1 ♂ , New England (AHC 41218).

G. thetidis was initially described by Johnston & Mawson (1939b) based on a specimen (AHC 41218) from *Thylogale thetis* from New England in New South Wales. Nevertheless, Beveridge (1979) placed this species as a synonym of *G. macropodis*, because descriptions were of a limited number of specimens and using unreliable characters. Beveridge et al. (1984) also argued that there had been a misidentification of the host.
In the present study, the specimen (AHC 41218) was re-examined and it was concluded that the host (*T. thetis*) was misidentified with *M. dorsalis*, because the species present in SAM was in fact *G. wallabiae*. Thus, *G. thetidis* is not a valid species but a synonym of *G. wallabiae*.
Although both *G. wallabiae* and *G. thetidis* were described by Johnson and Mawson in 1939 (Johnston & Mawson, 1939a,b), *G. macropodis* genotype 1 (from *M. dorsalis*) was named *G. wallabiae* since the description of *G. wallabiae* was published in July while that of *G. thetidis* was published in October. The name *G. wallabiae* therefore has priority.

3.3 Phylogenetic analysis: relationships between the genus *Globocephaloides* and selected trichostrongyloid genera

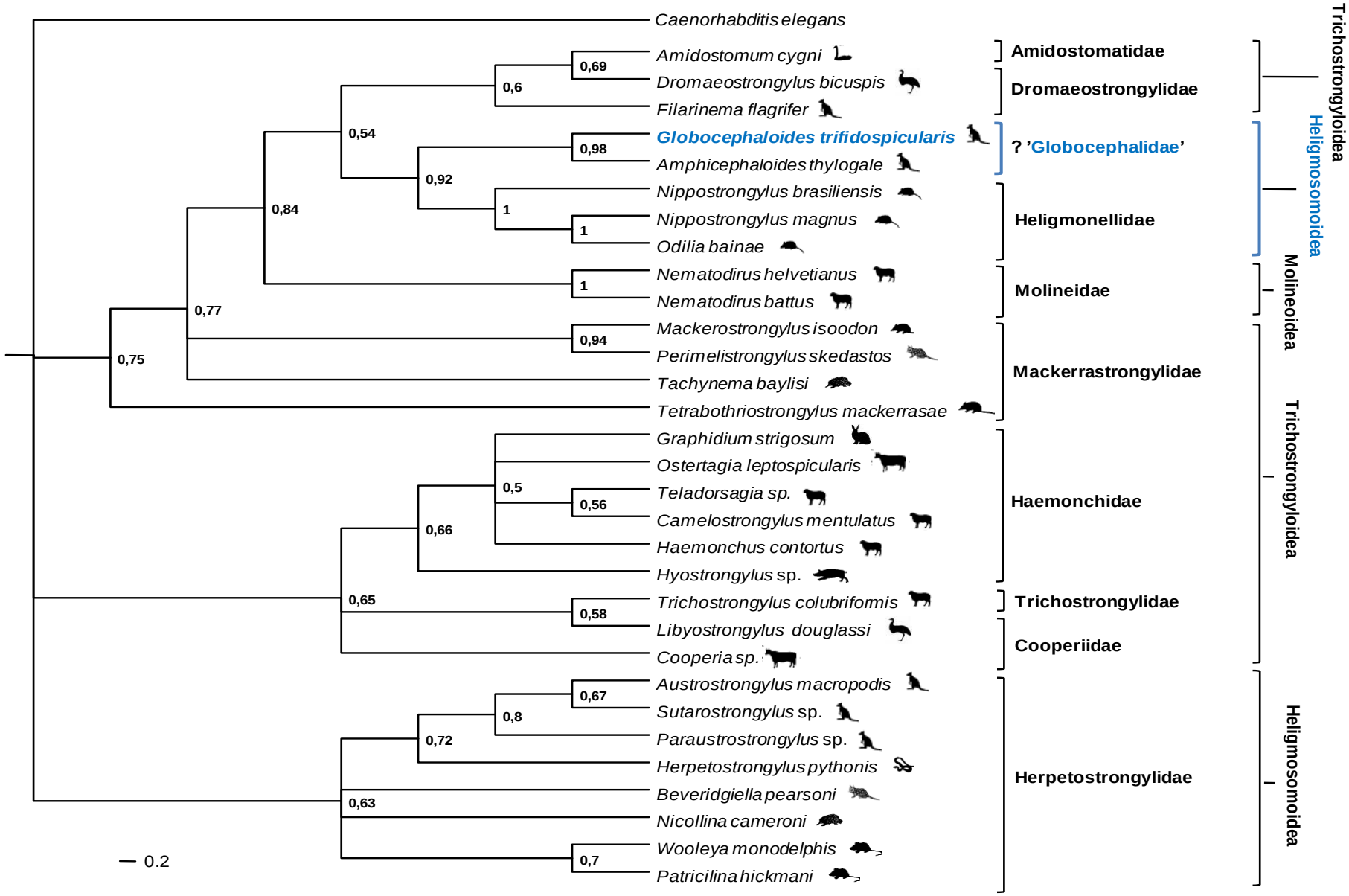
The partial sequences of the D1 and D2 domains of the 28S rRNA gene were used with Bayesian inference to determine the relationships between the genus *Globocephaloides* and 31 selected trichostrongyloid genera.

The phylogram (Fig. 27) shows that the genus *Globocephaloides* forms a monophyletic clade with the genus *Amphicephaloides*, having strong nodal support (pp = 0.98). This clade is the so-called subfamily Globocephaloidinae.

Moreover, the phylogram reveals that the Globocephaloidinae is more closely related to the Heligmonellidae (i.e., *Nippostrongylus brasiliensis*, *N. magnus* and *Odilia bainae*), which are gastrointestinal parasites of rats, than with the Herpetostrongylidae as previously suggested by Durette-Desset et al. (1981), based on morphological characters.

In the present study, the phylogenetic analysis using molecular data demonstrated that the genus *Globocephaloides* is correctly inserted in the superfamily Heligmosomoidea, but not in the family Herpetostrongylidae (Audebert et al., 2005). Therefore and to be consistent with current classifications, it would be necessary to elevate the subfamily Globocephaloidinae to family status, namely ‘Globocephalidae’.

Figure 27. Phylogram (Bayesian inference) showing the genetic differences between the genus *Globocephaloides* and selected trichostrongyloid genera, inferred from partial 28S rRNA gene sequence. *Caenorhabditis elegans* represents the outgroup. The posterior probability (pp) for each clade is given at each respective node. The respective host, family and superfamily are shown for each nematode species.


Caenorhabditis elegans
Amidostomum cygni
Dromaeostrongylus bicuspis
Filarinema flagrifer
Globocephaloides trifidospicularis
Amphicephaloides thylogale
Nippostrongylus brasiliensis
Nippostrongylus magnus
Odilia bainae
Nematodirus helvetianus
Nematodirus battus
Mackerostrongylus isoodon
Perimelistrongylus skedastos
Tachynema baylisi
Tetrabothriostrongylus mackerrasae
Graphidium strigosum
Ostertagia leptospicularis
Teladorsagia sp.
Camelostrongylus mentulatus
Haemonchus contortus
Hyostrongylus sp.
Trichostrongylus colubriformis
Libyostrongylus douglassi
Cooperia sp.
Austrostrongylus macropodis
Sutarostrongylus sp.
Paraustrostrongylus sp.
Herpetostrongylus pythonis
Beveridgiella pearsoni
Nicollina cameroni
Wooleya monodelphis
Patricilina hickmani
0,69
0,6
0,54
0,98
0,92
0,84
1
1
1
0,77
0,94
0,75
0,5
0,56
0,66
0,65
0,58
0,67
0,8
0,72
0,63
0,7
— 0.2
Amidostomatidae
Dromaeostrongylidae
? 'Globocephalidae'
Heligmonellidae
Molineidae
Mackerrastrongylidae
Haemonchidae
Trichostrongylidae
Cooperiidae
Herpetostrongylidae
Trichostrongyloidea
Heligmosomoidea
Molineoidea
Trichostrongyloidea
Heligmosomoidea

Chapter 4
Conclusions

In the present study, the genetic composition of the genus *Globocephaloides* is accessed for comprehensively the first time.

Globocephaloides species were characterized by the sequences of the ITS-1 and ITS-2 of nuclear rDNA using a mutation scanning-based approach coupled with sequencing. The results did not reveal the existence of cryptic species within *G. affinis* and/or *G. trifidospicularis* populations from different macropodid hosts and localities in Australia. However, this can not be a definitive conclusion since other studies employing multiple genetic loci (e.g., mitochondrial or microsatellite markers) with higher levels of intraspecific variation than the ITS region and/or sequencing of the whole genome, should be used to retest the presence of hidden species and co-speciation events in *G. affinis* and mainly in *G. trifidospicularis*.

By contrast, two distinct genotypes were detected for *G. macropodis* derived from *M. dorsalis* (*G. macropodis* genotype 1) and *M. agilis* (*G. macropodis* genotype 2), with further morphological examinations confirming the existence of two different phenotypes.

The molecular and morphological data from the present study culminated with the resurrection of *G. wallabiae* (= *G. macropodis* genotype 1) from the type-host *M. dorsalis*, which is as a valid sibling species of *G. macropodis* (= *G. macropodis* genotype 2) from its type-host *M. agilis*.

This discovery also underpinned a better understanding of host affiliation and geographical distribution of *Globocephaloides* spp.:

- *G. wallabiae* occurs mainly in *M. dorsalis* in the north-eastern Queensland;
- *G. macropodis* is present in *M. agilis* and *P. persephone* in the Northern Territory, north of Queensland and Papa New Guinea;
- *G. affinis* is relatively restricted to *M. dorsalis* in the northeast of Queensland;
- *G. trifidospicularis* infects a range of *Macropus* species in all southern Australia.

Moreover, the evolutionary relationships of the genus *Globocephaloides* were unmasked after 83 years of controversy by using the divergent domains of the 28S rRNA genes as genetic markers. The phylogenetic analyses excluded *Globocephaloides* species from the Herpetostrongylidae, with the suggestion of a new family, namely ‘Globocephalidae’, within the Heligmosomoidea.

Although the focus of the present study was on exploring the genetic composition of the genus *Globocephaloides* and to depict its evolutionary relationships, the mutation scanning-coupled analysis of specific nuclear markers, combined with morphological examination, continues to be a powerful approach for systematic studies and the detection and characterization of cryptic species in veterinary parasitology. This model provides unique prospects for the continued study of other wildlife parasitic nematodes and to gain further insights into their epidemiology, diagnosis, treatment and control methods.

Chapter 5

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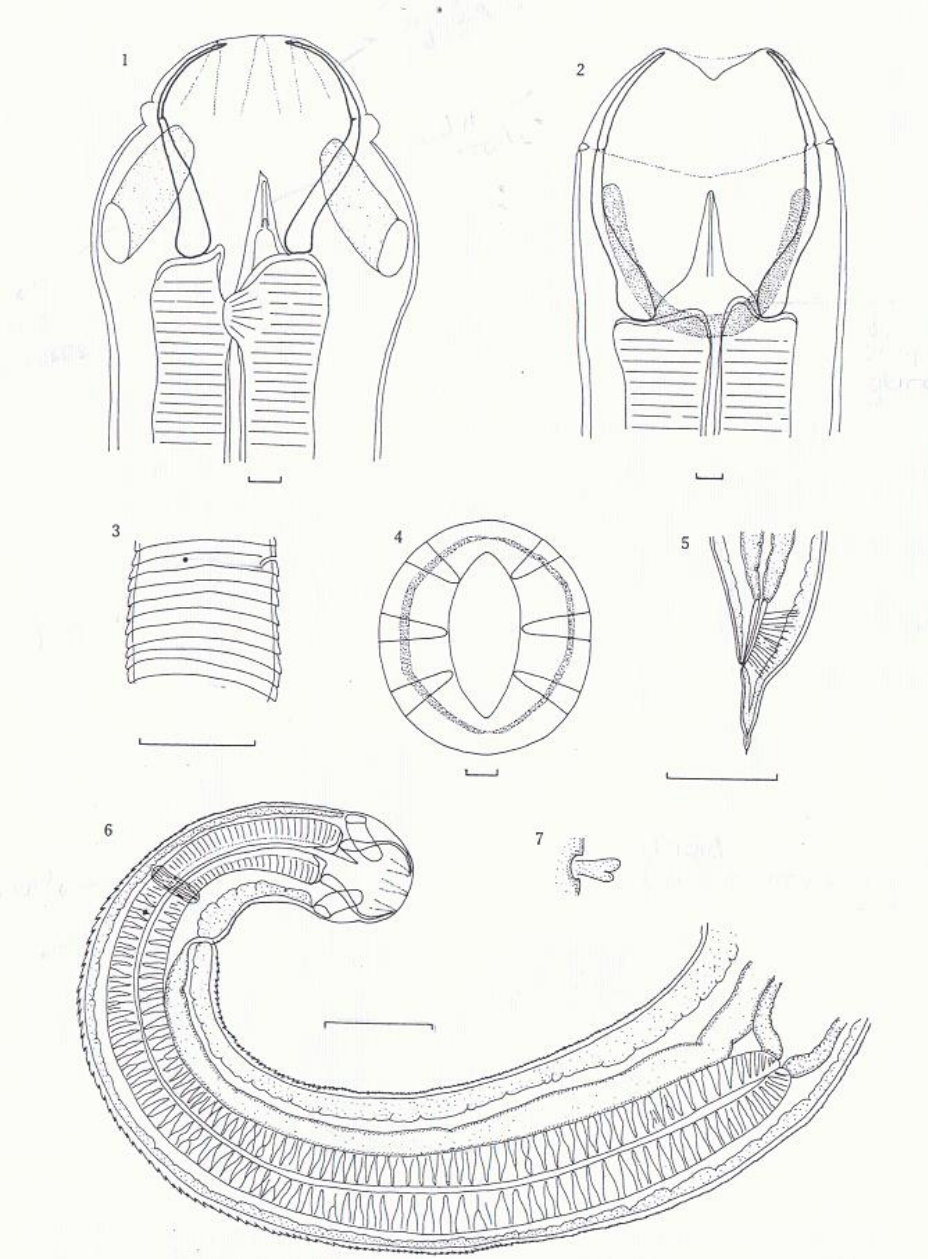
Appendix I

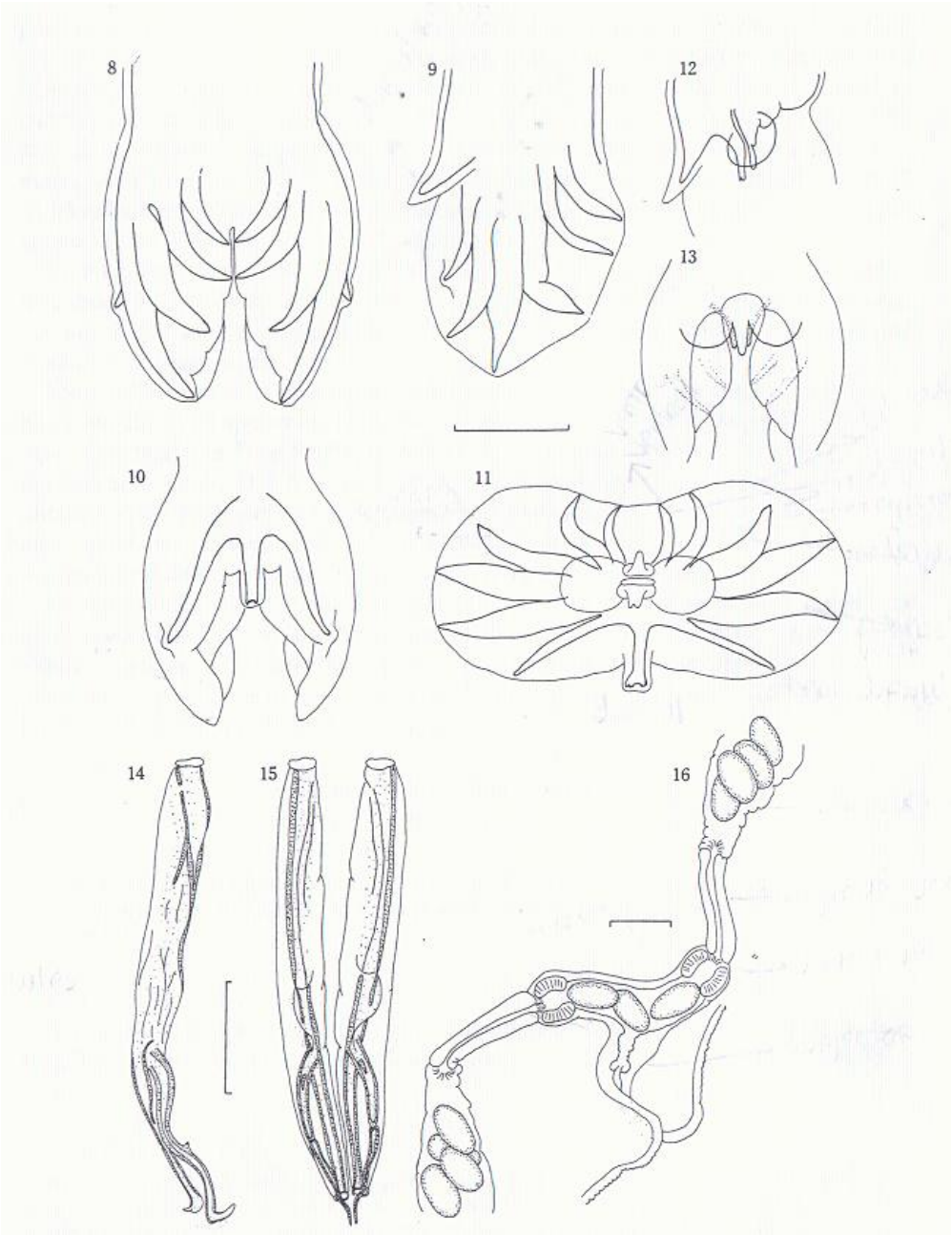
1. Globocephaloides *macropodis* Yorke & Maplestone, 1926. (Taken from Beveridge, 1979)



Figs 1-7. *Globocephaloides macropodis* Yorke & Maplestone, 1926: *1*, cephalic end male, lateral view; *2*, cephalic end male, dorsal view; *3*, transverse striations on body at level of deirid and excretory pore; *4*, cephalic end, *en face* view; *5*, posterior end, female, lateral view; *6*, anterior end male, lateral view; *7*, deirid, not to scale.

Scale lines: Figs 1, 2, 4, 0·01 mm; Figs 3, 5, 6, 0·1 mm.

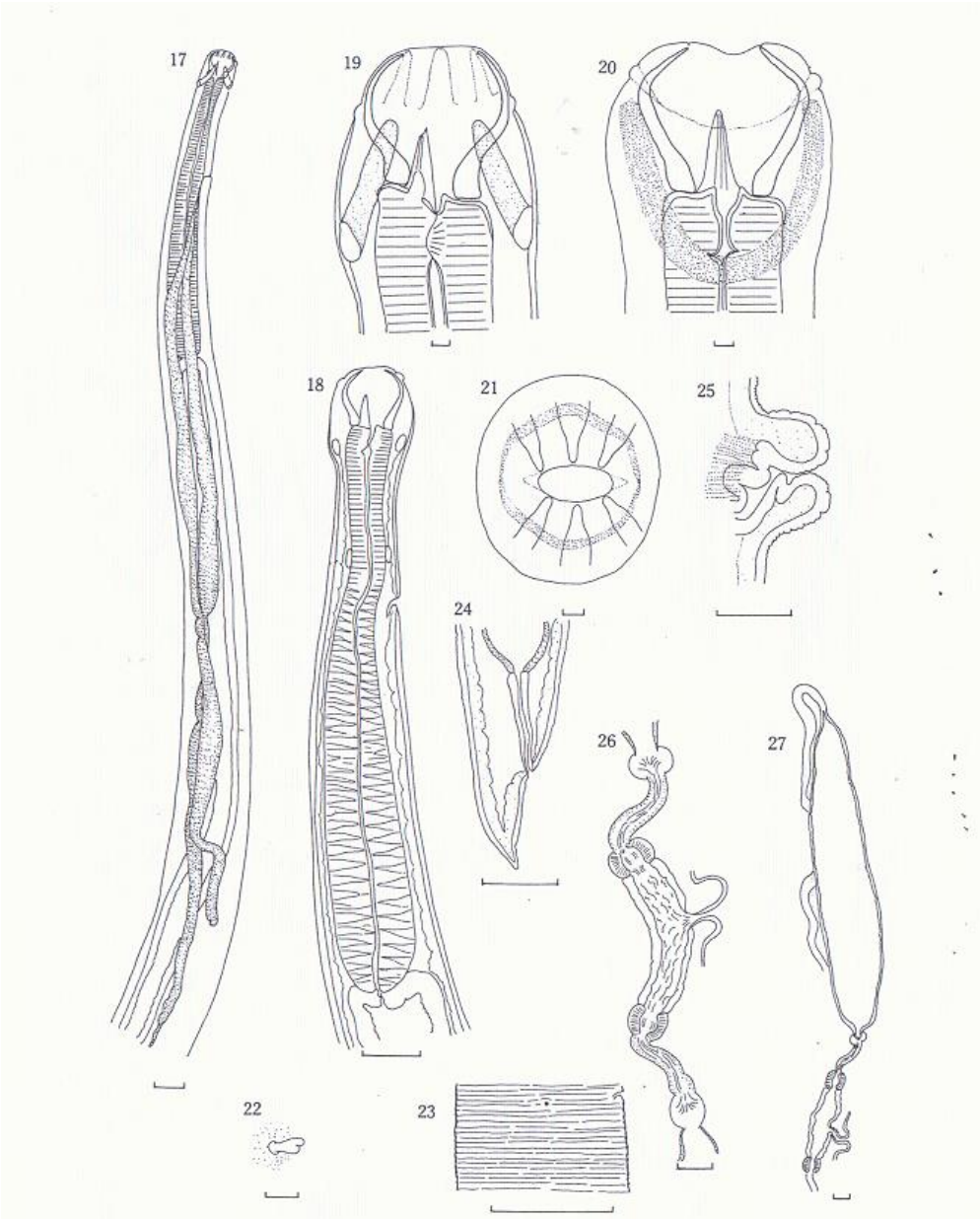
1. (Continuation)



Figs 8–16. *Globocephaloides macropodis* Yorke & Maplestone, 1926: *8*, bursa, ventral view; *9*, bursa, lateral view; *10*, bursa, dorsal view; *11*, bursa, apical view of flattened bursa; *12*, genital cone, lateral view; *13*, genital cone, ventral view; *14*, spicule, lateral view; *15*, spicules, ventral view; *16*, portion of female genital system.

Scale lines: Figs 8–13 to same scale, 0·1 mm; Figs 14–16, 0·1 mm.

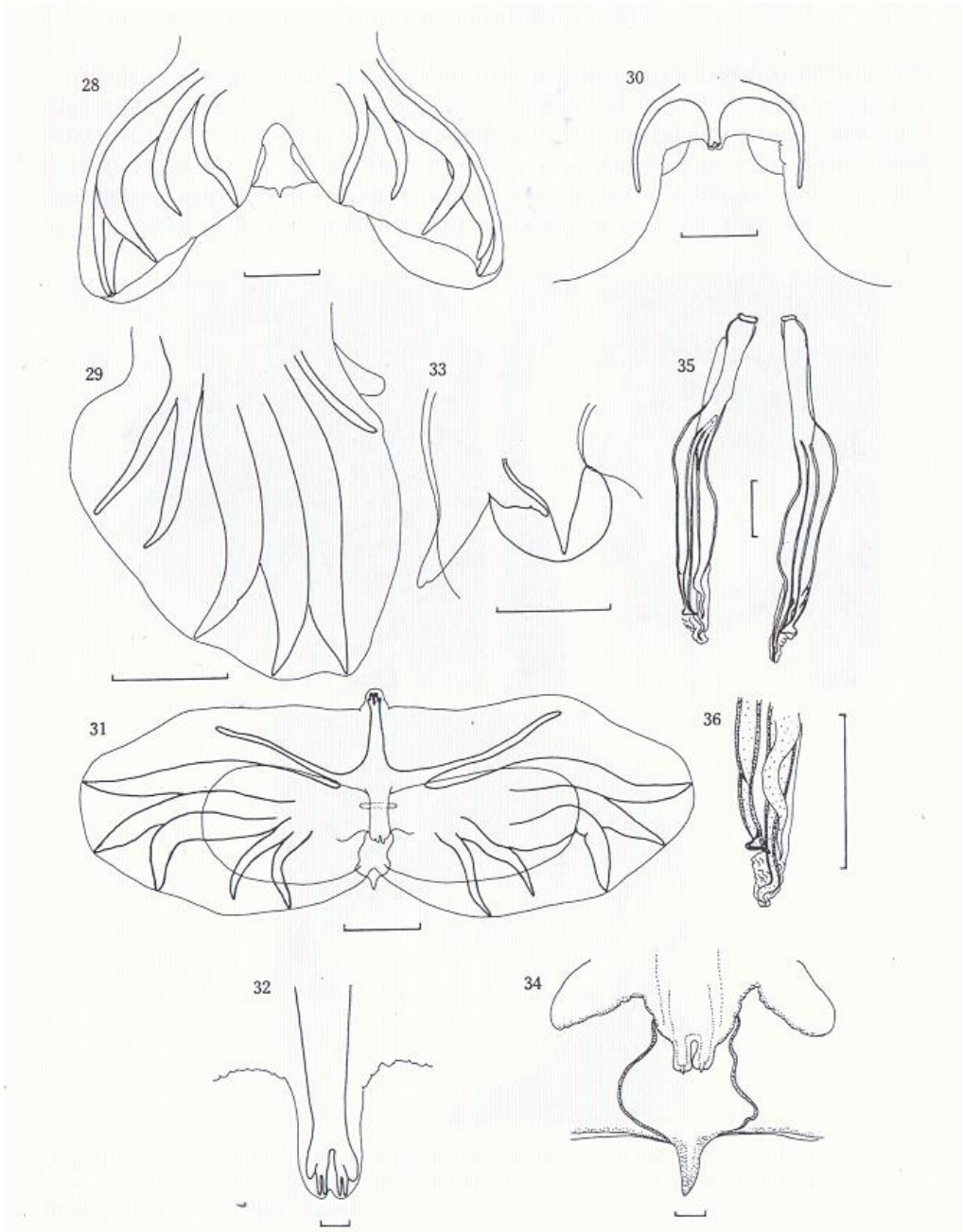
2. *Globocephaloides trifidospicularis* Kung, 1948. (Taken from Beveridge, 1979)



Figs 17–27. *Globocephaloides trifidospicularis* Kung, 1948: *17*, anterior end showing excretory glands; *18*, anterior end; *19*, cephalic end, lateral view; *20*, cephalic end, ventral view; *21*, cephalic end, *en face* view; *22*, deirid; *23*, transverse striations on body at level of deirid and excretory pore; *24*, posterior end, female, lateral view; *25*, vulva of female; *26*, vagina and ovejectors; *27*, portion of female genital system.

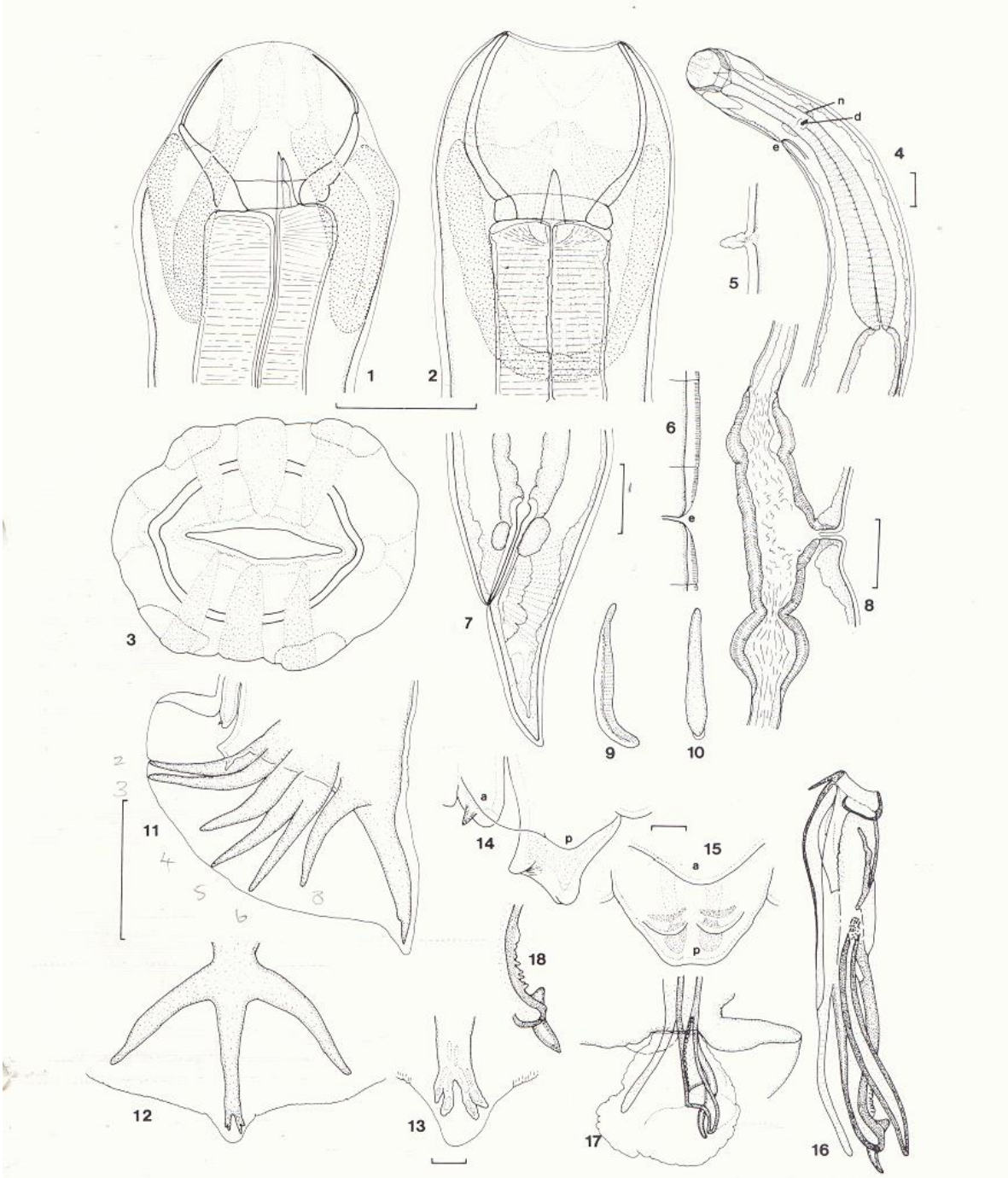
Scale lines: Figs 17, 18, 23–27, 0·1 mm; Figs 19–22, 0·01 mm.

2. (Continuation)



Figs 28–36. *Globocephaloides trifidospicularis* Kung, 1948: *28*, bursa, ventral view; *29*, bursa, lateral view; *30*, dorsal lobe and rays of bursa, dorsal view; *31*, apical view of flattened bursa; *32*, dorsal ray; *33*, genital cone, lateral view; *34*, genital cone, apical view; *35*, spicules, lateral view; *36*, spicule tips.
Scale lines: Figs 28–31, 33, 35, 36, 0·1 mm, Figs 32, 34, 0·01 mm.

3. *Globocephaloides affinis* Johnston & Mawson, 1939. (Taken from Beveridge et al., 1984)



Figs 1–18. *Globocephaloides affinis* Johnston & Mawson.
Fig. 1, Cephalic end, lateral view. Fig. 2, Cephalic end, ventral view. Fig. 3, Cephalic end, *en face* view. Fig. 4, Anterior end, lateral view. Fig. 5, Deirid, ventral view. Fig. 6, Transverse body striations in region of excretory pore. Fig. 7, Female tail, lateral view. Fig. 8, Vulva and ovejector, lateral view. Fig. 9, Gubernaculum, lateral view. Fig. 10, Gubernaculum, dorsal view. Fig. 11, Bursa, lateral view. Fig. 12, Dorsal lobe of bursa, dorsal view. Fig. 13, Termination of dorsal ray, dorsal view. Fig. 14, Genital cone, lateral view. Fig. 15, Genital cone, ventral view. Fig. 16, Spicule, lateral view. Fig. 17, Distal tip of spicule with surrounding cuticular flange, lateral view. Fig. 18, Main branch of spicule showing denticulate margin, lateral view. Scale lines: Figs 1–3, 5, 6, 9–12, 16–18 to same scale, 0.1 mm; Figs 4, 7, 8 to same scale, 0.1 mm; Figs 13–15 to same scale 0.01 mm.
Legend: a, anterior lip of genital cone; d, deirid; e, excretory pore; n, nerve ring; p, posterior lip of genital cone.

Appendix II

1. *Globocephaloides* specimens picked up by the author from the South Australian Museum. All specimens were analyzed under light microscopy and when possible identified to species.

SOUTH AUSTRALIAN MUSEUM,
NORTH TERRACE, ADELAIDE, SOUTH AUSTRALIA 5000
TELEPHONE: (08) 8207 7422. FACSIMILE (08) 8207 7222. CITES NO. AU035

LOAN OF NATURAL SCIENCE SPECIMENS

TO: **Prof. Ian Beveridge**

University of Melbourne,
Veterinary Clinical Centre,
250 Princes Highway,
WERRIBEE VICTORIA 3030

SECTION: PARASITOLOGY
LOAN NO.: **124**
DATE OF ISSUE: 02/iii/2009
EXPIRY DATE: 02/iii/2010
CITES Code No:
(Borrowing Registered Overseas Institution)
Loan Extended
Loan Returned:

The following specimens are consigned on loan for the purpose of study for a period of TWELVE (12) months from the date of issue.

Packed by: Leslie Chisholm Date sent: Picked up by hand on 03/iii/2009 by Inez for Ian Beveridge

Signed .....(Collection Manager)

Signed ..... (Head of Collections)

Loans of TYPE or UNIQUE material must be REGISTERED/INSURED and returned by AIR. Material returned from overseas must bear the words "RETURNING SCIENTIFIC MATERIAL FROM AUSTRALIA" clearly displayed on the outside of the package and must carry a correct CITES label.

Description Value

regno	current host name	pgrp	current parasite name	stat	no of slides
4898	Bettongia gaimardi •	N	Globocephaloides sp. Peterostrongylus	VOUCHER	W
5468	Wallabia bicolor • 3♀	N	Globocephaloides sp.	VOUCHER	W
6789	Macropus rufogriseus • 1♂1♀	N	Globocephaloides sp. Trapid.	VOUCHER	W
7131	Macropus rufogriseus	N	Globocephaloides sp.	VOUCHER	W
GM 7165	Macropus agilis • 1♂ 5♀	N	Globocephaloides macropodis	VOUCHER	W
7507	Macropus agilis	N	Globocephaloides macropodis	VOUCHER	W
7515	Macropus agilis • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 7517	Macropus agilis • 2♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
7524	Macropus agilis	N	Globocephaloides sp.	VOUCHER	W
GM 7565	Macropus agilis • 1♂ 3♀	N	Globocephaloides macropodis	VOUCHER	W
7586	Macropus agilis 1♀	N	Globocephaloides macropodis	VOUCHER	W
7854	Macropus giganteus • 1♀	N	Globocephaloides sp.	VOUCHER	W
GM 8841	Aepyprymnus rufescens • 2♂2♀	N	Globocephaloides macropodis	VOUCHER	W
9208	Macropus giganteus • 1♂	N	Globocephaloides sp. trapi	VOUCHER	W
9864	Macropus parma • 1♀	N	Globocephaloides sp.	VOUCHER	W
GM 9951	Macropus agilis 1♂ 3♀	N	Globocephaloides macropodis	VOUCHER	W
10180	Macropus agilis 2,5♀	N	Globocephaloides macropodis	VOUCHER	W
10533	Thylogale thetis •	N	Globocephaloides sp. ampica	VOUCHER	W
10767	Macropus robustus • 1♀	N	Globocephaloides sp.	VOUCHER	W
10772	Macropus agilis 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 10835	Petrogale brachyotis • 1♂	N	Globocephaloides macropodis	VOUCHER	W
11066	Macropus giganteus • 1♀	N	Globocephaloides macropodis	VOUCHER	W

empty

1. (Continuation)

regno	current host name	pgrp	current parasite name	stat	no of slides
11069	Macropus eugenii • 1♀	N	Globocephaloides sp.	VOUCHER	W
11104	Macropus dorsalis • 1♀	N	Globocephaloides macropodis	VOUCHER	W
11105	Petrogale inornata •	N	Globocephaloides macropodis	VOUCHER	W
GW 11141	Macropus dorsalis • 2♂	N	Globocephaloides macropodis	VOUCHER	W
11168	Macropus dorsalis • 1♀	N	Globocephaloides macropodis	VOUCHER	W
11702	Petrogale inornata • 1♀	N	Globocephaloides macropodis	VOUCHER	W
11703	Petrogale inornata • 1♀	N	Globocephaloides macropodis	VOUCHER	W
11704	Petrogale inornata • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 11840	Petrogale penicillata • 3♂ 5♀	N	Globocephaloides macropodis	VOUCHER	W
11931	Macropus parryi • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GW 12023	Macropus dorsalis •	N	Globocephaloides macropodis	VOUCHER	W
GW 12119	Macropus dorsalis • 1♂	N	Globocephaloides macropodis	VOUCHER	W
GW 12253	Macropus dorsalis • 1♂ 4♀	N	Globocephaloides macropodis	VOUCHER	W
12298	Macropus dorsalis • 4♀	N	Globocephaloides macropodis	VOUCHER	W
12334	Wallabia bicolor • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 12364	Macropus parryi • 1♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
12743	Macropus dorsalis • 1♀	N	Globocephaloides macropodis	VOUCHER	W
2GW GW,GM,GM 12744	Macropus dorsalis • 3♂ 13♀	N	Globocephaloides macropodis	VOUCHER	W
GW 12745	Macropus dorsalis • 1♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 12837	Lagorchestes conspicillatus •	N3♂	Globocephaloides macropodis	VOUCHER	W
empty 13101	Petrogale godmani	N5♀	Globocephaloides macropodis	VOUCHER	W
13169	Petrogale assimilis • 2♀	N	Globocephaloides macropodis	VOUCHER	W
13206	Petrogale sharmani • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 13422	Macropus giganteus • 3♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 15040	Macropus agilis G.M 1♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
19876	Macropus parryi • 2♀	N	Globocephaloides macropodis	VOUCHER	W
19883	Macropus parryi • 1♀	N	Globocephaloides macropodis	VOUCHER	W
empty 19917	Macropus dorsalis	N	Globocephaloides macropodis	VOUCHER	W
GM 23715	Petrogale persephone • 3♂ 3♀	N	Globocephaloides macropodis	VOUCHER	W
GM 24206	Petrogale persephone • 5♂ 5♀	N	Globocephaloides sp.	VOUCHER	W
GM 24207	Petrogale persephone • 1♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 24209	Petrogale persephone • 2♀ 1♂	N	Globocephaloides macropodis	VOUCHER	W
GM 24211	Petrogale persephone • 1♂	N	Globocephaloides macropodis	VOUCHER	W
24885	Macropus dorsalis • 1♀	N	Globocephaloides macropodis	VOUCHER	W
26172	Macropus dorsalis • 1♂ 1♀	N	Globocephaloides sp.	VOUCHER	W
26586	Macropus dorsalis • 3♀	N	Globocephaloides macropodis	VOUCHER	W
30423	Macropus dorsalis • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GM 30755	Petrogale persephone • 1♂	N	Globocephaloides macropodis	VOUCHER	W
GM 30758	Petrogale persephone • 4♂	N	Globocephaloides macropodis	VOUCHER	W
GM 30759	Petrogale persephone • 5♀ 4♂	N	Globocephaloides macropodis	VOUCHER	W
GM 30762	Petrogale persephone • 1♂ 1♀	N	Globocephaloides macropodis	VOUCHER	W
GW 32457	Macropus dorsalis • 1♂	N	Globocephaloides macropodis	VOUCHER	W
GW 33472	Petrogale mareeba • 1♂	N	Globocephaloides macropodis	VOUCHER	W
GM 33878	Macropus agilis • 2♂	N	Globocephaloides macropodis	VOUCHER	W
41217	Macropus dorsalis	N	Globocephaloides macropodis	TYPES	W
empty GW 41218	Thylogale thetis • 1♂	N	Globocephaloides macropodis	VOUCHER	W
41285	Wallabia bicolor • 1♀	N	Globocephaloides macropodis	VOUCHER	W
GW 41481	Macropus dorsalis 1♂	N	Globocephaloides macropodis	VOUCHER	W
41482	Macropus dorsalis	N	Globocephaloides macropodis	VOUCHER	W
GM 41483	Macropus agilis 2♂ 3♀	N	Globocephaloides macropodis	VOUCHER	W
41484	Macropus agilis	N	Globocephaloides macropodis	VOUCHER	W
41485	Macropus agilis	N	Globocephaloides macropodis	VOUCHER	W
41486	Macropus agilis	N	Globocephaloides macropodis	VOUCHER	W

1. (Continuation)

regno	current host name	pgrp	current parasite name	stat	no of slides
41487	Macropus agilis	N	Globocephaloides macropodis	VOUCHER	W

Conditions applying to the loan: Circle the appropriate conditions. The details of the conditions are listed overleaf.

Insurance:		**1a**	**1b**	**1c**	**1d**	Policy Number:				
②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	**11**	Lux levels
must not exceed:			**12**	**13**	**14**	**15**	**16**	**17**	**18**	
Destructive analysis permitted:				**Y**	**N**					

I have received the above material ..... Date .....

PLEASE SIGN AND RETURN PINK COPY ON RECEIPT OF MATERIAL