Genetic variation in the mitochondrial cytochrome *c* oxidase subunit 1 within *Progamotaenia festiva* (Cestoda: Anoplocephalidae) from macropodid marsupials

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SUMMARY

Genetic variation was examined in the anoplocephalid cestode *Progamotaenia festiva*, from Australian marsupials, in order to test the hypothesis that *P. festiva*, is a complex of sibling species and to assess the extent of host switching reported previously based on multilocus enzyme electrophoresis (MEE). Polymerase chain reaction (PCR)-based single-strand conformational polymorphism (SSCP) was used for the analysis of sequence variation in the cytochrome *c* oxidase subunit 1 (*cox1*) gene among 179 specimens of *P. festiva* (identified based on morphology and predilection site in the host) from 13 different host species, followed by selective DNA sequencing. Fifty-three distinct sequence types (haplotypes) representing all specimens were defined. Phylogenetic analyses of these sequence data (utilizing maximum parsimony and neighbour-joining methods) revealed 12 distinct clades. Other heterologous species, *P. ewersi* and *P. macropodis*, were used as outgroups and the remaining bile-duct inhabiting species, *P. diaphana* and *P. effigia*, were included in the analysis for comparative purposes. The latter 2 species were nested within the clades representing *P. festiva*. Most clades of *P. festiva* identified were restricted to a single host species; one clade primarily in *Macropus robustus* was also found in the related host species *M. antilopinus* in an area of host sympatry; another clade occurring primarily in *M. robustus* occurred also in additional kangaroo species, *M. rufus* and *M. dorsalis*. High levels of genetic divergence, the existence of distinct clades and their occurrence in sympatry provide support for the hypothesis that *P. festiva* represents a complex of numerous species, most of which, but not all, are host specific. Three distinct clades of cestodes were found within a single host, *M. robustus*, but there was no evidence of within-host speciation.

Key words: *Progamotaenia*, cestodes, marsupials, genetic variation, mitochondrial DNA, cytochrome c oxidase subunit 1 gene.

INTRODUCTION

Molecular genetic techniques such as multilocus enzyme electrophoresis (MEE) (reviewed by Andrews and Chilton, 1999) and DNA sequencing provide useful tools for exploring the existence of cryptic or sibling species in tapeworms (cestodes). The existence of such species appears to be relatively common, with examples elucidated to date utilizing MEE including the fish cestode genera *Bothriocephalus* (see Renaud *et al*. 1983, 1986; Renaud and Gabrion, 1984) and *Proteocephalus* (see Hanselová *et al*. 1995; Šnabel *et al*. 1996) as well as the ruminant cestodes *Moniezia expansa* and *M. benedeni* (see Ba *et al*. 1993; Chilton *et al*. 2007). In the economically important taeniid cestode *Echinococcus granulosus* (summarized by Thompson and McManus, 2002) and the anoplocephalid cestodes of holarctic rodents belonging to the genera *Anoplocephaloides* and *Paranoplocephala*, sibling species have also been identified using DNA sequence data (summarized by Wickström *et al*. 2005).

The anoplocephalid cestodes occurring in the bile duct of Australian marsupials have been investigated using MEE (Baverstock *et al*. 1985). Three species of *Progamotaenia* occur in the bile ducts of kangaroos, wallabies and wombats (families Macropodidae and Vombatidae, respectively) (Beveridge, 1976, 1980), but they differ markedly in their host ranges. *Progamotaenia diaphana* is restricted to the southern hairy nosed wombat, *Lasiorhinus latifrons*, *P. effigia* to the western grey kangaroo, *Macropus fuliginosus*, whereas *P. festiva* occurs in 14 species of kangaroos and wallabies as well as one species of wombat (Spratt *et al*. 1991). The wide host range of *P. festiva*, together with considerable morphological variation within the species, led to the speculation that it was a complex of sibling species (Beveridge, 1976). An MEE study (Baverstock *et al*. 1985) supported this hypothesis, with evidence that up to 12 genetically

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Table 1. Origin of specimens used in the study of p*cox*1 of *Progamotaenia festiva, P. diaphana* and *P. effigia* from kangaroos, wallabies and wombats

Host species Name	Locality	Lat (°S), Long (°E)	No. of specimens	Voucher no.	Month/yr of collection	Sequence no.
Onychogalea unguifera bridle nail-tailed wallaby	Springfield Stn via Mt Surprise, Qld	17° 58′ 144° 26′	1	28 950	ix.94	35
Lagorchestes conspicillatus spectacled hare wallaby	80 km N of Moorinya NP, Qld	21° 20′ 145° 03′	1	28 961	xi.02	39
	Blackbraes National Park, Qld	19° 32′ 144° 12′	6	28 946	x.03	40
Lasiorhinus latifrons southern hairy-nosed wombat	Swan Reach, SA	34° 34′ 139° 36′	2	21 511	v.98	52,53
Wallabia bicolor swamp wallaby	Rockhampton, Qld	23° 22′ 150° 32′	1		ix.89	36
	Woodlark Bay, Qld	20° 16′ 148° 43′	2		x.94	37
	Dixon's Creek, Vic.	37° 39′ 45° 32′	1		iii.92	38
Macropus agilis agile wallaby	30 km SE of Townsville, Qld	19° 25′ 147° 00′	1		vii.94	30
	Bluewater, Townsville, Qld	19° 16′ 146° 49′	3		vi.95	30
	Mt Louisa, Townsville, Qld	19° 53′ 147° 18′	4		vi.96	30
	5 km SE of Katherine, NT	14° 28′ 132° 16′	1	28 951	vii.03	30
	Scott Ck, 65 km SW of Katherine, NT	14° 53′ 131° 51′	3	28 952	vii.03	30
	25 km NW of Katherine, NT	14° 28′ 132° 16′	2	28 953	vii.03	30
Macropus antilopinus antilopine wallaroo	19 km E of Mt Surprise, Qld	18° 05′ 144° 30′	1		xi.94	8
	Limestone Ck, 50 km SW of Katherine, NT	14° 50′ 131° 55′	1	28 958	vii.03	21
	Matheson, Ck, 87 km SW of Katherine, NT	15° 04′ 131° 44′	1	28 954	vii.03	22
Macropus dorsalis black-stripe wallaby	Arlington Stn via Augathella, Qld	25° 35′ 146° 39′	3		vi.93	27
	2 km W of Jericho, Qld	23° 36′ 146° 07′	3		vi.93	27
	15 km NW of Marlborough, Qld	22° 49′ 149° 53′	2	28 945	vii.93	28
	Rockhampton, Qld	23° 22′ 150° 32′	1		vii.90	27
	7 km W of Beta, Qld	23° 38′ 146° 21′	1		vi.93	27
	Warrawee Stn via Charters Towers, Qld	20° 18′ 146° 38′	2	19 753	x.89	33
Macropus fuliginosus western grey kangaroo	Kwinana, Perth, WA	32° 14′ 115° 46′	1		x.04	29
Macropus giganteus eastern grey kangaroo	Dingo, Qld	23° 39′ 149° 20′	4		ix.89	13
	Mungallalla, Qld	26° 27′ 147° 33′	1		vii.92	13
	12 km S of Theodore, Qld	24° 57′ 150° 05′	2	23 637	vii.92	16
	Kumbarilla, 66 km E of Moonie, Qld	27° 19′ 150° 53′	8	25 694	vi.94	15
	Mangalore, 46 km S of Charleville, Qld	26° 42′ 146° 06′	2		vii.92	12,14
	3 km N of Miles, Qld	26° 40′ 150° 11′	1	23 641	vii.92	13
	Barradeen Stn, 40 km N of Charleville, Qld	26° 04′ 146° 25′	2		vi.93	13
	50 km E of Inglewood, Qld	28° 15′ 151° 33′	4	23 226	vii.92	13
	Belingra Stn, 30 km E of Bollon, Qld	27° 58′ 147° 51′	1	25 692	vi.94	15
	Belalie Stn, 95 km N of Bourke, NSW	27° 02′ 142° 57′	1	25 693	vi.94	17
	Calooma Stn, 50 km N of Bourke, NSW	29° 39′ 145° 54′	1		vi.92	15
	9 km S of Enngonia, NSW	29° 19′ 145° 51′	1		ix.95	16
	Trafalgar Stn, 24 km SE of Bourke, NSW	30° 18′ 146° 05′	2		ix.95	17

	Warraweena Stn via Bourke, NSW	29° 56′ 146° 14′	3	28 944	vi.98	18
	20 km NW of Nyngan, NSW	31° 25′ 147° 03′	3	27 265	ix.95	15
	38 km N of Moree, NSW	29° 10′ 150° 01′	1	23 246	vii.92	15
	70 km N of Coonabarabran, NSW	30° 38′ 149° 32′	1	23 248	vii.92	13
	West Wyalong, NSW	33° 55′ 147° 12′	1		vi.92	18
	Avalon, Vic	38° 02′ 144° 28′	2	23 243	x.91	18
	Research, Vic	37° 45′ 145° 13′	4		v.93	18
	Pomonal, Vic	37° 12′ 142° 37′	3	33 897	viii.99	19,20
	10 km N of Bacchus Marsh, Vic	37° 37′ 144° 27′	2		v.98	18
	Dadswell's Bridge, Vic	36° 55′ 142° 30′	2	33 896	vi.00	18
Macropus irma black-glove wallaby	Collie, WA	33° 22′ 116° 09′	1	28 948	xii.01	1
	Mt Trio, WA	43° 21′ 118° 07′	2	28 947	x.04	2
Macropus parryi whiptail wallaby	Thangool, Qld	24° 29′ 150° 35′	2	19 893	vii.90	3,5,6
	Yeppoon, Qld	23° 08′ 150° 44′	1	28 957	vii,01	3
	Darling Plains Stn via Banana, Qld	24° 25′ 150° 08′	5	17 982	vii.90	4
	Keppel Sands, Qld	23° 20′ 150° 48′	1		vii.93	7
Macropus robustus wallaroo	Yalgoo, WA	28° 21′ 116° 41′	1	28 959	xii.01	24
	Northampton, WA	28° 21′ 114° 38′	2	28 956	xii.01	23,25
	Nive Downs Stn via Augathella, Qld	28° 30′ 146° 35′	1		vi.93	26
	76 km W of Cloncurry, Qld	20° 42′ 139° 45′	4	33 900	vii.01	31
	70 km W of Cloncurry, Qld	20° 45′ 139° 49′	5	33 901	vii.01	31
	28 km E of Barcaldine, Qld	23° 34′ 145° 34′	4		vi.93	32
	Stockyard Creek, Bluewater Springs, Qld	19° 26′ 145° 38′	1	28 949	xi.94	32
	30 km N of Charters Towers, Qld	19° 53′ 146° 08′	1	28 955	vii.01	9
	Hillgrove Stn via Charters Towers, Qld	19° 38′ 145° 47′	1	28 960	vii.01	10
	Warrawee Stn via Charters Towers, Qld	20° 18′ 146° 38′	2	19 755	x.89	11
Macropus rufus red kangaroo	Kiora Stn via Pentland. Qld	20° 28′ 145° 27′	1	33 898	vii.01	34
	10 km E of Barcaldine, Qld	23° 34′ 145° 23′	1	33 899	vi.93	42
	Yanna, 64 km S of Charleville, Qld	26° 56′ 146° 03′	7		vii.92	44,45,47,49
	Coongoola, 47 km N of Cunnamulla, Qld	27° 39′ 145° 49′	17		vi.93	41
	Avoca Stn via Bourke, NSW	29° 13′ 145° 50′	1		vi.94	48
	Calooma Stn, 50 km N of Bourke, NSW	29° 39′ 145° 54′	2	23 199	vi.92	51,50
	18 km SE of Bourke, NSW	30° 15′ 146° 03′	1		vi.98	44
	Trafalgar Stn, 25 km SE of Bourke, NSW	30° 18′ 146° 05′	6		ix.95	46
	Mullagalah Stn, 40 km SE of Bourke, NSW	30° 27′ 146° 11′	10	24 186	vi.98	44
	Byrock, NSW	30° 40′ 146° 24′	1	24 864	vi.94	41
	22 km N of Jerilderie, NSW	35° 15′ 145° 50′	1		ix.95	46
	Wallerberdina Stn via Pt Augusta, SA	31° 46′ 138° 07′	4	19 798	vi.89	43
Total			182			

Fig. 1. Localities at which hosts of specimens of *Progamotaenia diaphana*, *P. effigia* and *P. festiva* were collected. Key: +, *Lagorchestes conspicillatus*; inverted open triangle, *Lasiorhinus latifrons*; solid triangles, *Macropus agilis*; open squares, *M. antilopinus*; closed circles, *M. dorsalis*; 5 pointed star, *M. fuliginosus*; inverted solid triangles, *M. irma*; asterisks, *M. parryi*; open triangles, *M. robustus*; open circle, *Onychogalea unguifera*; closed squares, *Wallabia bicolor*.

distinct groups existed within the taxon. However, this study was limited by relatively small sample sizes, and apparent instances of host switching complicated the interpretation of the findings. In addition, not all hosts of *P. festiva* were included.

In the present study, the hypothesis that *P. festiva* is a species complex is tested using a much larger number of samples of cestodes from a broad range of host species. The study was also designed to assess the extent of host switching suggested in the earlier study (Baverstock *et al*. 1985). Since recent genetic or phylogenetic studies of other species of *Progamotaenia* (see Hu *et al*. 2005) and of other anoplocephalid genera, *Anoplocephaloides* and *Paranoplocephala*, from rodents (*e.g.* Wickström *et al*. 2005) have demonstrated the usefulness of sequence data sets of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1), this gene was chosen for the analyses herein.

MATERIALS AND METHODS

Collection of cestodes

Cestodes were collected opportunistically from fresh, road-killed kangaroos and wallabies, from road-killed animals which had been frozen before examination for parasites or from animals shot commercially. Cestodes from freshly killed animals or from defrosted carcasses were washed in distilled water or saline and frozen either at −20 °C or in liquid nitrogen. They were subsequently stored at −70 °C until used. Parts of most of the cestodes used for molecular analysis were also fixed in AFA (alcohol, formalin, acetic acid) (Pritchard and Kruse, 1982), stained with Celestine blue and mounted in Canada balsam. Voucher slides have been deposited in the South Australian Museum, Adelaide (SAM). Liver samples from each host examined were also frozen in liquid nitrogen. Although not used in the present study, the host tissues have been deposited in the collections of the Evolutionary Biology Unit of SAM. Collection localities, hosts and registration numbers are given in Table 1 and in Figs 1 and 2. The following abbreviations or contractions have been used for Australian States and territories: NSW: New South Wales; NT: Northern Territory; Qld: Queensland; SA: South Australia; Vic: Victoria; WA: Western Australia.

Isolation of genomic DNA and enzymatic amplification

Total genomic DNA was isolated from individual worms by sodium dodecyl-sulphate/proteinase K

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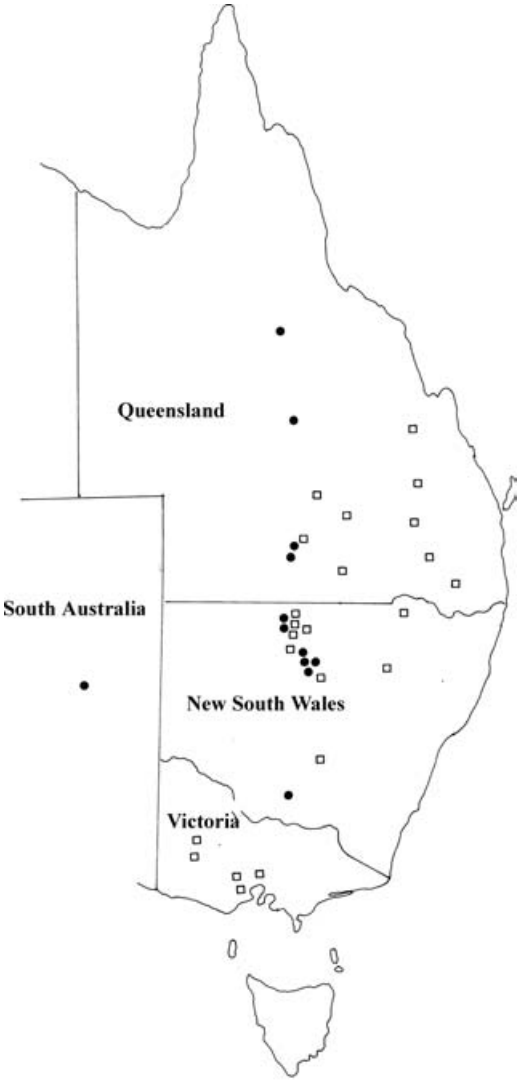

Fig. 2. Localities at which hosts of specimens of *Progamotaenia festiva* were collected from *Macropus giganteus* and *M. rufus*. Key: open squares, *M. giganteus*; closed circles, *M. rufus*.

digestion (37 °C for 12 h) (Gasser *et al.* 1993), purified over a spin column (Wizard™ DNA CleanUp, Promega) and eluted in 30 μl of H_20. From 5–20 ng of genomic DNA, a portion of the *cox*1 (~450 bp; p*cox1*) was amplified by PCR from 10–20 ng of genomic DNA using primers JB3 (forward: 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (reverse: 5′-TAAAGAAAGAACATAATGAAAATG-3′) (Bowles *et al.* 1992), known to be conserved for platyhelminths (Garey and Wolstenholme, 1989). The PCR was performed in 50 μl using 50 pmol of each primer, 250 μM of each dNTP, 4 mM $MgCl_2$ and 1 U *Taq* polymerase (Promega) under the following cycling conditions. After an initial denaturation at 94 °C for 5 min, reactions were subjected to 30 cycles of 94 °C for 30 sec (denaturation), 50 °C for 30 sec (annealing) and 72 °C for 30 sec (extension), followed by a final extension at 72 °C for 5 min in a 480 thermocycler (Perkin Elmer Cetus). Control samples without DNA were included in each PCR run.

Single-strand conformation polymorphism (SSCP) analysis

After thermocycling, 10 μl of individual amplicons (~100–200 ng of DNA) were mixed with an equal volume of loading buffer (10 mM NaOH, 95% formamide, 0·05% of both bromophenol blue and xylene cyanole) and shown to represent single bands on ethidium bromide-stained 2% (w/v) agarose gels. After denaturation at 94 °C for 15 min and snap cooling on a freeze block (−20 °C), individual samples (10 μl) were loaded into the wells of precast GMA™ gels (96 × 261 mm; S-2 × 25; product no. 3548; Elchrom Scientific AG) and subjected to electrophoresis for 14 h at 72 V and 7·2 °C (constant) in a horizontal SEA2000 apparatus (Elchrom Scientific) connected to a MultiTemp III (Pharmacia) cooling system. After electrophoresis, gels were stained for 15 min with ethidium bromide (5 mg/ml), destained in H_20 for the same time and then photographed upon ultraviolet transillumination.

Sequencing and phylogenetic analyses

For each species of host and each geographical locality, amplicons representing each distinct SSCP profile were selected and purified over spin-columns (Wizard™ PCR Prep, Promega), eluted in 30 μl of H_2O and subjected to automated sequencing (from both strands) employing BigDye® Chemistry (Applied Biosystems) and primers JB# and JB4.5, and 2 internal primers (CoX_1F 5′-GGTTTAGATGTTAAGACTGC-3′ and CoX_1R 5′-CCAATAATCATAGTAACAGA-3′). In order to exclude the possibility that any of the sequences obtained represented pseudogenes and to demonstrate an open reading frame, each *cox*1 sequence was conceptually translated into an amino acid sequence. The *cox*1 sequence data have been deposited in the EMBL, GenBank™ and DDJB databases under the Accession numbers AM495418-70. Pairwise comparisons of sequence differences (D) of particular interest were determined using the formula D = 1−(M/L), where M is the number of alignment positions at which the 2 sequences have a base in common, and L is the total number of alignment positions over which the 2 sequences are compared (Chilton *et al.* 1995).

Phylogenetic analyses were conducted using the nucleotide and amino acid sequence data sets. A maximum parsimony (MP) analysis was conducted using PAUP* 4.0b10 (Swofford, 1999). Characters were treated as unordered and were equally weighted; alignment gaps were treated as 'missing' in all

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Table 2. Nucleotide variation among 53 p*cox1* haplotypes (S1–S53) of the bile duct cestodes, *Progamotaenia diaphana*, *P. effigia* and *P. festiva* from Australian marsupials (dot indicates identical base compared with S1)

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      0000000000000000000000000000000011111111111111111111111111112222222222222222222222222222222233333333333333333333333333333333
      0001112222333444455566677788899001112222333445555566777888000111123334444555556666777778889999001111122223333444555556667777888999
      3462584567139258914803757847806581470369258170367923147069147036984780379024581270136905812466702568147036925815678039025814703 6

S1    ATTAAGTGTAGTTTGGTAAGTAGTTTTGCCTTTGTGATTTGATTTGAATATGAGTTTATTCGTTTGTATATTATAGCTTGCGAATTAGGGATCTGCTTAATGTTTATTATTGTGCATTTATTTGTAAT
S2    ..A.....................................G.....................................................................................G.............
S3    .CG.T....G....A...G..G.......T....C.G...AC...T..C.......CG.CT..G...G......G.T...T...C..A...A.A.......A...G.......ATG...G...A..G.
S4    .C..T....G.C.CA...G..G.......T....C.G...AC...T..C.......CG.CT..G...G......G.T...T...C..A...A.A.......A...........ATG...G...A..G.
S5    .CG.T....G.C..A...G..G.......T....C.G...AC...T..C.......CG.CT.CG...G......G.T...T...C..A...A.A.......A...........ATG...G...A..G.
S6    .CG.T....G.C..A...G..G.......T....C.G...AC...T..........CG.CT..A...G......G.T...T...T..A...A.A.......A...G.......ATG...G...A..G.
S7    ..A......G.C..............A.T...A.AG....T..CT.......A......TA..CA........G.T..AT.......A..A.........T...........ATG.....C.A.G..
S8    G...TA...G....A..GG..G.......T......G...ATC..T...GC..A.C.G.C.A...A.G......G.T...T......A...A.C...........GA......ATG..C....A.G..
S9    G...TA...G....A..GG..G.......T......G...ATC..T...GC..A.C.G.C.A...A.G......G.T...T......A...A.C...........GA......ATG..C....A.G..
S10   G.G.TA...G....A..GG..G.......T......G...ATC..T...GC..A.C.G.C.A.C.A.G......G.T...T......A...A.C...........GA......ATG..C....A.G..
S11   G.Z.TA...G....A..GG..G.......T......G...AT...T...G...A.C.G.C.A...A.G......G.T...T..G...A.A.A.C...........GA......ATG..C....A.G..
S12   ...GG....G..C..........C.....T...A.....AT...T.GC........G..T......G.G...G.T..........A...A.........A.CT..G.C..ATG......A....
S13   ..AGG....G..C..........C.....T...A.....AT...T.GC........G..T...C..G.G....G.T..........A...A.........A.C....G.C..ATG......A....
S14   ..AGG....G..C..........C.....T...A.....AT...T.GC........G..T...C..G.G....G.T..........A...A.........A.C....G.C..ATG......A....
S15   ..AGG....G..C..........C.....T...A.....AC...T.GC........G..T...C..G.G....G.T..........A...A.........A.C....G.C..ATG......A....
S16   ...GG....G..C..............T...A.....AT...T.GC.......CG..T...C..G.G....G.T..........A...A.........A.C....G....ATG......A....
S17   ...GG....G..C..............T...A.....AT...T.GC.......CG..T...C..G.G....G.T..........A...A.........A.C....G....ATG......A....
S18   ..AGG....G..C..........C.....T...A.....AT...T.GC........G..T...C..G.G....G.T...T.....A...A.........A.C....G....ATG......A....
S19   ..AGG....G..C..........C.....T...A.....AT...T.GC........G..T...C..G.G....G.T...T.....A...A.........A.C....G....ATG.....C.A....
S20   ..AGG....G..C..........C.....T...A.....AT...T.GC........G..TA..C..G.G....G.T...T.....A...A.........A.C....G....ATG......A....
S21   ...GC....G.A..AA.....G.C.....T...AC.....T...T.....GA...GCCTA.....G.G....G.....T......A...A.......G..A.......C..ATG....C.CA.G..
S22   ..AGC....G.A..AA.....G.C..C..T...AC.....AT...T..C...GA...G.C.A.....G.G....G.....T......A...A.......G..G..........ACG......CA.G..
S23   ...GC......A..AA.....G.......T...AC.....AC...T.......A...G.C.A.G...GCG....G.T...T......A...A.........C......A....TG.C.....A....
S24   ..AGC......A..AA.....G.......T...AC.G...AT...T.......A...G.C.A.G...GCG....G.T...T......A...A.........C......A....TG.C.....AC...
S25   ..AGC......A..AA.....G.......T...AC.G...AT...T.......A...G.C.A.G...GCG....G.T...T......A...A.........C......A....TG.C.....AC...
S26   ..AGT......A..AA.G...G.......T...A.....AT...T.......A...G.C.A.A...G.G....G.T...T......A...A.........G..C..........ATG......A....
S27   ..A......G.C..............A.T...A.AG....T..CT.......A......TA..CA........G.T..AT.......A..A.........T...........ATG.....C.A.G..
S28   ..A.T....G.C..............A.T...A.AG....T...T.......A......TA...........CG.T..AT......A...A......C.CTA.........ATG.......A.GG.
S29   ..AGG....G....A.....G....C..T...A..G...AT.C.TG..G...A...G..T..A.A.G..C.G.GGT...T..G...A...A.......G........G.....TG...........
S30   T..GT............G...G..A.CA.T.....A....AT...TGG.G..GA..AG.A.A..G....G..G.G.T...T..G...A...A.......G.TG.......A..ATG...G.....GG.
S31   ..AG...A......A..GG....G....TG...A.AG.......TG.....G.C.AT.ATA..........G.G.T...T..G....A.GG..A....T.T........A...TG.......A..G.
S32   T.AG...A......A..GG....G....TG...A..G.......TG.....G.C.AT.ATA.......A..G.G.T...T..G....A.GG..A....T.T........A...TG.........G.
S33   T.AG...A......A..GG....G....TG...A..G.......TG.....G.C.AT.ATA..........G.G.T...T..G....A.GG..A....T.T........A...TG.........G.
S34   T.AG...A......A..GG....G....TG...A..G.......TG.....G.C.AT.ATA..........G.G.T...T..G....A.GG..A....T.T........A...TG.........G.
S35   G.A.G..A...G.....GC...AA....TA.G.A.AGGAGAC...TGT.....A..AG..TCCAC..G.G..G.GAT...T..G.AGA...A..........GCC....A...TTG.....C..C.G.
S36   T.G.G....GA.......GA.G.A.....T.....AT....G...T.G.....AG.AG...AG.A..G.G.....AT...T..G..G.A..A.T.TA..G.T..C........ATG...G.......
S37   T.G.G....GA.......GA.G.A.....T.....AT....G...T.G.....AG.AG...AG.A..G.G.....AT...T.....G.A..A...TA..G.T..C........ATG...G.......
S38   T.G.CA...G....A...GA...G...A.T.....ATC...GCC.A........G.AG..TAG.A..G.G....GAT...T...C.G.A..A.......G.TC..G.......TT.C..G.......
S39   T.AGG.CA......A.C......G...A.TGA..CAT...AG.....T.....A..AG..TA.CAA.GCT.....AT...T...C.G..A.A..A....T.T...G.C.....TT........A.G..
S39   T.AGG.CA......A.C......G...A.TGA..CAT...AG.....T.....A..AG..TA.CAA.GCT.....AT...T...C.G..A.A..A....T.T...G.C.....TT........A.G..
S40   T.AGC..A......A.C......G.C.A.TG....AG...AG.....T.....A..AG..TAC.AAAGCT.....AT...T...C.G.AA.A..A....T.T.C.G.......TT........A.G.C
S41   T.AG...A......A.C......A...CA.TG.....G....G...T.T.....A..AG..TAG.AACGC.CCG.GAC...TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S42   T.AG...A......A.C......A...CA.TG.....G....G...T.T.....A..AG..TAG.AACGC.CCG.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S43   T.AG...A......A.C......A...CA.TG.....G....G...T.T....GA..AG..TAG.AA.GC.CCG.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S44   T.AG...A......A.C...Y..A...CA.TG.....G....G.C.T.T....GA..AG..TAG.AA.GC.CCG.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S45   T.AG...A......A.C......A...CA.TG.....G....G...T.T....GA..AG..TAG.AA.GC.CCG.GA....TAG..AG....AT.A..C.G.T...G.....ACTTG.........G.
S46   T.AG...A......A.C......A...CA.TG.....G....G...T.T....GA..AG..TAG.AA.GC.CCG.GA....TA...AG....AT.A..C.G.T...G.....ACTT..........G.
S47   T.AG...A......A.C...C..A...CA.TG.....G....G...TGT....A..AG..TAG.AA.GCGCCG.GAT...TA...AG....A..A..C.G.T...G.....ACTTG.........GC
S48   T.AGG..A......A.C......A...CA.TG.....G....G...T.T....A..AG..TAG.AA.GC.CCG.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S49   T.AG.A.A......A.C......A...CA.TG.....G....G...T.T....A..AG..TAG.AA.GC.CCG.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........
S50   T.AG...A......A.C......A...CA.TG.....G....G...T.T....GA..AG..TAG.AA.GC.C.G.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S51   T.AG...A......A.C......A...CA.TG.....G....G...T.T....GA..AG..TAG.A..GCACCG.GA....TA...AG....AT.A..C.G.T...G.....ACTTG.........G.
S52   T.A.GA..C.....A..GG....G..CA.T......T...AG.C.T.T...AGA..GG.CTAC.GA.G.......ATA..T..G....A.A..A..C.T.A......A.....T.........G..
S53   T.A.GA..C.....A..GG....G..CA.T......T...AG.C.T.T...AGA..GG.YTAC.GA.G.......ATA..T..G....A.A..A..C.T.A...........T.........G..
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analyses. Exhaustive searches with TBR branch-swapping were used to infer the shortest trees. The length, consistency index, excluding uninformative characters, and the retention indices of each most parsimonious tree were recorded. Bootstrap analyses (1000 replicates) were conducted using heuristic searches and tree bisection–reconnection (TBR) branch-swapping, with the MulTrees option, to determine the relative support for clades in the consensus trees. Sequence data were also analysed using the neighbour-joining (NJ) method in PAUP. *Progamotaenia ewersi* and *P. macropodis* were used as outgroups.

RESULTS

DNA was extracted from each of 182 specimens of *P. diaphana*, *P. effigia* and *P. festiva* from 13 host species (Table 1) and subjected to PCR-based SSCP analysis of p*cox*1. Fifty-three different SSCP profiles were recorded, representing the entire spectrum of variation among all 182 amplicons. Aliquots of the amplicons representing the 53 profiles were subjected to sequencing, and 53 distinct p*cox*1 sequences (haplotypes) were obtained (Table 2). Alignment of the 53 sequences (all 396 bp) revealed nucleotide variations at 129 alignment positions (Table 2).

Analyses using both neighbour-joining (Fig. 3) and parsimony methods (not shown) produced trees of essentially the same topology. In the parsimony tree, 109 characters were informative. The tree length was 443, the consistency index 0·433 and the retention index 0·805. Using bootstrap values >85% in the maximum parsimony tree as the criterion for identifying distinct clades in the parsimony tree, 14 clades were resolved (Fig. 3). For 13 of these clades, the bootstrap values exceeded 83% for the neighbour-joining tree and 95% for the maximum parsimony tree. In only 1 instance, the clade containing specimens from *W. bicolor*, were the values lower, namely 64% for the neighbour-joining tree and 86% for the maximum parsimony tree. *P. diaphana* in *Lasiorhinus latifrons* and *P. effigia* in

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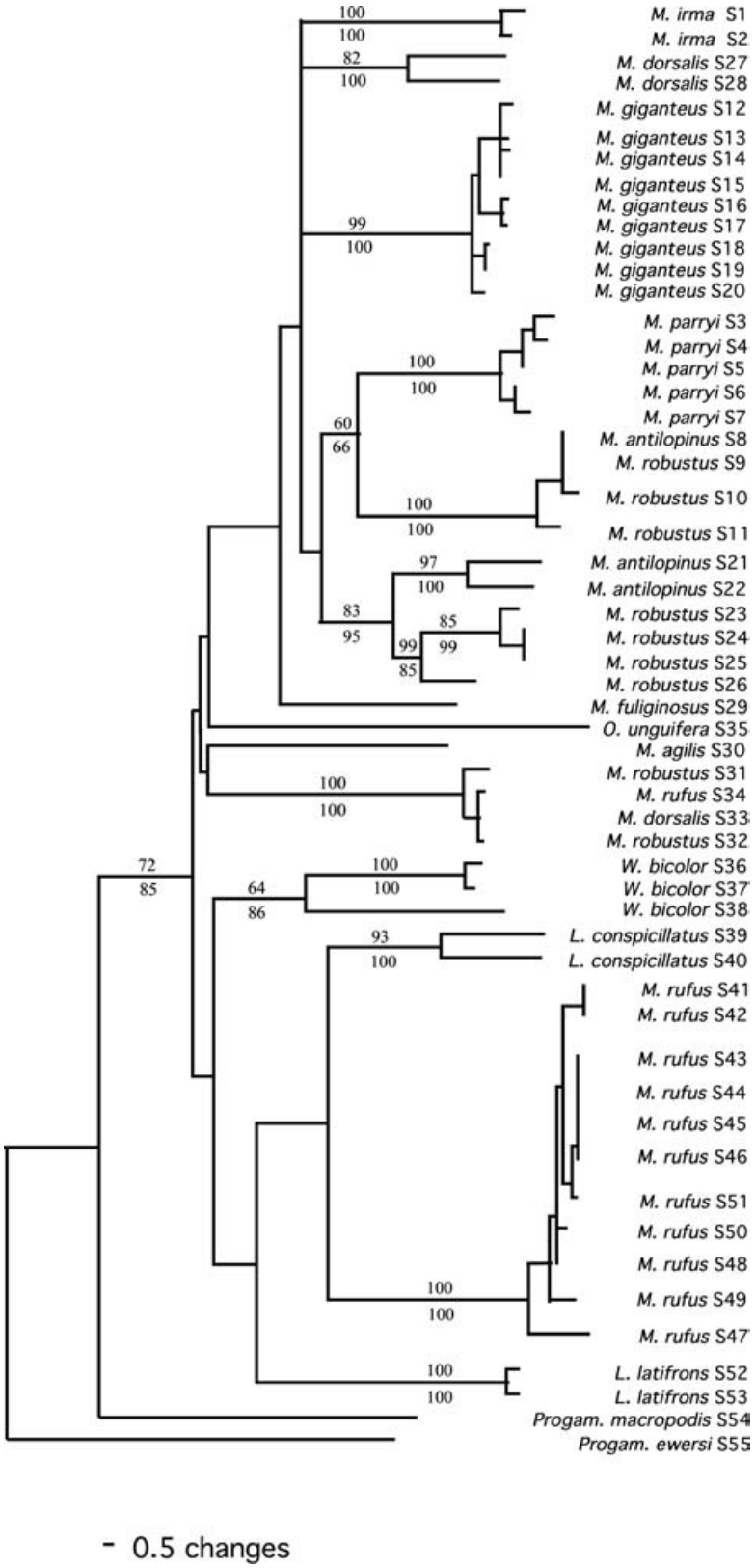

Fig. 3. Neighbour-joining tree depicting the relationships between the specimens of *Progamotaenia festiva*, *P. diaphana* (S52–53) and *P. effigia* (S29), based on analyses of the p*cox*1 nucleotide sequence and with *Progamotaenia ewersi* and *P. macropodis* used as outgroups. Numerals above branches represent bootstrap values in the neighbour-joining analysis, those below branches bootstrap values in the maximum parsimony analysis.

Macropus fuliginosus occurred in distinct clades, while specimens currently identified as *P. festiva* were distributed among 12 clades. In 9 of these clades, the cestodes occurred in a single species of host (*Lagorchestes conspicillatus*, *Macropus irma*. *Macropus parryi*, *Macropus giganteus*, *Macropus*

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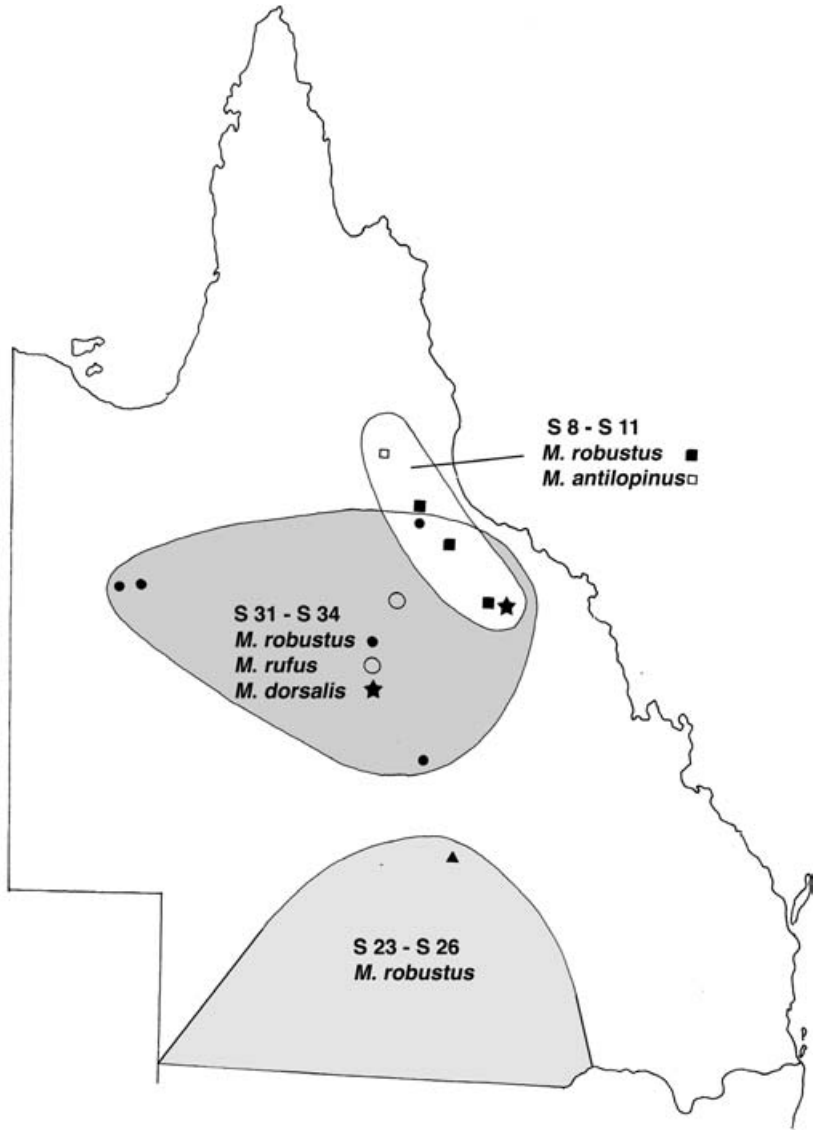

Fig. 4. Distribution of three genetic groups of *Progamotaenia festiva* found primarily in *Macropus robustus* in Queensland. Group 1: from *M. robustus* (triangle); group 2: closed circles, *M. robustus*, open circle, *M. rufus*, star, *M. dorsalis*; group 3: squares: closed squares, *M. robustus*, open squares, *M. antilopinus*.

dorsalis, *Macropus agilis*, *Macropus rufus*, *Onychogalea unguifera* or *Wallabia bicolor*). Representing the remaining clades, 2 cestode clades were found in the closely related host species *M. robustus* and *M. antilopinus*, whereas the third clade was found primarily in *M. robustus*, but occurred also in both *M. rufus* and *M. dorsalis*.

Macropus robustus was the only host species with multiple clades of cestode. One clade was found in south-western Queensland and Western Australia, a second occurred in north-eastern Queensland, from Mount Surprise to Charters Towers, and a third extended from north-western Queensland (Mount Isa) to north-eastern Queensland, overlapping with the second clade in the region of Charters Towers, Queensland (Fig. 4).

Nucleotide variation was low (0–3·0%) within clades containing specimens from *M. agilis*, *M. dorsalis*, *M. giganteus*, *M. irma*, *M. robustus* and *M. rufus* (Table 3). Within clades containing specimens from *M. parryi* and *L. conspicillatus*, the extent of variation was greater (7–10%), even though these samples were collected over relatively small geographical ranges (Table 3). In the case of specimens collected from *W. bicolor*, the differences found between specimens collected in Queensland were 0·05%, whereas those collected in Victoria, 2800 km to the south, differed by 6·3% (Table 3).

The analysis of the amino acid sequences (Table 4) produced a neighbour-joining tree similar in topology to that produced by the nucleotide sequences (not shown), except that specimens from *M. irma*, *M. giganteus*, *M. parryi*, *M. fuliginosus*, *M. antilopinus* and *M. robustus* (S9–S11, S23–S26) were not resolved because of their identical amino acid sequences.

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Table 3. Nucleotide variation (%) within clades of cestodes, collected from a single host species, compared with the geographical ranges over which specimens were collected

Host species	Sequence haplotype (Table 2)	No. specimens	Variation (%)	Distance over which collections were made (km)
Macropus agilis	S30	16	0	1700
M. dorsalis	S27, 28	10	3·0	390
M. giganteus	S12–20	52	1·5	1600
M. irma	S1, 2	4	1·0	200
M. parryi	S3–7	9	7·8	190
M. robustus	S23–26	4	3·0	3800
M. rufus	S41–51	52	1·3	1500
Lagorchestes conspicillatus	S39–40	7	9·3	200
Wallabia bicolor (Qld)	S36–37	2	0·05	460
(Vic)	S38	1	6·3	2800

Table 4. Amino acid variation among 53 p*cox1* haplotypes of *Progamotaenia* species from the bile ducts of marsupials (Dot indicates identical amino acid compared with S1.)

Haplotype	000|000|000|000|000|111|222|222|222|222|222|222|222|333|333|333| 222|333|556|666|777|666|555|666|667|777|888|999|999|000|111|555| 567|123|890|789|678|345|345|234|890|123|012|234|567|789|678|567|
S1-25, 26, 29	V G A V F V S V K S I I A V T V
S30	. . . . Y . . . .
S28	. . . . I . . . . . . P .
S27,33,34	. . . . I . . . .
S31	I . . . . V . I . .
S35	I . . I . . N . . . V . . . . .
S36,37	. S T . . . N . . . V . . . . .
S39,40	I . . . . . N . . . V . . I . .
S41-44,46,48,49	I . . . . . N . N . V . V I . T
S47,50,51	I . . . . . N . N . V . . I . T
S38	. . T . . . N . . . V . . . . .
S45	I . . . . . N . N G V . V I . T
S52,53	A . . . . I N . . . . . I . .
S32	I . . . . V V I . .

DISCUSSION

The phylogenetic trees constructed based on the *cox*1 sequence data were generally well resolved, with high bootstrap support for most of the clades. In addition, samples within most clades were obtained from a single host species, and there was relatively little nucleotide variation within a clade. Specimens collected from *M. agilis* over a geographical range of over 1700 km exhibited no nucleotide variation, whereas specimens collected from *M. giganteus* between Queensland and Victoria, a geographical range of 1600 km, exhibited nucleotide variation of 1·5% among specimens from this host species and 0·8% variation among 52 specimens from *M. rufus* collected over a geographic range of 1500 km. For most collections from the same host species over a wide geographical range, nucleotide variation was limited. In the case of worms from *Wallabia bicolor*, specimens collected in Queensland exhibited very limited (0·05%) nucleotide variation but differed at 6·3% of nucleotides when compared with those collected in Victoria. This difference may be due to geographical variation. By contrast, specimens from *M. parryi* and *L. conspicillatus*, collected within a relatively limited geographical area in central Queensland varied at up to 7·8 and 9·3% of nucleotide positions, respectively, indicating that low levels of variation within specimens from a single host species were not recorded in all cases.

Genetically distinct groups of cestodes, currently identified as *P. festiva*, exhibited a high degree of host specificity, with a single, distinct clade of cestodes usually occurring in each individual host species examined. In addition, the morphologically distinct species, *P. diaphana* in *Lasiorhinus latifrons* and *P. effigia* in *Macropus fuliginosus* also occurred in distinct genetic clades. In *M. robustus*, cestodes representing 3 distinct genetic groups were recorded, and host switches were inferred in 2 of these clades. In spite of these exceptions, the level of host specificity was usually high. Additional evidence of host specificity is provided by the extensive series of specimens collected from *M. giganteus* and *M. rufus*

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in north-western New South Wales and south-western Queensland where the two species occur in broad sympatry. In this area, no evidence of host switching was detected.

By contrast, host switching was detected in 2 cases. In north-eastern Queensland, 1 clade of cestodes (S8–S11), occurring in *M. robustus* in the Charters Towers region, was also found in *M. antilopinus*. In the area in which the specimens were collected from *M. antilopinus*, *M. robustus* is also abundant (Beveridge *et al.* 1998) and the 2 host species are closely related phylogenetically (Flannery, 1989). At this collection site, many nematode parasites are also shared between these 2 host species (Beveridge *et al.* 1998), and the sequences from the specimens from the 2 hosts (S8 and S9) were identical. Consequently, these findings were interpreted as a case of host switching.

In a second clade (S31–34), cestodes presumed to be parasitic primarily in *M. robustus*, since they are found only in this host in the Mount Isa region of north-western Queensland (unpublished observations), were also found in both *M. rufus* and *M. dorsalis* in the Charters Towers region. The host distributions of these cestodes are complicated by the occurrence of the 2 genetic groups of cestode in the same principal host, *M. robustus*, in the same region. The existence of different genetic groups of cestode in *M. robustus* in this region was first observed in the MEE study of Baverstock *et al.* (1985). The results of the MEE study also provided evidence that one of the genetic groups identified in the MEE study was capable of host switching into *W. bicolor*. It seems likely that the same genetic group identified as switching hosts between *M. robustus* and *W. bicolor* in the latter study is the same taxon that switches from *M. robustus* to sympatric *M. rufus* and *M. dorsalis* in the present study. Therefore, the present results confirm and extend the findings of the earlier MEE study.

A third clade included specimens from *M. robustus* from south-western Queensland, southern Western Australia and *M. antilopinus* from the Northern Territory. In this case, the clade was subdivided into specimens from the 2 host species, with high bootstrap support for the subdivision. In this instance, the cestodes in *M. robustus* and *M. antilopinus* represent distinct genetic groups. Within the clade representing specimens from *M. robustus*, the cestodes from central Queensland (S26) differed genetically from those collected in Western Australia (S23–S25). There was insufficient cestode material to determine whether specimens S23–S26 represented 2 distinct genetic populations, and additional collecting is required in central Queensland to define the distribution of this genetic population, particularly in the area to the north of current collection sites, to establish whether all 3 genetic groups co-occur in some regions of Queensland. Although 3 distinct genetic groups of *P. festiva* were found in *M. robustus* in Queensland, the available evidence points towards allopatric speciation rather than speciation within a host, as has been inferred for some monogeneans (Littlewood *et al.* 1997; Simková *et al.* 2004).

The findings from the present analysis of the *cox*1 data sets confirm and expand the earlier MEE studies of *P. festiva* (see Baverstock *et al.* 1985), but the pectinate nature of the phylogenetic tree provides little insight into the evolution of members of the *P. festiva* complex. Wickström *et al.* (2005), also working with anoplocephalid cestodes, showed that addition of data from the first internal transcribed spacer (ITS 1) and the large subunit of nuclear ribosomal DNA genes did not provide increased resolution of clades compared with an analysis of the data derived from the *cox1* gene alone. Further studies on the phylogeny of this cestode complex may therefore require the examination of genes other than those examined to date.

This simplest explanation of the results presented herein would be a virtually simultaneous colonization of the bile ducts of a wide range of marsupial hosts by a common but as yet unidentified ancestor. The other possible explanation of a co-evolutionary expansion can be eliminated, since the data include *P. diaphana*, a parasite of vombatid marsupials, which evolved much earlier than the macropodids (Archer, 1984). The MEE study by Baverstock *et al.* (1985) also included cestodes from the common wombat, *Vombatus ursinus*, which are closely related to *P. diaphana* from the hairy-nosed wombat, *Lasiorhinus latifrons*, and which clustered within the *P. festiva* complex. Thus the MEE and *cox* 1 sequence data from cestodes from the 2 genera of wombats, *Vombatus* and *Lasiorhinus*, preclude an hypothesis of coevolution between cestodes and their hosts, because wombats are only distantly related to the kangaroos and wallabies (Archer, 1984).

The distinct clades resolved in trees constructed in this study (based on genetic differences) probably represent distinct species. The basis for this proposal is 3-fold. Firstly, the level of genetic diffences between clades is high. Second, if a phylogenetic approach is taken (Nadler, 2002), each clade can be considered to represent a distinct species. Third, many of the genetic groups of cestode occur in broad sympatry, with the potential for transfer among host species, yet retaining their genetic differences. Not only do the morphologically distinct species *P. diaphana* and *P. effigia* occur in sympatry with *P. festiva* found in *M. giganteus*, *M. robustus* and *M. rufus*, but the differentiation maintained between specimens from *M. giganteus* and *M. rufus* in areas of close sympatry is striking. In addition, cestodes from *M. agilis*, *M. dorsalis*, *M. parryi* and *M. robustus* in north-eastern Queensland retain their genetic

identities, in spite of the fact that all host species are broadly sympatric.

In the case of *M. robustus*, three clades of cestodes were identified in a single host species and at least two of them can occur in sympatry. This provides compelling evidence that the relevant clades identified herein represent distinct species. Based on these arguments, it appears that the clades identified within *P. festiva*, based on genetic data, represent distinct species. The results presented here need to be compared with morphological data before any definitive conclusions can be drawn. Beveridge (1976) recognized 2 broad morphological groups within the complex, but more detailed morphological studies are clearly required to determine whether morphological characteristics are present to support the genetic data.

The present results suggest that *P. festiva*, as currently recognized morphologically, may represent at least 12 genetically distinct species. In the MEE study by Baverstock *et al*. (1985), additional samples were studied from the common wombat, *Vombatus ursinus*, and the quokka, *Setonix brachyurus*. No samples from either host were available for the current study, but the extent of genetic differences in the MEE data suggested that the cestodes from these hosts represented 2 additional species of *Progamotaenia* within the complex. The *P. festiva* complex is therefore potentially one of the largest complexes of sibling cestode species known. In addition, observations of host specificity suggest that, while most of these sibling species are highly host specific, some may exhibit relatively little host specificity. This range of host specificity among members of a sibling species complex indicates caution in any generalizations concerning host specificity in species of anoplocephalid cestodes.

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