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ORIGINAL PAPER

Genetic markers to distinguish *Moniezia expansa* from *M. benedeni* (Cestoda: Anoplocephalidae) and evidence of the existence of cryptic species in Australia

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Abstract Multilocus enzyme electrophoresis (MEE) was used to genetically compare *Moniezia* spp. collected from sheep and cattle in southern Australia. Fifteen enzyme loci were established for the genetic characterisation of individual specimens of *Moniezia*, which represents a significant increase in the number of loci that was previously available for these cestodes. Another four enzyme loci were also identified as providing potential genetic markers. There were no differences in staining activity or banding patterns of different body regions of individual cestodes. Fixed genetic differences between *Moniezia expansa* and *Moniezia benedeni* were detected at 10 (77%) loci. Individuals that lacked the diagnostic morphological characters (i.e. the arrangement pattern of the interproglottidal glands) could be assigned to either morphospecies based on their MEE profiles. Genetic differences were also detected among individuals of the two morphospecies. One specimen of *M. expansa* had fixed genetic differences at 33% of enzyme loci when compared to all other specimens of *M. expansa*. Fixed genetic differences were also detected between the two groups of *M. benedeni* at 12 of 13 (92%) enzyme loci. This magnitude of genetic difference was greater than that detected between the two morphospecies. This provides evidence for the existence of cryptic species in the genus *Moniezia*.

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Introduction

Anoplocephalid cestodes of the genus *Moniezia* Blanchard, 1891 occur in the intestines of ruminants, suids, rodents, primates and ratite birds (Beveridge 1994). The type species, *Moniezia expansa*, is a widely distributed cestode of ungulates in Europe, Asia, Africa, America and Australia. At least seven species of *Moniezia* have been described from ruminants; however, only *M. expansa* and *Moniezia benedeni* were considered to be valid species by Spasskii (1951). These two species can be distinguished from one another based on differences in the arrangement of the interproglottidal glands (Spasskii 1951). In *M. expansa*, these glands are grouped in a rosette pattern around depressions opening onto the posterior surface of the segment, while in *M. benedeni*, they are arranged in a linear pattern. However, it is not uncommon to find individuals that lack interproglottidal glands (Taylor 1928; Spasskii 1951), which prevents their identification to species. Therefore, other methods are required to identify such individuals.

Multilocus enzyme electrophoresis (MEE) has been used effectively to define genetic markers for a wide range of parasitic helminth species (see review by Andrews and Chilton 1999) even when the diagnostic morphological

features are present in only one sex or one life-cycle stage of the parasite (e.g. Andrews et al. 1988, 1992; Norman and Chilton 1994). MEE was used in the study of parasitic nematodes of the genus *Physaloptera*, where female specimens, which lack the diagnostic morphological characters of their male counterparts, were assigned to a particular species based on 15 diagnostic enzyme loci (Norman and Chilton 1994). Although MEE and another biochemical technique, isoelectric focussing (ISF), have been used to determine enzyme activity levels in *Moniezia* (see Hermoso et al. 1982; Balasubramanian et al. 1984; Dhandayuthapani et al. 1984; Khera and Arora 1984) and/or to compare *M. expansa* with *M. benedeni* (see Hermoso et al. 1982; Johnson and Hoberg 1989; Ba et al. 1993), such studies employed only a small number (1–6) of enzyme loci. It is important for any taxonomic and/or population studies on parasitic helminths to compare individuals at a greater number of enzyme loci (Andrews and Chilton 1999). The aim of this paper was to increase the number of genetic markers that can be used to examine the population genetics of *Moniezia* from sheep and cattle and for the specific identification of specimens that lack interproglottidal glands.

Materials and methods

Specimens of *Moniezia* were removed from the intestines of sheep (*Ovis aries*) or cattle (*Bos taurus*) collected from nine locations in southern Australia (Table 1, Fig. 1). Each cestode was washed in saline and divided into three sections, designated as scolex/neck, mid-body and posterior end. A part of each section was placed into a separate microcentrifuge tube, frozen and stored at −70°C until required for electrophoretic analysis. Other mature segments were relaxed in water for several hours and fixed in AFA solution (Pritchard and Kruse 1982) for subsequent

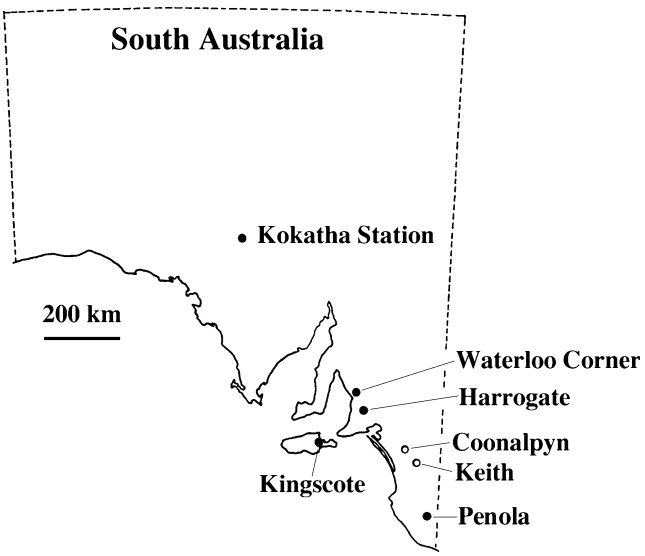

Fig. 1 Geographical localities within South Australia from where *Moniezia* were collected from *Ovis aries* (*filled circles*) and *Bos taurus* (*open circles*)

morphological examination of the interproglottidal glands. They were stained with Celestine blue, cleared in methyl salicylate, and mounted in Canada balsam. Mature segments of each cestode were examined by light microscopy to determine whether the interproglottidal glands were arranged as a row of rosette-like glands extending across the width of the segment in *M. expansa* or arranged in a short, continuous row close to the midline of each segment in *M. benedeni*. On this basis, seven specimens from sheep were identified as *M. expansa*, and six specimens from cattle were identified as *M. benedeni* (Table 1). Five specimens of *Moniezia* (two from sheep and four from cattle) could not be assigned to a particular species on morphological grounds because they lacked interproglottidal glands. These were designated as "unknowns" for the electrophoretic examination (Table 1).

Table 1 Geographical localities in South Australia (SA) and Victoria (V) at which *Moniezia* specimens were collected from sheep and cattle for this study

Specimen number	Locality		Host	Interproglottidal glands	Identification based on morphology
Ov1	Kokatha Station (SA)	31°16′S, 135°14′E	*Ovis aries*	Present	*M. expansa*
Ov2–4	Kingscote, Kangaroo Island (SA)	35°39′S, 137°38′E	*O. aries*	Present	*M. expansa*
Ov5	Waterloo Corner (SA)	34°40′S, 138°34′E	*O. aries*	Absent	Unknown
Ov6	Kingscote, Kangaroo Island (SA)	35°39′S, 137°38′E	*O. aries*	Absent	Unknown
Ov7–8	Harrogate, Adelaide Hills (SA)	34°57′S, 139°01′E	*O. aries*	Present	*M. expansa*
Ov9	Penola (SA)	37°23′S, 140°50′E	*O. aries*	Present	*M. expansa*
Bt1–3	Keith (SA)	36°06′S, 140°21′E	*Bos taurus*	Absent	unknown
Bt4	Coonalpyn (SA)	35°46′S, 140°01′E	*B. taurus*	Absent	unknown
Bt5–10	Geelong Abattoir[a] (V)		*B. taurus*	Present	*M. benedeni*

[a] Precise origin of each host uncertain

Individual cestodes were prepared for electrophoresis by adding an equivalent volume of lysing solution (100 µl *b*-mercaptoethanol, 10 mg nicotinamide-adenine dinucleotide phosphate and 100 ml distilled water) to the thawed samples before homogenisation by hand. Small pieces of intestine were also removed from one *O. aries* and one *B. taurus*, washed in saline, placed into separate microcentrifuge tubes and frozen at −70°C for use on all electrophoretic gels as a control to distinguish parasite-specific enzymes from host enzymes. These were prepared by adding an equivalent volume of lysing solution to the thawed sample, which was then sonicated and centrifuged at 5,000×*g* for 10 min at 4°C. Electrophoresis was conducted on cellulose acetate, "Cellogel" (Malta s.r.l., Milan). Electrophoretic gels were run at 200 V for 2–3 h. Histochemical staining of gels follows methods described in detail by Richardson et al. (1986). The electrophoretic banding patterns of samples at each enzyme locus were interpreted allozymically (i.e. allele with the least electrophoretic mobility from the cathode designated as allele *a*). The proportion of loci showing 'fixed' allelic differences (i.e. where groups of samples do not share any alleles at a locus) was used to compare cestodes from different geographical localities and host species. A phenogram was constructed using an unweighted pair group method with arithmetic means (Sneath and Sokal 1973).

Results and discussion

MEE is an effective method for the delineation of parasite species but only when a sufficient number of enzyme loci are employed (Andrews and Chilton 1999). In the present study, a total of 14 enzymes, encoded by 15 presumptive loci, gave sufficient staining intensity and resolution to allow reliable genetic comparisons of individual *Moniezia*. This represents a significant increase in the number of characters available to genetically compare individuals of this genus. The enzymes used and their enzyme commission (E.C.) numbers were: adenylate kinase (AK, E.C. 2.7.4.3), citrate synthase (CS, E.C. 4.1.3.7), enolase (ENOL, E.C. 4.2.1.11), fructose-1,6-diphosphatase (FDP, E.C. 3.1.3.11), glyceraldehyde-3-phosphate (GA3PD, E.C. 1.2.1.12), glucose-phosphate isomerase (GPI, E.C. 5.3.1.9), hexokinase (HK, E.C. 2.7.1.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), mannose-phosphate isomerase (MPI, E.C. 5.3.1.8), purine nucleoside phosphorylase (NP, E.C.2.4.2.1), peptidase valine–leucine (PEP-A, E.C. 3.4.13.11), phosphoglycerate kinase (PGK, E.C. 2.7.2.3), phosphoglucomutase (PGM, E.C. 5.4.2.2) and triose-phosphate isomerase (TPI, E.C. 5.3.1.1). There was also an activity in *Moniezia* sonicates for the enzymes, aspartate aminotransferase (GOT, E.C. 2.6.1.1), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), alanine aminotransferase (GPT, E.C. 2.6.1.2) and isocitrate dehydrogenase (IDH, E.C. 1.1.1.42). However, further modification is required before they could be used as potential genetic markers. Hermoso et al. (1982) also reported activity for lactate dehydrogenase (LDH, E.C. 1.1.1.27) and glucose-6-phosphate dehydrogenase (6GPD, E.C. 1.1.1.49) in their study of *Moniezia*. However, we found the activity of these two enzymes to be too low for reliable genetic interpretation. Strong activity was also detected in specimens for the enzyme peptidase leucine–glycine–glycine (PEP-B, E.C. 3.4.11.4). However, the banding patterns of each specimen were identical to their respective patterns for PEP-A. Although the substrates for each peptidase are usually specific (Richardson et al. 1986), we have considered the electrophoretic results for PEP-A and PEP-B to represent the same enzyme locus. For the enzymes CS and TPI, there was evidence on the electrophoretic gels of host contamination in some parasite tissues. However, the parasite-specific locus could be distinguished unequivocally from that of the host. Two parasite-specific loci were detected for MDH, with *Mdh-1* defined as the locus with the least electrophoretic mobility from the cathode.

Genetic variation among *Moniezia* samples was detected at all 15 enzyme loci. However, for each cestode, the scolex/neck and segments from the mid-body and posterior end did not differ in their relative staining intensity nor in their electrophoretic profiles at all loci examined. This is in contrast to Balasubramanian et al. 1984 who detected changes in isozyme pattern of alkaline phosphatase from *M. benedeni* in relation to growth and differentiation of the segments. This enzyme was not examined in our study. Multiple banding patterns in individual samples were detected at six (40%) loci. The patterns at these loci were consistent to that expected for heterozygous individuals at loci for monomeric (two bands; AK, HK and MPI), dimeric (three bands; MDH) and trimeric enzymes (four bands; NP). The quaternary structure of PEP-A could not be reliably determined due to insufficient resolution of electrophoretic bands on gels. In other parasitic helminths, PEP-A is typically a dimeric enzyme (Andrews and Chilton 1999). Although cestodes are capable of self-fertilization, the individuals used in this study were heterozygous for one or more loci, suggesting that cross-breeding occurs in this genus.

A comparison of the allelic profiles of specimens identified morphologically as either *M. expansa* or *M. benedeni* revealed fixed genetic differences between the two morphospecies at 10 of the 13 (77%) enzyme loci (Table 2). However, there were also fixed genetic differences among specimens of each morphospecies. The six specimens identified morphologically as *M. benedeni* could be separated into two distinct groups (Fig. 2), with fixed genetic differences at 12 (92%) of 13 loci. This magnitude of genetic difference is significantly greater than that

Table 2 Alleles detected in *Moniezia* specimens at 15 enzyme loci

Morphological identification (specimen number)	Enzyme locus														
	Ak	*Cs*	*Enol*	*Fdp*	*Ga3pd*	*Gpi*	*Hk*	*Mdh-1*	*Mdh-2*	*Mpi*	*Np*	*Pep-A*	*Pgk*	*Pgm*	*Tpi*
M. expansa (Ov1)	*a*	*b*	*a*	*c*	*a*	*c*	*b*	*a, b*	*a*	*d*	*c*	*d*	*a*	*c*	*c*
M. expansa (Ov2, 3, 4, 7, 8 and 9)	*b*	*b*	*b*	*b*	*a*	*c*	*b*	*a*	*b*	*a*	*c, d*	*c*	*c, d*	*c*	*c*
Unknown (Ov6)	*b*	*b*	*b*	*b*	*a*	*c*	*b*	*a, b*	*a*	*c*	*c*	*c*	*c*	*c*	*d*
M. benedeni (Bt6, 7, 8 and 9)	*c*	*c*	*b*	*d*	*c*	*b*	?[a]	*c*	*a*	*a*	*a*	?	*c*	*a*	*a*
Unknowns (Bt1, 2, 3, and 4)	*c*	*c*	*b*	*d*	*c*	*b*	*b, c*	*c*	*a*	*a, b*	*a*	*a, b*	*c*	*a*	*a*
M. benedeni (Bt5 and 10)	*d*	*a*	*c*	*a*	*b*	*a*	?	*d*	*b*	*e*	*a, b*	?	*b*	*b*	*b*
Unknown (Ov5)	*d, e*	–[b]	–	*a*	–	*a*	*a*	–	*b*	–	*b*	*d*	*b*	–	*b*

[a] Not examined for this enzyme
[b] Insufficient staining activity

between morphospecies suggesting that *M. benedeni* in southern Australia consists of more than one species. Specimens Bt6–9 were genetically more similar to *M. expansa* than to specimens Bt5 and Bt10 (Fig. 2). Both groups of *M. benedeni* were collected from cattle at the Geelong Abattoir. However, their precise geographical origins in southern Australia were unknown. There were also fixed genetic differences among *M. expansa* in sheep from different geographical areas in South Australia. *Moniezia expansa* from Kangaroo Island (Ov2-4) shared alleles in common with *M. expansa* from the adjacent mainland (Ov7-8; Adelaide Hills) and in the southeast of South Australia (Ov9; Penola), localities that are approximately 110–320 km apart. However, all these individuals had fixed differences at five (33%) loci when compared with the single specimen of *M. expansa* (Ov1) from Kokatha Station in the Far North of South Australia, situated ~540 km to the north of the Adelaide Hills. Thus, the extent of fixed genetic differences between these two groups of *M. expansa* could either represent two extremes of the population variation or indicate that there are two genetically distinct but morphologically similar (i.e. cryptic) species within the currently recognised *M. expansa*. However, caution is advised in over-interpreting the allelic data based on a single specimen from Kokatha Station for electrophoretic comparison. Therefore, a large number of samples from this area and other geographical regions need to be examined electrophoretically to distinguish between these two alternative hypotheses.

The concept that *M. expansa* and *M. benedeni* may each represent a species complex has been suggested previously by Ba et al. (1993) based on a comparison of *Moniezia* from France and Africa using six enzyme loci. They found fixed genetic differences at five loci between the two groups of *M. benedeni* from cattle, which was greater than the difference between the two morphospecies. It was further speculated

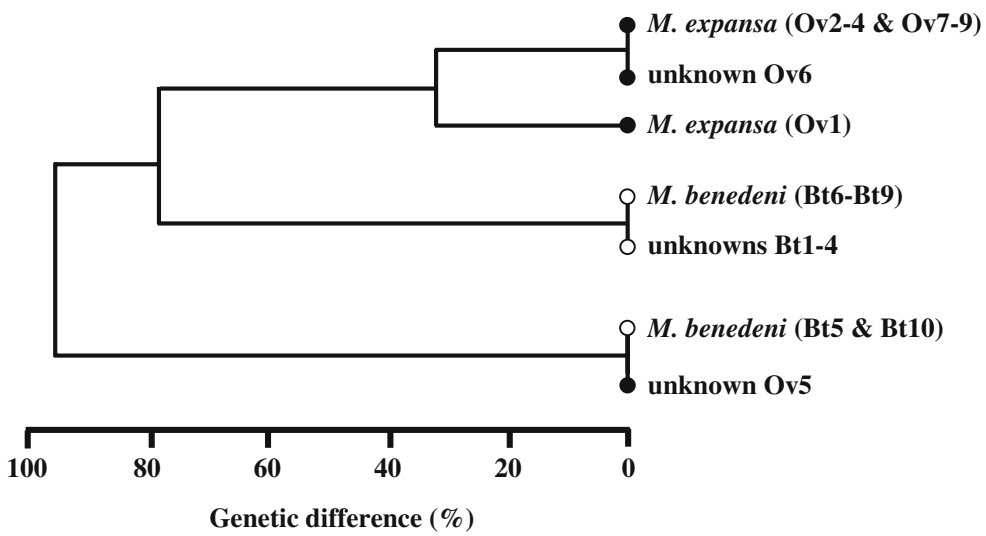

Fig. 2 Phenogram depicting the genetic differences between specimens of *Moniezia* from *Ovis aries* (*filled circles*) and *Bos taurus* (*open circles*)

that one of the *M. benedeni* species may have been derived from wild African ruminants. This is unlikely to explain the presence of more than one species of *M. benedeni* in cattle from southern Australia because of the lack of native wild ruminants, whereas their ecological equivalents, the macropodid marsupials (kangaroos and wallabies), are not infected with species of *Moniezia* (see Spratt et al 1991). The magnitude of the electrophoretic differences between the two groups of *M. benedeni* (92%) and between the two groups of *M. expansa* (33%) from southern Australia is similar to those reported by Baverstock et al. (1985) for the species complex in another anoplocephalid cestode, *Progamotaenia festiva*, parasitic in macropodid and vombatid marsupials. The existence of a species complex in *P. festiva* has also been supported by mitochondrial DNA sequence data (Beveridge et al., unpublished data). Other species complexes have also been recently detected in *P. ewersi*, *P. macropodis* and *P. zschokkei* (see Hu et al. 2005), suggesting that this phenomenon may be frequent in anoplocephalid cestodes.

Ba et al. (1993) suggested that the existence of cryptic species in *M. benedeni* and *M. expansa* questions the validity of the arrangement of the interproglottidal glands as a diagnostic morphological feature. One advantage of the MEE approach is that individuals that lack interproglottidal glands can be assigned to one of the morphospecies (and species contained therein) based on comparisons of their allelic profiles. The four specimens of unknown identity collected from cattle at Keith (Bt1–3) and Coonalpyn, South Australia (Bt4), shared alleles at 13 loci with individuals Bt6–9 identified morphologically as *M. benedeni*. This provides strong evidence that these three unknown specimens belong to the morphospecies, *M. benedeni*. Similarly, one of the two unknown specimens from sheep (Ov6: Kangaroo Island) shared alleles at all 15 loci as other individuals (Ov2–4) from the same location identified as *M. expansa*, thus confirming the identity of specimen Ov6 as *M. expansa*. In contrast, the allelic profile of the other unknown specimen from sheep (Ov5; Waterloo Corner, South Australia) could only be established at nine loci (Table 2). It is interesting to note that this unknown individual had a unique allele at one locus (*Ak*) but had different alleles to all specimens of *M. expansa* from sheep at eight (89%) of the nine loci. When compared with *M. benedeni* from cattle, it shared alleles at the seven loci with specimens Bt5 and Bt10, suggesting that this specimen from sheep (Ov5) belonged to the morphospecies, *M. benedeni*. The presence of *M. benedeni* in Australian sheep is possible given that both *M. benedeni* and *M. expansa* are capable of parasitising a similar range of hosts (Spasskii 1951) and that *M. benedeni* has been detected in both cattle and sheep from Senegal, Africa (Ba et al. 1993). In the present study, we found *M. expansa* in sheep but not cattle, which is consistent with the findings of Ba et al. (1993) for this species in Africa and France.

It is important that genetic markers be established for the accurate identification of *Moniezia* species and to provide the basis for taxonomic and population-based studies, particularly given that *M. expansa* and *M. benedeni* are capable of parasitising the same hosts (Spasskii 1951; Ba et al. 1993) and that they can have an impact on the health of domestic animals, particularly young individuals, when present in large numbers (e.g. Spasskii 1951; Ershov 1956; Juyal 1987; Dorchies 1999). Moreover, other independent loci within the genome, such as those of the mitochondrial genes (as used for the anoplocephalid cestode *Progamotaenia*; see Hu et al. 2005) could be used together with MEE to examine the population structure and taxonomy of the genus. In conclusion, this study highlights the advantage using the technique of MEE, together with a significant increase in the number of genetic markers (i.e. enzyme loci), to accurately identify species of parasitic helminths, particularly where there are a limited number or no morphological characters to discriminate between individuals of different species.

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