

# Combining data from morphological traits and genetic markers to determine transmission cycles in the tape worm, *Echinococcus granulosus*

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(Received 10 December 1997; revised 25 February 1998; accepted 18 March 1998)

## SUMMARY

Species of *Echinococcus* (Cestoda: Taeniidae) require 2 mammalian hosts to complete their life-cycle; a carnivorous definitive host, and a herbivorous or omnivorous intermediate host. For most species of *Echinococcus*, the definitive host range is restricted to 1 or a few species, but the intermediate host range is very broad. Programmes to control hydatid disease attempt to break the life-cycle of the parasite and their effectiveness is therefore enhanced by an understanding of local patterns of transmission. Although it is known that the rostellar hooks of protoscoleces may be influenced by the species of intermediate host in which they develop, the application of this knowledge to infer transmission cycles has been limited, because the intermediate host effect has not been isolated from other environmental and genetic components of phenotypic variance. This study presents a method for separating these potentially confounding genetic and environmental effects, by combining quantitative genetic analyses of hook traits with data on population structure from neutral genetic markers. The method was applied to 5 hook traits (hook number, total length of large hooks, blade length of large hooks, total length of small hooks, blade length of small hooks) measured on protoscoleces from 2 intermediate host types (sheep and macropod marsupials) in Australia. Although genetic variance was similar for all traits, they differed markedly in the extent of environmental variance attributed to development in different host types. Total length of small hooks was the trait most affected, with 49–60% of phenotypic variance being explained by environmental differences between intermediate host species. Blade length of small hooks was least affected, with none of the phenotypic variance due to intermediate host origin. These data suggest that hook measurements of adult worms from naturally infected definitive hosts could be used to determine the intermediate host species from which infection was acquired, if the appropriate traits are measured.

**Key words:** *Echinococcus granulosus*, transmission cycles, population genetics, quantitative genetics, allozymes, morphology.

## INTRODUCTION

Species in the genus *Echinococcus* Rudolphi, 1801 (Cestoda: Taeniidae) cause hydatid disease. There are currently 4 recognized species of *Echinococcus*, *E. granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli*, although recent molecular genetic studies have suggested that *E. granulosus* should be split into at least 5 separate species (Bowles, Blair & McManus, 1995; Thompson, Lymbery & Constantine, 1995). All species require 2 mammalian hosts to complete their life-cycle. The adult develops in the small intestine of a carnivorous definitive host, while the metacestode develops in the viscera of a herbivorous or omnivorous intermediate host. Definitive hosts become infected by eating the organs of intermediate hosts which contain protoscoleces produced by asexual reproduction in the meta-

cestode. Intermediate hosts become infected by eating eggs which are shed in the faeces of definitive hosts. For all species of *Echinococcus*, the definitive host range is restricted to 1 or a few species, usually of canids, but the intermediate host range is very broad and often includes humans (Rausch, 1995).

The only species of *Echinococcus* which occurs in Australia is the taxon currently designated the sheep strain of *Echinococcus granulosus* (Batsch, 1768). In Australia, the parasite is believed to be maintained in 2 cycles of transmission: a domestic cycle principally between sheep and domestic dogs, and a wildlife cycle involving mainly macropod marsupials and feral dogs or dingoes (Kumaratilake & Thompson, 1982). On the mainland of Australia, allozyme and DNA studies have found only minor genetic differences, consistent with extensive gene flow, between populations in different species of intermediate host (Lymbery, Thompson & Hobbs, 1990; Hope, Bowles & McManus, 1991; Lymbery, Constantine & Thompson, 1997). By contrast,

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Kumaratilake & Thompson (1984) and Thompson & Kumaratilake (1985) found major differences in morphological traits between these populations, particularly in the hooks which ring the rostellum and help to anchor the adult worm in the intestine of the definitive host.

Hobbs, Lymbery & Thompson (1990) suggested that rostellar hook traits of protoscolecids may be directly influenced by the intermediate host in which they develop, and also found that the larval hook outline remains visible within the adult hook and is unchanged by passage through the definitive host. Constantine *et al.* (1993) proposed that, taken together, these findings suggest that the hook traits of adult worms from naturally infected definitive hosts could be used to determine the intermediate host species from which infection was acquired. This may provide an important tool in epidemiological studies of hydatid disease (Rausch, 1995; Ponce Gordo & Cuesta Bandera, 1997). Mathematical modelling of the life-cycles of species of *Echinococcus* has suggested that the transmission dynamics of the parasite should make it vulnerable to control intervention (Gemmell & Roberts, 1995). The aim of control programmes is to break the life-cycle of the parasite, usually by a combination of educational and legislative measures designed to prevent access by definitive hosts to sources of infection. Their effectiveness is therefore enhanced by a detailed understanding of local patterns of transmission (Thompson, 1992; Schantz *et al.* 1995).

Constantine *et al.* (1993) attempted to define criteria for such 'transmission typing' of adult worms from dogs in Australia and showed how measurements of hook number and hook length could be used to infer transmission cycles in an area where sheep and macropods occurred together. The accuracy of their conclusions was constrained, however, by the fact that they assumed that all the observed variation in hook number and hook length was of environmental (host) origin. It is possible that genetic differences between parasite populations in different intermediate host species may also contribute to variation in hook morphology. For example, although populations of *E. granulosus* from sheep and macropod hosts in Australia are clearly not different strains, analysis of rapidly evolving gene regions, such as allozymes, have consistently found 2–6% of total genetic variance to be distributed between host populations (Lymbery *et al.* 1990, 1997). Even this low level of between-host variation, if it also occurred in the genes which determine hook traits, may affect the accuracy with which intermediate host origin can be determined.

My aim in this paper is to show how the potentially confounding genetic and environmental influences on rostellar hook traits can be separated by combining a quantitative genetic analysis with data on population structure from genetic markers. I will

first describe the theoretical approach, and then give an example, using the data of Constantine *et al.* (1993) and Lymbery *et al.* (1997), of how the approach may be used to determine the most appropriate hook trait or combination of traits for accurate transmission typing.

#### THEORY

Rostellar hook traits, being quantitative in nature, are influenced by both genetic and environmental factors. As almost all of the life-cycle of *Echinococcus* is spent within mammalian hosts, I will assume that all environmental effects are of host origin. Ignoring genotype–environment interactions and covariance, the phenotypic variance ( $V_P$ ) of hook traits can be partitioned in the standard way (e.g. Falconer, 1981) into a portion due to the effects of genetic differences between individuals ( $V_G$ ) and a portion due to environmental differences ( $V_E$ ):

$$V_P = V_G + V_E. \quad (1)$$

For species of *Echinococcus*, which have a clonal stage in their life-cycle,  $V_G$  may be simply estimated as the among-clone component of variance and  $V_E$  as the within-clone component. In addition, where protoscolecids develop in different species of intermediate host,  $V_G$  and  $V_E$  may be further partitioned into components due to differences between isolates from the same host species ( $V_{GW}$  and  $V_{EW}$ ) and between isolates from different species ( $V_{GB}$  and  $V_{EB}$ ):

$$V_P = V_{GW} + V_{EW} + V_{GB} + V_{EB}. \quad (2)$$

My aim is to separate these causal components of variance in hook traits. In particular, I wish to estimate  $V_{EB}$ , that part of the phenotypic variance due to environmental differences between host species, because the magnitude of this component determines the usefulness of any particular trait for transmission typing. If hook measurements are obtained from  $k$  protoscolecids from each of  $i$  isolates (metacestodes) taken from each of  $h$  host species, then the causal components of variance in equation (2) may be partly separated by equating observed mean squares in a nested analysis of variance (Table 1) with their expectations. The observational component of variance,  $\sigma_W^2$  corresponds to the causal component  $V_{EW}$ , and  $\sigma_I^2$  corresponds to  $V_{GW}$ , assuming that there are no environmental effects due to different hosts of the same species (see Discussion section).

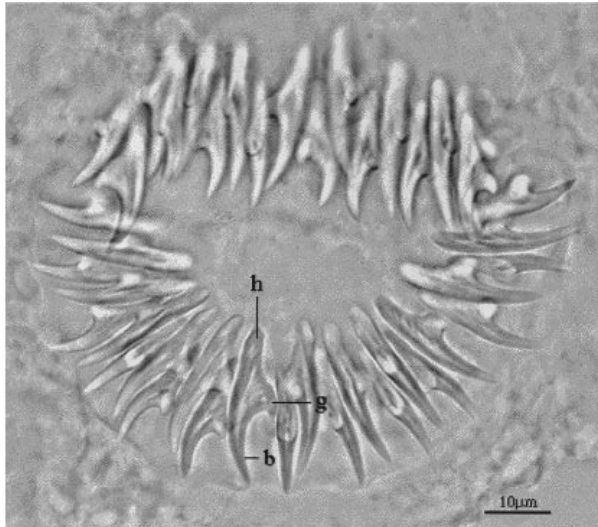
$V_{EB}$  and  $V_{GB}$  are confounded within the observational component  $\sigma_B^2$ . To separate these 2 components, I use the relationship described by Wright (1965) between the components of genetic variance ( $V_{GW}$ ,  $V_{GB}$ ) and fixation indices for quantitative traits ( $Q_{IT}$ ,  $Q_{ST}$ ):  $V_{GB} = 2Q_{ST} V_A$ ;  $V_{GW} = (1 + Q_{IS}) (1 - Q_{ST}) V_A$ , where  $V_A$  is the additive genetic variance for a population in Hardy Weinberg and linkage

Table 1. Nested analysis of variance table for computing observational components of variance for rostellar hook traits

Source of variation	D.F.	Mean square	Composition of mean square
Between hosts	$h-1$	$MS_B$	$\sigma_W^2 + k\sigma_I^2 + ik\sigma_B^2$
Between isolates (within hosts)	$h(i-1)$	$MS_I$	$\sigma_W^2 + k\sigma_I^2$
Within isolates	$hi(k-1)$	$MS_W$	$\sigma_W^2$

Table 2. Causal components of phenotypic variance for rostellar hook traits and formulae for estimating them from observational components of variance (see Table 1) and  $F_{ST}$ 

Causal components	Estimation
Genetic variance within hosts $V_{GW}$	$\sigma_I^2$
Environmental variance within hosts $V_{EW}$	$\sigma_W^2$
Genetic variance between hosts $V_{GB}$	$F_{ST}\sigma_I^2/(1-F_{ST})$
Environmental variance between hosts $V_{EB}$	$\sigma_B^2 - F_{ST}\sigma_I^2/(1-F_{ST})$

Fig. 1. Photograph of rostellar hooks from protoscolex of *Echinococcus granulosus*, showing alternating large and small hooks, and the characteristic morphological features of the handle (h), guard (g) and blade (b) of each hook.

equilibrium, and non-additive genetic effects are assumed to be small. Assuming self-fertilization in Australian populations of *Echinococcus* ( $Q_{IS} = 1$ ; Lymbery *et al.* (1997)), these equations reduce to:

$$Q_{ST} = V_{GB}/(V_{GB} + V_{GW}). \quad (3)$$

The neutral expectation for  $Q_{ST}$  is the value of  $F_{ST}$ , the standardized variance in allelic frequencies for neutral, single-locus genes in the same populations

(Felsenstein, 1986; Lande, 1992). Assuming that differences in both rostellar hook traits and genetic markers between host species are due to genetic drift (see Discussion section), we can substitute  $F_{ST}$  calculated from neutral genetic markers, and rearrange equation (3):

$$V_{GB} = F_{ST} V_{GW}/(1-F_{ST}) \quad (4)$$

or

$$V_{GB} = F_{ST} \sigma_I^2/(1-F_{ST}). \quad (5)$$

$V_{EB}$  may now be calculated from the observational components of variance and equation (5) as:

$$V_{EB} = \sigma_B^2 - F_{ST} \sigma_I^2/(1-F_{ST}). \quad (6)$$

In summary, Table 2 lists the 4 causal components of variance in rostellar hook traits, and the formulae for estimating them from observational components of variance and  $F_{ST}$ .

## MATERIALS AND METHODS

### Populations studied

A population sample is defined as the isolates (metacystodes or hydatid cysts) of *E. granulosus* obtained from sheep slaughtered at 1 or more abattoirs in the same locality, or from 1 or more species of macropods (*Macropus giganteus*, *M. fuliginosus*, *Wallabia bicolor*, *W. rufogrisea*) shot in the same locality. Preliminary analyses showed no differences in rostellar hook traits or genetic markers among different macropod species, so they are treated here as 1 host species type. For simplicity, I will refer throughout the rest of the paper to 2 intermediate host 'species': sheep and macropod marsupials. Cysts from sheep were collected by meat inspectors at the abattoirs, while macropods were shot by government wildlife officials in state forests. I consider here sympatric sheep and macropod populations from Western Australia and New South Wales, 2 localities separated by approximately 4000 km. These populations have been described more fully by Lymbery *et al.* (1997). All share the same species of definitive host (domestic or feral dogs) and there is no evidence from either locality of any geographical separation between cycles in different intermediate host types.

Table 3. Number of isolates sampled from sheep and macropod intermediate hosts at each locality for rostellar hook and genetic marker measurements

	New South Wales		Western Australia	
	Sheep	Macropods	Sheep	Macropods
Rostellar hook measurements	16	12	12	6
Genetic marker measurements	46	13	23	25

### Rostellar hook traits

The rostellum of *Echinococcus* has a row of small hooks and a row of alternating large hooks. The hooks are made primarily of keratin (Smith & Richards, 1991) and consist of a blade, a guard and a handle (Fig. 1). The data used here comprise total hook numbers, and the mean total length and blade length for 3 large and 3 small hooks, measured for each of 6 protoscoleces per isolate. The raw data were obtained by Hobbs *et al.* (1990) and Constantine *et al.* (1993) and they describe the methods of measurement. The number of isolates measured for each host population in each locality are shown in Table 3.

### Genetic markers

Enzyme electrophoresis was used to estimate  $F_{ST}$  for single-locus genes. All the protoscoleces (except those fixed for morphological studies) from 1 cyst were regarded as a single isolate and were examined for 6 variable enzyme loci: esterase (*Est*); glucose-6-phosphate dehydrogenase 2 (*G6pd-2*); isocitrate dehydrogenase 1 (*Idh-1*); nucleoside phosphorylase 2 (*Np-2*); peptidase (*Pep*); 6-phosphogluconate dehydrogenase 2 (*6pgd-2*). The raw data were obtained by Lymbery *et al.* (1997), where electrophoretic techniques are described. Table 3 shows the number of isolates measured for each host population in each locality. Although the isolates used for morphological and genetic marker studies were not identical, there was substantial overlap. Fifty-four per cent of the isolates measured for morphology were also analysed electrophoretically, while 21 % of the isolates used in electrophoretic studies were also measured for morphological traits.

### Analysis

Observational components of variance for each rostellar hook trait were obtained by least squares from the nested linear model:

$$y_{hik} = \mu + \alpha_h + \beta_{hi} + \epsilon_{hik},$$

where  $y_{hik}$  is the rostellar measurement for the  $k$ th protoscolex of the  $i$ th isolate from the  $h$ th host

species,  $\mu$  is the overall mean,  $\alpha_h$  is the effect of the  $h$ th host species,  $\beta_{hi}$  is the effect of the  $i$ th isolate within the  $h$ th host species and  $\epsilon_{hik}$  is the residual, representing variation among protoscoleces within an isolate. Both isolate and host species were considered random effects. Because the data were unbalanced, Satterthwaite's (1946) approximation was used to synthesize denominator mean squares and degrees of freedom. Preliminary analyses, on a subset of the data, found no significant added variance component due to individual hosts of the same species which contained multiple isolates, so this level of variation was not considered in the final model.

$F_{ST}$  for enzyme loci was calculated by the method of Nei & Chesser (1983), implemented with the program GENESTRUT (Constantine, Hobbs & Lymbery, 1994). The departure of  $F_{ST}$  from 0 was tested by  $\chi^2$  (Workman & Niswander, 1970).

For comparisons between rostellar hook traits, estimated causal components of variance ( $V_{GW}$ ,  $V_{EW}$ ,  $V_{GB}$  and  $V_{EB}$ ) were expressed as a proportion of total phenotypic variance ( $V_P$ ). Standard errors of the estimates were obtained by jackknifing over isolates, and 95 % confidence intervals were calculated assuming a normal error distribution for the jackknife estimates (Crowly, 1992). The confidence intervals for  $V_{GB}$  and  $V_{EB}$  did not include any variance in the estimate of  $F_{ST}$ .

## RESULTS

Table 4 shows estimates of the standardized variance in allelic frequencies at allozyme loci, between populations of *E. granulosus* from different host species in each locality. There were significant differences in allelic frequencies between populations for 3 loci in New South Wales and 1 locus in Western Australia. Mean  $F_{ST}$  values were significantly greater than 0 for both localities, indicating that host populations were genetically different, with around 5 % of the allozyme variance distributed between them.

Table 5 shows the genetic and environmental components of variance within and between host species for rostellar hook traits in each locality. The within-host genetic variance was similar for all traits

Table 4. Estimates of the standardized variance in allelic frequencies ( $F_{ST}$ ) between populations of *Echinococcus granulosus* from sheep and macropod hosts in 2 localities

(—, Indicates locus was not variable.)

Locus	Locality	
	New South Wales	Western Australia
<i>Est</i>	0	0.062*
<i>G6pd-2</i>	0.043*	—
<i>Idh-1</i>	0.035*	—
<i>Np-2</i>	0.029	0.031
<i>Pep</i>	0	0.010
<i>6pgd-2</i>	0.177*	—
Mean	0.055*	0.043*

\*  $F_{ST}$  significantly different from 0 ( $P < 0.05$ ).

in each locality, contributing 20–33% of total phenotypic variance in New South Wales and 16–45% in Western Australia. Between-host genetic variance was low for all traits, and not significantly greater than 0 for large blade length in Western Australia or for small blade length in either locality.

Small and large blade lengths had large within-host environmental variance (55–68%), but very little or no environmental variance between host species. Hook number and total hook lengths, by contrast, had less within-host environmental variance (14–45%), but significantly more between-host environmental variance (26–62%). There were differences between localities in the distribution of environmental variance, with isolates from New South Wales having relatively less within-host and more between-host variance for hook number and

total length traits. Between-host environmental variance ( $V_{EB}/V_P$ ) was greatest for the total length of small hooks in Western Australia, and for the total length of large and small hooks (not significantly different at the 5% level) in New South Wales.

#### DISCUSSION

Hook size and shape have long been used for classification in *Echinococcus* and other cestodes. Although a number of studies have suggested that hook traits are influenced by the host species in which development occurs (Raush, 1953; Vogel, 1957; Lubinsky, 1960; Sweatman & Williams, 1963; Hobbs *et al.* 1990), this is the first time that the different genetic and environmental factors acting during hook development have been partitioned. The extent of within-population genetic variance (technically defined as broad-sense heritability) was similar for all traits measured in this study, but traits differed markedly in the extent to which they were influenced by development in different intermediate host species. Blade lengths of large and small hooks were unaffected by different hosts, and may therefore be more reliable traits than hook number and total hook length for differentiating taxa which occur in a range of intermediate host species. This conclusion must be tempered by the large within-host environmental variance in blade length traits, which will decrease their precision as taxonomic tools.

The host-induced variation in hook number and total hook length may, as suggested by Constantine *et al.* (1993), be used to infer intermediate host origin of adult worms. The usefulness of morphological traits for accurate transmission typing is given by  $V_{EB}$ , that portion of the phenotypic variance due to

Table 5. Estimates (with jackknife s.e.s in parentheses) of genetic and environmental components of variance for rostellar hook traits within and between host species in 2 localities, expressed as a proportion of total phenotypic variance

( $V_{GW}$  = genetic variance within host species;  $V_{EW}$  = environmental variance within host species;  $V_{GB}$  = genetic variance between host species;  $V_{EB}$  = environmental variance between host species;  $V_P$  = total phenotypic variance. NSW = New South Wales; WA = Western Australia. HN = hook number; TL = total length of large hooks; BL = blade length of large hooks; TS = total length of small hooks; BS = blade length of small hooks.)

Trait	$V_{GW}/V_P$		$V_{EW}/V_P$		$V_{GB}/V_P$		$V_{EB}/V_P$	
	NSW	WA	NSW	WA	NSW	WA	NSW	WA
HN	0.27 (0.003)	0.28 (0.006)	0.16 (0.010)	0.45 (0.004)	0.02 (0.001)	0.01 (0.001)	0.55 (0.004)	0.26 (0.007)
TL	0.23 (0.003)	0.35 (0.005)	0.14 (0.002)	0.32 (0.006)	0.01 (0.001)	0.02 (0.001)	0.62 (0.004)	0.31 (0.008)
BL	0.33 (0.003)	0.45 (0.004)	0.59 (0.004)	0.55 (0.003)	0.02 (0.001)	0	0.06 (0.004)	0
TS	0.20 (0.002)	0.16 (0.004)	0.18 (0.002)	0.34 (0.006)	0.02 (0.001)	0.01 (0.001)	0.60 (0.009)	0.49 (0.007)
BS	0.32 (0.003)	0.32 (0.006)	0.68 (0.002)	0.68 (0.006)	0	0	0	0

environmental differences between host species. The simplest method of assigning isolates to most probable host categories is on the basis of their value for the trait with maximum  $V_{EB}/V_P$ . From the data presented here, small hook length would be the trait of choice for determining intermediate host origin in Australia. If we define the accuracy of host assignment, i.e. the correlation between trait value and true host origin, by  $\sqrt{V_{EB}/V_P}$ , then using small hook length for host assignment would result in about 20–30% of isolates being mis-classified (accuracy = 0.70–0.79).

Where more than 1 trait has been measured, they could be used for host assignment in an independent culling fashion, i.e. only assigning isolates which unambiguously meet the criterion for host origin for every trait. This was, in effect, the approach taken by Constantine *et al.* (1993), who estimated most probable intermediate host origin on the basis of trait values for hook number and hook length. Independent culling may improve accuracy of assignment, but at the cost of increasing the number of unassigned isolates. Alternatively, the traits could be combined to produce a discriminant function (e.g. Ponce Gordo & Cuesta Bandera, 1997), but traditional discriminant analysis would not take account of differences in assignment accuracy among traits. Of more value would be the construction of an index, analogous to those used in multiple trait artificial selection (Rönningen & Van Vleck, 1985). This will require estimates not only of genetic and environmental variances, but also of covariances between traits, so that appropriate weightings for each trait may be obtained.

The conclusions of Constantine *et al.* (1993) with respect to the sources of infection of feral dogs and foxes in Australia are not substantially affected by the results presented here. This is because, for populations of *E. granulosus* in sheep and macropod hosts in Australia, they happened to choose those traits with the greatest between-host environmental variance ( $V_{EB}/V_P$ ). The real value of the current approach is to provide a means of determining the most appropriate traits to use as transmission markers in future epidemiological studies. The type of transmission cycle described here, of a restricted definitive host range but a wide range of sympatric intermediate hosts, is common not only for species of *Echinococcus*, but also for many other cestodes (Mackiewicz, 1988). We cannot assume, however, that the traits which serve as accurate transmission markers for *Echinococcus granulosus* in Australia will be appropriate for other populations of *E. granulosus*, for other species of *Echinococcus* or for more distantly related cestodes. Before the approach of Constantine *et al.* (1993) can be applied more generally, then the magnitude of between-host environmental variance for putative transmission markers needs to be determined.

The approach outlined here to estimate between-host environmental variance for morphological traits is relatively straightforward, and does not require complex *in vitro* or *in vivo* experimentation. The estimation formulae in Table 2, however, depend on 2 assumptions: (1) that differences in hook traits among isolates from different hosts of the same species are of genetic, rather than environmental origin; (2) that  $F_{ST}$  calculated from genetic markers can be equated with  $Q_{ST}$ .

The first assumption is not inherent in the methodology, and among-isolate environmental effects could be incorporated into the linear model by which variance components were estimated. The empirical justification for ignoring them in this study was that preliminary analyses found multiple isolates from the same individual host to be no more alike for any hook traits than isolates from different hosts of the same species. Biologically, this implies that the sheep and macropod intermediate host environments in which cysts reside in Australia are similar in all hosts of the same species. This assumption may not be valid in other localities or for other host or parasite species, and would need to be checked in the linear model used to derive observational components of variance.

The second assumption implies that differences in both marker gene frequencies and rostellar hook traits between isolates in different intermediate host species are due to genetic drift rather than selection. Selection appears unlikely to be responsible for the allozyme differences observed here between isolates from different intermediate hosts. The value of  $F_{ST}$  between isolates from sheep and macropod marsupials is very similar to that found in other allozyme (Lymbery *et al.* 1990) and more recent RAPD (M. Hankinson and R. C. A. Thompson, unpublished data) studies, implying a similar degree of differentiation across the genome. Selection also seems unlikely to be responsible for differences in the rostellar hook traits of protoscoleces between intermediate host species. Rostellar hooks are used to anchor the adult worm in the intestine of the definitive host. In the intermediate host, the rostellum serves no function, as it is invaginated within the protoscolex, which is in turn protected within the cyst (Thompson, 1986). Selection could still act if cysts in different intermediate host species were transmitted to different definitive host species or geographically isolated populations. There is no evidence that this occurs in Australia and it is not common in other parts of the world where cycles of *Echinococcus* are found (Rausch, 1995). The constancy of the selective environment in the definitive host means that rostellar hook traits of species of *Echinococcus* are very unlikely to be under differential selection pressure in different intermediate host species. The assumption may be generally valid for the attachment organs of other cestode species,

which, like the rostellar hooks of *Echinococcus*, appear to be adapted to the intestinal conditions of the definitive host, but serve no function in intermediate hosts (Hayunga, 1991).

Thanks to Clare Constantine, Ian Dadour, Russ Hobbs and Mike Johnson for comments on the manuscript. The raw data were collected in studies supported by the Australian Research Council.

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