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Morphometric and Cladistic Analyses Of The Phylogeny of *Macropodinium* (Ciliophora: Litostomatea: Macropodiniidae)

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Summary. Phylogenetic studies of the genus *Macropodinium* were conducted using two methods; phenetics and cladistics. The phenetic study of morphometrics suggested that the genus could be divided into 3 groups attributable mostly to cell size and shape. The cladistic study also split the genus into 3 groups related to cell size but groups were further distinguished by patterns of ornamentation. Reconciliation of both approaches revealed considerable congruence, however, it also suggested the existence of convergences in the phenetic study and a lack of resolution in the cladistic study. The morphological diversity of *Macropodinium* is probably due to evolutionary trends such as increasing body size, allometry and polymerisation of structures. None of these trends, however, was uniformly directional and differential effects were observed in different regions of the phylogenetic tree. Comparison of the phylogeny of *Macropodinium* to a consensus phylogeny of the macropodids revealed limited incongruence between the 2 trees. The ciliate groups could be related to 2 host groups; the wallaby genera and the kangaroo and wallaroo subgenera. The association with these host groups may be the result of phyletic codescent, ecological resource tracking or a combination of both. Further studies of both host and ciliate phylogeny are necessary to resolve these effects.

Key words: Ciliophora, cladistic analysis, endosymbionts, phylogeny, *Macropodinium*, *Megavestibulum*.

INTRODUCTION

The Macropodiniidae are endosymbiotic ciliates which inhabit the enlarged, fermentative forestomachs of macropodid marsupials (kangaroos and wallabies) (Dehority 1996; Cameron *et al*., 2001, 2002; Cameron and O'Donoghue 2002, 2003a). Two genera have been described: *Macropodinium* Dehority, 1996 with 13 species; and *Megavestibulum* Cameron *et* O'Donoghue, 2003 with 2 species. Of the two genera *Megavestibulum* is clearly more plesiomorphic and grossly similar to other trichostome ciliates inhabiting the macropodid stomach, such as the amylovoracids or polycostids (Cameron and O'Donoghue 2003a). In contrast, *Macropodinium* species have a highly derived and variable morphology which made species diagnosis easy and suggested the possibility of studying the evolution of the genus by phylogenetic studies. The aim of this study was therefore to elucidate the phylogeny of the genus *Macropodinium* using continuous morphometric characters in a phenetic analysis and discrete cell features in a cladistic analysis and to use the resultant phylogenies to investigate character trends within the genus and the evolution of host relationships.

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MATERIALS AND METHODS

For full details of collection, preservation and staining methods see Cameron *et al*. (2001, 2002). Terminology used throughout is that of Cameron *et al*. (2002).

Morphometric analyses. A morphometric analysis of 10 standardised linear measurements [cell length, cell width, macronucleus length, macronucleus width, vestibulum width, vestibulum depth, cytostome width, ventral bar (VB) width, dorsal bar (DB) width, width between longitudinal grooves] and 2 count variables (number of longitudinal grooves left and right) was conducted to assess phenetic distinctiveness within *Macropodinium* (see Fig. 1 for definitions). Species which lacked either VB or DB were scored as having a width of 0 for this feature. The measurements of individual cells used to generate summary morphometric statistics in our previous papers (Cameron *et al*. 2001, 2002) were used again in this study. Only individual cells from which all linear measurements could be obtained were used in this study. The 12 *Macropodinium* species described or redescribed in Cameron *et al*. (2001, 2002) were used: *M. setonixium*, *M. titan*, *M. petrogale*, *M. ocallaghani*, *M. yalanbense*, *M. ennuensis*, *M. moiri*, *M. hallae*, *M. marai*, *M. bicolor*, *M. tricresta* and *M. spinosus*. *M. baldense* Dehority, 1996, was the only species in the genus omitted due to a lack of appropriately stained specimens. Within-species variation was assessed by inclusion of the 3 host groups of *M. yalanbense* (eastern-grey kangaroo, EG; western-grey kangaroo, WG; and Kangaroo Is. grey kangaroo, KI) and the two forms of *M. ennuensis* (forma *ennuensis* and *dentis*). Discriminant analysis was performed to determine the morphometric distinctiveness of each species or subspecies group. Cluster observations were then performed to determine the phenetic similarity of each species or subspecific group. Multivariate statistics were performed using the software package Minitab® ver. 11 (1996).

Morphological cladistics. The thirteen species of *Macropodinium* were examined by light and electron microscopy and a matrix of 21 characters developed (Table 2). A generalised *Megavestibulum* sp. was used as an outgroup and characters which did not occur in *Megavestibulum* were coded as a separate character state "not applicable"; otherwise character polarity was determined relative to *Megavestibulum*. Characters for which the character state could not be determined were coded as '?'. Taxa with multistate characters were scored as possessing both character states. The list of characters and their transformations are listed in Appendix 1. Characters sets were coded as either unordered or ordered and duplicate analyses performed in PAUP 4.0b10 (Swofford 2002) and character state changes assigned to nodes by MacClade ver4 (Maddison and Maddison 2000).

RESULTS

Multivariate analysis using cluster observations yielded 9 discrete groups. Of the 15 taxa included, only 3 (*M. tricresta*, *M. titan*, *M. moiri*) formed monophenetic groups. The remaining species appeared intermingled in the dendrogram and did not form distinct clusters (not shown). For this reason, a discriminant analysis of the data set was performed to determine the distinctiveness of each taxon. Discriminant analysis compares *a priori* defined groups, in this case the species or subspecific groups, against the groupings supported by the morphometric data. The results of the discriminant analysis are presented in Table 1. In this analysis, 9 of the 12 species were found to be distinct and supported by all specimens assigned to that species. *M. marai*, *M. hallae* and *M. ocallaghani* each had a single specimen which appeared to be aberrant and be more similar to members of another group, *M. setonixium*, *M. petrogale* and *M. yalanbense* KI respectively. The *M. ennuensis* forms, despite slight overlap in the cluster observation, were found to be completely distinct in the discriminant analysis. In contrast, the three host groups of *M. yalanbense* were highly overlapping in the cluster observations and indistinct in the discriminant analysis. The cluster observation suggested that the genus has three broad groups: group 1 comprising *M. titan* and *M. moiri*; group 2 comprising *M. setonixum*, *M. marai*, *M. bicolor*, *M. hallae* and *M. tricresta*; and group 3 comprising *M. ennuensis*, *M. ocallaghani*, *M. petrogale*, *M. spinosus* and *M. yalanbense*. There are also strong phenetic associations between taxa within these groups. The unrooted network of phenetic relationships suggested by the morphometric analysis is presented in Fig. 2.

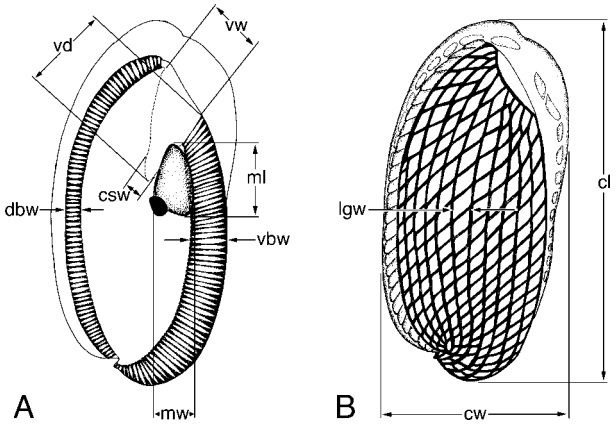

Fig. 1. Diagram of measurements. **A** - internal cell features. **B** - external cell features. cl - cell length, csw - cytostome width, cw - cell width, dbw - dorsal bar width, lgw - lateral groove width, ml - macronucleus length, mw - macronucleus width, vbw - ventral bar width, vd - vestibulum depth, vw - vestibulum width.

The results of both cladistic analyses are presented in Fig. 3 (ordered data set) and Fig. 4 (unordered data set).

Table 1. Summary of classifications suggested by discriminant analysis of *Macropodidium* morphometric data.

Group	Suggested group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*M. petrogale*	12														
2	*M. titan*		3													
3	*M. spinosus*			22												
4	*M. tricresta*				28											
5	*M. marai*					14										
6	*M. yal.* EG						25	3	1							
7	*M. yal.* KI						1	21	6					1		
8	*M. yal.* WG						3	5	20							
9	*M. enn. dentis*									20						
10	*M. enn. ennuensis*										20					
11	*M. bicolor*											10				
12	*M. hallae*												9			
13	*M. ocallaghani*													28		
14	*M. moiri*														18	
15	*M. setonixium*					1										15
Total		12	3	22	28	15	30	30	27	20	20	10	10	30	18	15
Nº. Correct		12	3	22	28	14	25	21	20	20	20	10	9	29	18	15

Heuristic analysis of the ordered data set yielded 6 equally parsimonious trees, 69 steps long, CI = 0.522, RI = 0.593 and RC = 0.309. The analysis of the unordered data set yielded 7 equally parsimonious trees with a length of 61 steps, CI = 0.590, RI = 0.615 and RC = 0.363. The unordered tree is significantly less structured than the ordered tree with a large basal polytomy within the genus and only recovering 2 clades which were both in common with the ordered consensus tree clade 6 (*M. ocallaghani* + *M. yalanbense* + *M. ennuensis*) and clade 4 (*M. bicolor* + *M. tricresta* + *M. spinosus*).

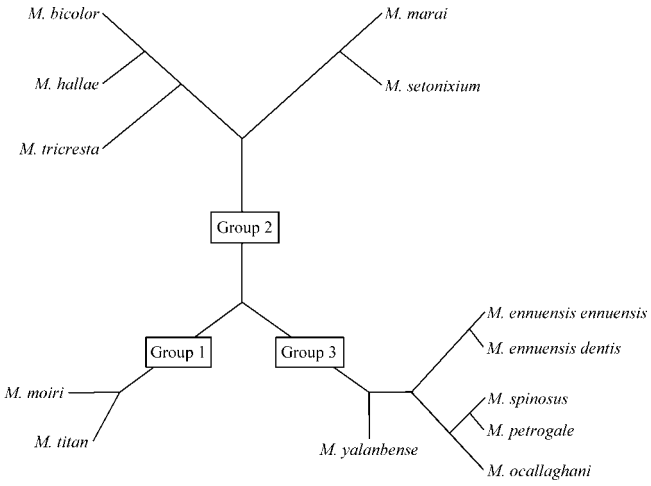

Fig. 2. Phenetic cluster diagram based on morphometric similarity.

Table 2. Character matrix - *Macropodinium* (*M.*) morphology.

	5	10	15	20	
Megavestibulum	00000	00000	00000	00000	0
M. setonixium	01000	12101	12210	10000	1
M. marai	01001	11202	22210	01000	1
M. tricresta	12111	11112	22111	11001	2/3
M. spinosus	12011	12212	22111	01000	2
M. bicolor	02021	12211	22211	01100	2/3
M. hallae	21001	12112	22210	10000	1
M. ocallaghani	22011	11111	11100	00000	0
M. petrogale	01111	12211	12100	00000	0
M. yalanbense	22011	21111	11100	00010	0
M. ennuensis	21111	21111	21200	00000	0
M. moiri	02311	11212	22210	00010	1
M. baldense	210?1	111?2	22210	10000	3
M. titan	02201	13101	21200	00110	0

DISCUSSION

Both methods used to analyse the phylogeny of *Macropodinium* in this study have methodological concerns which affect the conclusions that can be drawn.

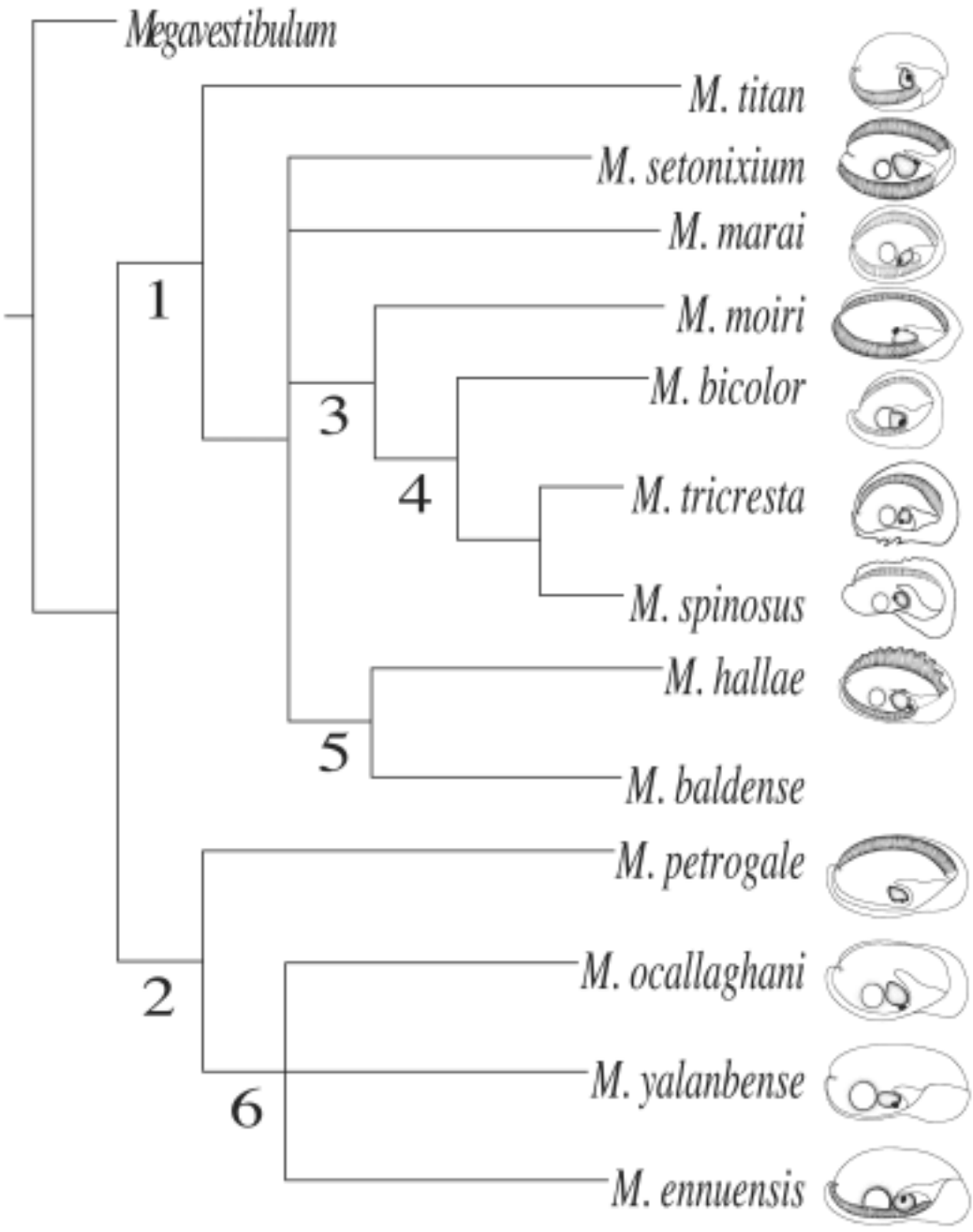

Fig. 3. Cladistic analysis, ordered data set. Numbers indicate clades of interest. Figures are internal right side views of each species except *M. baldense* and are not drawn to scale.

Cluster observation is a phenetic method of analysing morphometric data. Phenetic methods cannot be used to directly infer phylogenies because they group taxa based on total similarities between them. It is insensitive to the differences between shared derived features (synapomorphies) and shared primitive features (sympleisomorphies). For this reason, cladistic methods are more applicable to discrete data sets, and phenetic methods for continuous data sets such as morphometrics. The resultant dendrogram thus cannot be interpreted as

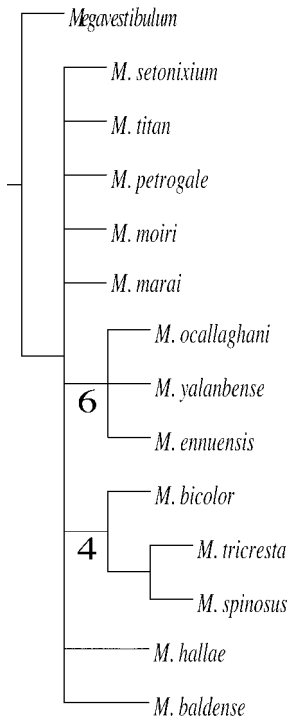

Fig. 4. Cladistic analysis, unordered data set. Numbers indicate clades shared with Fig. 3.

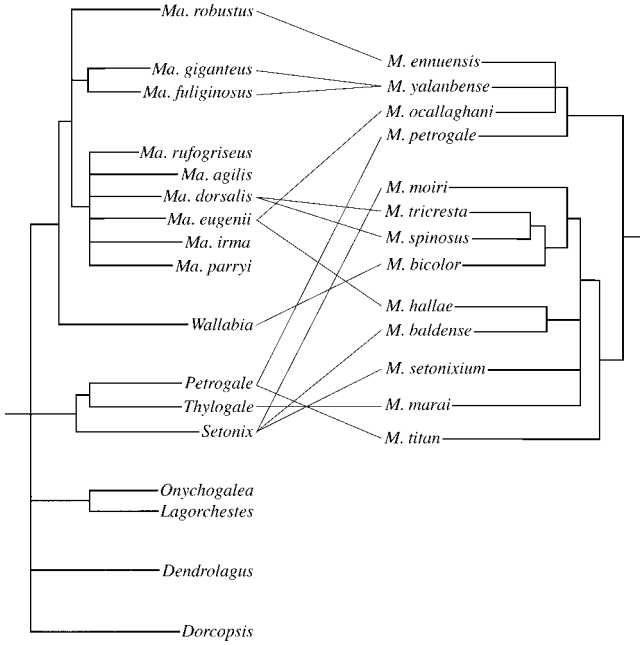

Fig. 5. Co-phylogenetic comparison of *Macropodinium* species (right hand tree) and macropodid hosts (left hand tree). Lines linking taxa represent host/parasite relationships. Macropodid phylogeny redrawn after Flannery (1989).

indicative of phylogenetic relationships between the specimens included (and therefore is omitted). If the dendrogram was read in a directly phylogenetic context, the considerable intermingling between the clusters would suggest that few of the *Macropodinium* species are monophyletic. This is not the case as demonstrated by the discriminant analysis in which the distinctiveness of each of the species was well supported despite a few outlying specimens which resembled other species.

The cluster observation result can be used to produce a network of phenetic similarities (Fig. 2). The groups within this network correspond to broadly similar morphotypes. Group 1 (*M. moiri*, *M. titan*) is composed of the largest species within the genus and may be an artefactual grouping based mostly on the extremely large size of the individual cells in these two species. Group 2 (*M. setonixium*, *M. marai*, *M. tricresta*, *M. hallae*, *M. bicolor*) is composed of species broadly oval in outline with prominent ventral and dorsal bars. Group 3 (*M. yalanbense*, *M. ennuensis*, *M. ocallaghani*, *M. petrogale*, *M. spinosus*) is composed of elongate species, most of which lack or have short ventral and dorsal bars and have large vestibular openings. The lack of solid resolution within the dendrogram precludes making conclusions on branching orders within the groups.

One of the tenets of Darwinian evolution is gradualism; species diverge from each other slowly in response to directional selection within the environment (Patterson 1978). Darwinian gradualism is well demonstrated in comparisons of body form between closely related taxa such as this morphometric analysis of the genus *Macropodinium*. It is common to see both the trends observed in this study. First, closely related species overlap in body form; only 3 of the 12 species did not overlap with any other species in the analysis. Secondly, internal groups can be related to particular correlated characters e.g. the 3 superspecific groups identified above. Morphometric analysis of *Macropodinium* suggests the diversity of body form within the genus may be the result of Darwinian gradualism. The closely related species have been diverging for a shorter period and so still overlap in morphospace (MacLeod 1999). It is also equally possible that the observed pattern is the result of morphological convergences. The weakness of phenetic methods, such as cluster observations, is that they are unable to indicate which effect best explains the data.

Analysis of the two cladistic datasets (ordered *vs.* unordered) differed mostly in the degree of resolution recovered, the unordered dataset resulting in a significantly less structured tree. There are however similarities in the clades which are recovered in each tree, the only clades recovered by the unordered analysis (clades 4 and 6) are also recovered by the ordered analysis. This suggests that these relationships are insensitive to data manipulation but the other proposed relationships described in Fig. 3 need further exploration. The use of *Megavestibulum* as an outgroup is less than ideal due to the absence of many of the characters used in this analysis from this genus. Five of the 21 characters are coded as not applicable or absent in *Megavestibulum* while all *Macropodinium* species possess that character. While coding of this type reduces signal within a data set, as the comparison to outgroup taxa has no resolving power within the ingroup (Strong and Lipscomb 1999), it is superior to either of the 2 alternatives. A hypothetical taxon in which all character states are set to 0 could be used. In this case, character polarity is subjective and the resolving power of comparison to an outgroup absent. The risk of circularity in such an approach is very high. Secondly, species within the ingroup could be used arbitrarily as outgroups. In the absence of some phylogenetic information this is entirely subjective. Small, simple species are often chosen as most evolutionary patterns proceed from small and simple to large and complex, but what if that is not the case? These analyses indicate that both very small and large *Macropodinium* species, *M. petrogale* and *M. titan*, are early branching within the two major clades within the genus. Use of either would significantly alter the result as they are virtually diametric opposites; *M. petrogale* is small and simple whereas *M. titan* is large and complex. Arbitrary choice of outgroup is thus almost certain to bias a phylogenetic analysis. Despite its faults, *Megavestibulum* is the best outgroup available at this time. The most notable feature of the ordered analysis (Fig. 3) is the suggestion of a 2 sub-groups within the genus which broadly correspond to general morphological types and host occurrence. Node 1 is composed of ornamented with marginal spines or crenullations which have small oral apertures and cover the range of cell sizes for the genus. Node 2 is composed of medium sized elongate species, totally lacking in marginal ornamentation which have large oral apertures.

Both the cladistic and cluster observation methods suggested that the genus *Macropodinium* could be divided into species groups on the basis on their morphology. Phenetic clustering suggested groups which corresponded strongly to cell size and shape whereas the cladistic analysis suggested groups which corresponded to cell ornamentation and size. There was considerable overlap in the two studies; Node 1 corresponds to Group 1 plus Group 2 and Node 2 corresponds to Group 3. The only major difference is in the position of *M. spinosus* which is strongly supported as the sister group of *M. tricresta* in both cladistic analyses but falls well within Group 3 (= node 2) in the phenetic analysis. In both analyses, *M. titan* is a highly divergent species and its association with *M. moiri* in the phenetic cluster is loose and probably only due to the large size of both species rather than indicative of a close relationship.

The major difficulty confronting the use of morphological data in the inference of relationships between taxa is the potential existence of convergent evolution. Phenetic methods are particularly susceptible to convergence as the directionality of evolution (as implied in cladistics by the use of outgroups or character ordering) is omitted. The similarity of *M. spinosus* to *M. petrogale* and *M. ocallaghani* in the phenetic clusters is probably the result of convergent evolution; all 3 are small, elongate species. *M. spinosus*, however, shares many characters with *M. tricresta*, cell ornamentation, pellicular windows and possession of a DB but no VB. The cladistic analysis shows that the features shared with *M. tricresta* are derived features whereas those shared with *M. petrogale* and *M. ocallaghani* are either convergent or pleisiomorphic.

Lynn (1978) applied theories about evolutionary patterns within lineages to ciliates and identified 3 main patterns within colpodids: size increase, body allometry and polymerisation. The phylogeny of *Macropodinium* suggests that size increase has not been a universally directional force with the evolution of this genus. Two taxa are consistently basal within the 2 major clades of the genus, *M. petrogale* and *M. titan*, the former species is one of the smallest within the genus whereas *M. titan* is the largest. It is thus possible that the genus has either increased in size through evolutionary time (evolution from forms similar to *M. petrogale*) or decreased in size (evolution from forms similar to *M. titan*). Early branching taxa are not necessarily representative of the primitive forms within a group (Yeates 1995); early branching taxa can be very derived due to long periods of directional divergence. It is probable that *M. titan* is one such early branching, highly

derived taxon. Even within the *Macropodinium* groups, size direction has not been uniform. In Node 3, the earliest diverging species, *M. moiri*, is large whereas in Node 2, the basal taxon, *M. petrogale*, is the smallest in the node and in Node 4 there is no apparent size difference for any of the members. Because the factors favouring size increase in ciliates proposed by Lynn (1978) (predator avoidance, food acquisition and metabolic efficiency) are probably not acting uniformly across all the diverse ecological range of hosts of *Macropodinium* spp., a trend towards size increase is not apparent.

Body allometry is the evolutionary trend of differential growth rates of body parts relative to total body size. It has been demonstrated in ciliates for colpodids (Lynn 1978) and *Paramecium* spp. (Fokin and Chivilev 2000) and it is apparent in *Macropodinium*. Mouth size is proportionately larger in the Node 6 species relative to the remaining *Macropodinium* species. Polymerisation is a net increase in the number or complexity of a particular organelle through evolutionary time. Polymerisation was observed in relation to the number of longitudinal grooves (*M. titan* and the Node 3 species), presence and extent of marginal ornamentations (Node 1 species) and the dorso-ventral groove (*M. tricresta*). A special case of polymerisation is metamerism, the complete duplication of a complex structure and all its constituent parts. The evolution of *M. tricresta* from a species resembling *M. spinosus* is best explained as a case of serial metamerisation. The main difference between the two species is the presence of a second complete dorsal groove, complete with identical marginal ornamentation and somatic ciliary bands associated with each groove. Net increase in the diversity of form within *Macropodinium* can be related to all of the factors identified by Lynn (1978), however, none of these factors provides a strong unidirectional trend. The absence of such a trend is probably due to environmental heterogeneity (the species are spread across 9 host species with the full range of herbivorous diets and the entire Australian continent) which fails to produce unidirectional selective pressures.

A sophisticated array of computer programs and algorithms have been proposed to examine parasite phylogenies in relation to host phylogenies including Brooks parsimony (Brooks 1981), Treemap (Page 1994) and generalised parsimony methods (Ronquist 1995). All of these methods require accurate, resolved phylogenies of both parasite and host, prerequisites which are lacking for both *Macropodinium* and the macropodids. For both host and ciliate, the phylogenetic trees have areas of uncertainty in the form of polytomies. For this reason, two phylogenies have simply been mapped together and linkages between the two indicated rather than use inappropriate computer models (Fig. 5). We compared the most resolved and robust phylogeny (the ordered cladistic analysis) against the most widely accepted phylogeny of the macropodids (Flannery 1989). There is poor concordance between the two phylogenies, however, patterns of host association are apparent. Node 1 species are all associated with wallabies, members of the genera *Setonix*, *Thylogale*, *Wallabia* and *Macropus* (*Notamacropus*). The wallabies are all small bodied (< 10 kg), mostly mixed foragers and rarely feed in mobs. Phylogenetically, the wallabies form a paraphyletic assemblage, are considered basal within the macropodids and have an early appearance in the fossil record (Archer 1984). Node 6 species are mostly associated with the kangaroos and wallaroos, *Macropus* (*Macropus*) and *Macropus* (*Osphranter*). The hosts are large bodied macropodids (>15 kg), selective grazers and often live in mobs. The exception is *M. ocallaghani* which is an obvious host switch probably facilitated by the usually close habitat ranges of *Ma. eugenii* (the tammar wallaby) and *Ma. fuliginosus fuliginosus* (Kangaroo Is. Grey Kangaroo) on Kangaroo Island, South Australia. The phylogenetic relationships of the two subgenera are unresolved as is their origin from one of the wallaby taxa (Kirsch *et al*. 1997, Burk *et al*. 1998). Interestingly the most basal branch of both clades 1 and 2, *M. titan* and *M. petrogale* respectively, are associated with rock-wallabies of the genus *Thylogale* a group which has been proposed as one of the more primitive macropodid groups (Flannery 1989) The associations of the ciliate groups with host groups are broadly consistent with phylogenetic branching order only because of the lack of resolution within the macropodid phylogeny. Additional resolution in both trees will greatly enhance our understanding of the phyletic associations of *Macropodinium* and its hosts.

A second possibility is that the association of ciliates with hosts is not the result of phylogenetic co-descent, but rather due to resource tracking. The diets of the wallaby genera are broadly similar; they are all classified as intermediate feeders (Langer 1988) and selectively consume browse and fresh graze. In contrast, both the kangaroos and wallaroos are specialist grazers which exclusively consume grasses both fresh and moderately

desiccated. The association of the ciliates with one host group or the other may be in response to these dietary differences. The host switching event evident in Node 6, *M. ocallaghani*, suggests that resource tracking, if any, is not absolute as the putative source host, *Ma. fuliginosus* is a grazer and the new host, *Ma. eugenii* is a browser. The observed pattern of host association is probably a product of 3 factors: deficiencies in the phylogenies of both host and ciliate; phylogentic codescent and ecological based host tracking.

The evolution of parasite species is currently considered to result from a balance between 4 factors: host switching; failure to colonise descendent host species; sympatric speciation (in relation to host species not necessarily habitat); and co-speciation (Paterson and Gray 1997). We have good evidence that all 4 factors contributed to the evolution of *Macropodinium* species. Host switching between macropodids and their ciliates definitely occurred with *Cycloposthium edentatum* (Cameron *et al*. 2000a) and is suggested by the 3 macropodiniid species associated with the quokka. The detection of the equid associated ciliate, *C. edentatum*, in the black-striped wallaby suggests two extreme positions on the possibility of ciliate host switching in macropodids. First, host switching may be quite easy as it was accomplished despite radical differences between hosts, transmission strategy and gut structures. Alternatively, host switching between similar hosts may be quite difficult due to competition with resident ciliates whereas host switching between diverse hosts (e.g. horse → wallaby) may have been favoured in this instance by unique factors (e.g. vacant niche, lack of competitors, specialised diet). In all probability, reality is somewhere between these two poles of "easy" and "hard" host switching.

Amongst endemic ciliate species, the 3 *Macropodinium* species from quokkas (*M. moiri*, *M. baldense* and *M. setonixium*) are widely divergent and none formed bigeminate pairs suggesting that at least 2 colonised this host as the result of host switching. *M. setonixium* was revealed as one of the earliest branching species and if the quokka is as primitive a macropodid as is presently believed (Archer 1984, Flannery 1989, Kirsch *et al*. 1997) then *M. setonixium* is likely to be the original species and *M. baldense* and *M. moiri* are the products of subsequent switches. The bigeminate pair *M. baldense* and *M. hallae* (Fig. 4 Node 5) suggests geographical host switching. Both hosts, the quokka and tammar wallaby respectively, occur on the offshore islands of south-western Australia and were probably sympatric on areas of the continental shelf which were exposed during the Pleistocene ice ages when sea levels dropped (Frakes *et al*. 1987). Another case of geographical host switching appears to be the close phyletic association of *M. ocallaghani* and *M. yalanbense*. Their hosts, the tammar wallaby and western-grey kangaroo, occur sympatrically only on Kangaroo Island off South Australia. Examination of tammar wallabies from mainland sites, and additional offshore sites would aid our understanding of these host switching events including which are source, and which are sink, host species.

There are also examples of host speciations which have failed to result in ciliate speciation. Most extreme is the red-necked wallaby, *M. rufogriseus*, which lacks ciliates altogether in the wild, although it is a suitable host of ciliates in a captive situation (Cameron and O'Donoghue 2003b). Comparison with nematodes reveals that it has a depauperate fauna relative to other *Notamacropus* wallabies entirely lacking in *Cloacina* spp., the most speciose strongyloid genus (Spratt *et al.* 1991). It does seem that there is something "odd" in the development of this macropodid. The biogeographic history of the red-necked wallaby (see Littlejohn *et al.* 1993) provides no clue. Many other macropodid species lack a *Macropodinium* species symbiont. These absences do not conform to a phyletic pattern and thus it appears that failure to colonise descendent species or "missing the boat" (Paterson *et al*. 1999) was a relatively common event during the evolution of the contemporary Australian trichostome fauna.

A range of speciation responses have occurred in response to host vicariation events. *M. yalanbese* has apparently failed to speciate in response to the speciation of its hosts *Ma. giganteus* and *Ma. fuliginosus*, a bigeminate pair which diverged due to Pleistocene ice age separation of populations to the south eastern and south western fringes of the Australian continent respectively (Flannery 1989). There were no discriminating differences between populations of *M. yalanbense* recovered from either host or from the 2 subspecies of *Ma. fuliginosus*. A similar pattern was found with the other trichostomes found in these hosts, *Amylovorax dehorityi* and *Bitricha oblata* (Cameron *et al*. 2000b). In contrast, *M. ennuensis* associated with the wallaroo, *Ma. robustus*, appears to have diverged more despite less conspicuous host divergence. Two distinct forms of *M. ennuensis* (forma *ennuensis* and forma *dentis*)

were found in the western and eastern subspecies (*Ma. r. erubescens* and *Ma. r. robustus* respectively). Divergence between these two forms of *M. ennuensis* is modest in comparison to the differences found between *Macropodinium* species (Cameron *et al*. 2001, 2002) however they were found to be completely distinct by discriminant analysis (Table 1). *Ma. robustus* is more xerically adapted than either *Ma. giganteus* or *Ma. fuliginosus* (Strahan 1995) and so was probably less effected by ice age expansions of Australia's central deserts. *M. ennuensis* appears to match the divergence of it's host in that there is modest differentiation but apparent failure to speciate.

Speciation in the absence of host speciation (sympatric parasite speciation) appears to have occurred in the case of *M. tricresta* and *M. spinosus*. Combes and Théron (2000) outlined a process by which sympatric speciation could occur within a host species by aggregation within different organs to ensure breeding success. This would result in strengthening organ specificity and eventual speciation. We have no evidence of habitat segregation for any species of trichostome ciliate. Furthermore, Combes and Théron's (2000) scheme is most applicable to parasites which use short range pheromones to attract mates. There is some evidence that pheromones are used as mate recognition factors in ciliate conjugation (Dini and Luporini 1985), however, in the absence of compartmentalisation of the macropodid stomach such aggregation based speciation appears unlikely. A second method compatible with sympatric speciation is the so-called 'instant chromosomal evolution' (Eldridge and Close 1993). Under this process, chromosome duplications or fusions can instantly create differences in ploidy such that the homologous pairs cannot join and fertilisation fails; sexual isolation is thus instantaneous. However, conjugation does not involve the joining of homologous chromosomal pairs thus changes in chromosome number should have no effect on conjugation success. Instead changes to the genes responsible for conjugation compatibility could result in instantaneous speciation within ciliates; asexual reproduction would increase the population size necessary to sustain the new species.

The accepted method of parasite speciation is, as with most animals, allopatric speciation; small populations of hosts become isolated from their conspecifics and because of their shorter generation times the parasites have greater capacity to speciate than their hosts. Subsequent shifts which remove the isolation reincorporate a new parasite species into the larger host population (Inglis 1971). Parasite speciation can thus appear to be sympatric when the actual process was allopatric. The differences in generation time should result in larger numbers of parasite species than host species; this method was suggested by Beveridge and Spratt (1996) as responsible for a large proportion of nematode speciations in macropodid stomachs and the excess of nematode species relative to host species. The biogeographical history of *Ma. dorsalis* has not been studied extensively but its present restriction to thick hill scrub (Strahan 1995) suggests that it would have been sensitive to habitat changes during the Pleistocene. Ice age desertification would most likely have split the habitat range into isolated montane scrublands along the spine of the Great Dividing Range separated by xeric grasslands. There would have been ample opportunities for a small population to become isolated and speciation of ciliates to result. *M. tricresta* is probably derived from an ancestor morphologically similar to *M. spinosus* by metamerism of the dorsal element of the DVG. The remaining changes are ones of scale; the cells are of similar size but *M. tricresta* is stouter, and the marginal ornamentations are similar but thicker in *M. tricresta*. A few basic changes in development could account for major morphological difference, the extracalary groove, and the remaining differences, size and spines, could be the result of random genetic drift. If this were true, the two putative species may still be conjugation intercompatible but have radically different forms.

The three examples presented show the range of possible outcomes of host isolation events on parasite evolution. Host vicariance may result in host speciation in the seeming absence of parasite speciation or even morphological divergence (e.g. *Ma. giganteus*/ *Ma. fuliginosus* and *M. yalanbense*). Secondly, host vicariance may result in host division into subspecific groups and increased morphological diversity within the parasite possibly to the extent of semicryptic speciation (e.g. *Ma. robustus* subspecies and the *M. ennuensis* forms). Finally, host vicariance may fail to result in host speciation but still result in parasite speciation (e.g. *Ma. dorsalis* and *M. tricresta*/ *M. spinosus*). This diversity of parasite response to host vicariance suggests that isolation is not a generalised force which will always favour parasite speciation at a faster rate than host speciation. The characteristics of the isolation must be taken into consideration. As has been found for animal and plant taxa "bottleneck effects" can significantly

increase the chances of speciation (Page and Holmes 1998); *M. tricresta*/ *M. spinosus* may be the result of just such an isolation of a small number of hosts. In contrast, continental scale vicariation events, such as occurred for *Ma. giganteus*/*Ma. fuliginosus* in the last ice age, may capture all of the parasite species' genetic diversity in both host populations and buffer against genetic drift. If isolation events do not result in net changes in habitat or diet of the host there will not be differential selective pressure acting on the two parasite populations. Speciation is thus limited to what can be achieved by random genetic drift alone. The period of isolation will determine the extent of genetic drift and thus speciation. As parasites and hosts respond to different components of the environment, different selective pressures can act independently on the two and generation time cannot be taken as directly indicative of speciation chance.

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Appendix 1. Characters and their transformation states used to construct the character matrix used for morphological cladistics in Table 2. Character state transformations are optimized on the tree presented in Figure 4.

1. Body shape	0	Oval
	1	Wedge
	2	Reniform
2. Body symmetry	0	Not applicable
	1	Equal
	2	Unequal
3. Cell curvature	0	Absent
	1	Left anterior
	2	Left concave
	3	Left posterior ventral
4. Anterior window shape	0	Absent
	1	Single and strap-like
	2	Bilobed and triangular
5. Mouth orientation	0	Anterior
	1	Anterio-ventral
6. Mouth size	0	Not applicable
	1	Limited
	2	Entire
7. Longitudinal groove numbers	0	Absent
	1	Equal numbers right and left
	2	More left than right
	3	More right than left
8. Cytoproct shape	0	Hole
	1	Slot
	2	Cup
9. Preoral cilia	0	Absent
	1	Present
10. DVG depth dorsal	0	Absent
	1	Shallow
	2	Deep
11. DVG depth ventral	0	Absent
	1	Shallow
	2	Deep
12. Dorsal bars	0	Not applicable
	1	Absent
	2	Present
13. Ventral bars	0	Not applicable
	1	Absent
	2	Present
14. Ornamentations right dorsal	0	Absent
	1	Present
15. Ornamentations left dorsal	0	Absent
	1	Present
16. Ornamentations right ventral	0	Absent
	1	Present
17. Ornamentations left ventral	0	Absent
	1	Present
18. Tail bulge	0	Absent
	1	Present
19. Posterior spine	0	Absent
	1	Present
20. Intercalary row	0	Absent
	1	Present
21. Ornamentation type	0	None
	1	Crenulations
	2	Spines
	3	Teeth