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A DNA-sequence based phylogeny for triculine snails (Gastropoda: Pomatiopsidae: Triculinae), intermediate hosts for *Schistosoma* (Trematoda: Digenea): phylogeography and the origin of *Neotricula*

S. W. Attwood[1]*, E. S. Upatham[2], Y.-P. Zhang[3,4], Z.-Q. Yang[3] and V. R. Southgate[1]

[1] Wolfson Wellcome Biomedical Laboratories, Department of Zoology, The Natural History Museum, London SW7 5BD, U.K.
[2] Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[3] Key Laboratory of Cellular & Molecular Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, P.R. China
[4] Laboratory for Conservation and Utilization of Bio-resource, Yunnan University, Kunming 650091, P.R. China

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Abstract

Partial (DNA) sequences were examined for one nuclear (28S rRNA gene) and one mitochondrial (16S rRNA) locus for nine species of pomatiopsid snail (Gastropoda: Rissooidea: Pomatiopsidae) from south-east Asia and south-west China. Fresh field samples were collected for the following taxa: *Delavaya dianchiensis* (Triculinae: Triculini) from Dianchi lake, Yunnan Province, China; *Neotricula aperta* (Triculinae: Pachydrobiini) from north-east Thailand; *Neotricula burchi* from northern Thailand; *Oncomelania hupensis robertsoni* (Pomatiopsinae: Pomatiopsini) from south-west China; *Tricula bambooensis* (Triculinae: Triculini) from Dianchi lake; *Tricula bollingi* from northern Thailand; *Tricula hortensis* from Sichuan Province, China; *Tricula ludongbini* from Dianchi lake; and *Tricula xiaolongmenensis* from Dianchi lake. This work represents the first published DNA-sequence data for the Dianchi lake taxa and the first 28S sequence data for all nine taxa. All of these taxa with the exception of *N. burchi* and the Dianchi taxa transmit *Schistosoma* in nature; *N. aperta* and *O. h. robertsoni* transmit *Schistosoma* to humans. The data were used to estimate phylogenies using the maximum likelihood method, a Bayesian method, and the maximum parsimony method. The paper aims to examine the relationships between *N. aperta, N. burchi* and *T. bollingi* and the relationship between the Chinese taxa and the south-east Asian taxa; these relationships being important in evaluating certain historical biogeographical hypotheses. Good congruence was found between the phylogenies estimated by the three methods for both the 16S and 28S loci. However, poor congruence was found between the phylogenies based on the 16S and 28S data when the maximum likelihood and Bayesian methods were used. The lack of congruence is explained as a consequence of a rapid, recent, endemic radiation of the Yunnanese *Tricula* spp.; this hypothesis is consistent with current historical biogeographical models for the Triculinae. The paper puts forward dispersal hypotheses, based on palaeogeographical changes, as possible explanations for the phylogenetic relationships estimated here and for the current biogeography of these taxa.

Key words: phylogeography, mitochondrial 16S, 28S large subunit (LSU), rRNA genes, Asian *Schistosoma*

INTRODUCTION

The Pomatiopsidae Stimpson, 1865 are conservative rissooidean snails, eight species of which are known to act as intermediate host for species of *Schistosoma* Weinland, 1858 (Trematoda: Digenea). The Pomatiopsidae comprises two subfamilies, the Pomatiopsinae Stimpson, 1865 and the Triculinae Annandale, 1924. The Pomatiopsinae include *Oncomelania hupensis* sub-spp. Gredler, 1881, the intermediate hosts of *Schistosoma japonicum* Katsurada, 1904 a parasite of humans in central and southern China (and other areas, see Rollinson & Southgate, 1987). The Triculinae include *Neotricula aperta* (Temcharoen, 1971), the intermediate host of *Schistosoma mekongi* Voge, Buckner & Bruce, 1978, a parasite of humans along the Mekong river of Laos and Cambodia; together the total number of people already infected by these two parasites is estimated to be over 50 million (see Attwood, 2001). In addition to their obvious public health importance, triculine snails (Triculinae) are important in studies of the historical biogeography and evolutionary radiation of *Schistosoma* in Asia. The small size of triculine snails (often < 2 mm in length) leads to morphological simplicity and technical difficulties in the laboratory. Consequently, in spite of the importance of this group taxonomic questions remain at all systematic levels. The problem of small size, together with a high prevalence of convergent evolution and a relatively high degree of intraspecific variation among anatomical

*All correspondence to: S. W. Attwood.
E-mail: s.attwood@nhatt.ac.uk

characters, suggests that DNA-sequence variation will be useful in resolving ambiguous anatomical relationships. The present work aimed to use DNA-sequence based phylogenies to address questions of historical biogeography surrounding those triculine lineages bearing taxa of medical importance. The two main (related) problems to be addressed were the occurrence of two species of *Neotricula* in south-east Asia, far from the main radiation of this genus in Hunan, China; and, the possibility that the Pleistocene phylogeographies of *Neotricula* and *Tricula* in south-east Asia were independent of one another. Solving such problems will also shed light on the phylogeography of *Schistosoma* by revealing potential sources and routes of colonization during the Plio-Pleistocene radiation of this genus in Asia.

The Pomatiopsidae appear to have arisen on the Indian Plate and colonized south-east Asia, from north-east India, during the mid-Miocene (Davis, 1979). Today the more generalized (i.e. showing generally plesiomorphic morphological character states) Pomatiopsidae inhabit streams and minor rivers draining hillsides and other highland areas. This ecological habit may reflect the ancestral habitat during the early radiation of this clade in the highland areas of northern India and Burma. The habitat requirements of these taxa, which appear to preserve past biogeographical patterns, have led to their use in historical biogeographical studies of not only the Rissooidea but also of *Schistosoma* (see Davis, 1979, 1992; Attwood, 2001; Attwood & Johnston, 2001; Attwood *et al*., 2002*b*). The extant Triculinae are found along a tract running from northern India into southern China and south-east Asia. In addition to *N. aperta*, several other Triculinae are known to act as intermediate host for *Schistosoma*. *Robertsiella kaporensis* Davis & Greer, 1980 transmits *S. malayensis* Greer, Ow-Yang & Yong, 1988 in peninsular Malaysia, this is a parasite of rodents but also infects the aboriginal peoples of the region. *Tricula bollingi* Davis, 1968 transmits *S. ovuncatum* Attwood, 2002 (and possibly also a sympatric strain of *S. sinensium*) to rodents in northern Thailand (Attwood *et al*., 2002*a*). *Tricula hortensis* Attwood & Brown, 2003 from Sichuan, China, transmits another rodent *Schistosoma, S. sinensium* Bao, 1958 on the Yangtze river platform (Bao, 1958; Attwood *et al*., 2002*a*, 2003). Clearly, triculine snails are important in the evolution, radiation and transmission of south-east Asian *Schistosoma*. The relatively low dispersal capabilities and the habitat requirements of these snails suggest that something may be learnt regarding the radiation of *Schistosoma* from a study of the phylogeography of the Triculinae. Attwood *et al*. (2002*b*) considered palaeogeography and the historical biogeographies of both the Asian *Schistosoma* and their intermediate hosts in arriving at a phylogeographical hypothesis for this group. The present work is part of an ongoing program aimed at examining the reciprocal illumination between snail and schistosome phylogenies, following on from the ideas of Wright (1973) and Davis (1979).

The first studies of DNA-sequence variation among the Pomatiopsidae were restricted to the *O. hupensis* complex and involved partial sequences from the mitochondrial (mt) cytochrome *c* oxidase subunit 1 (COI) and cytochrome *b* genes (Hope & McManus, 1994; Spolsky, Davis & Yi, 1996; Rosenberg *et al*., 1997). Partial sequences are now available for the mt16S, COI and nuclear 18S rRNA genes of *Oncomelania hupensis robertsoni* Bartsch, 1946 and *Erhaia jianouensis* (Liu & Zhang, 1979), both Pomatiopsinae, and for the Triculinae, *Lacunopsis* sp. Deshayes, 1876, *Tricula* sp. Benson, 1843 and *Gammatricula* spp. (Davis & Liu, 1990) (see Davis *et al*., 1998; Wilke *et al*., 2000, 2001). More recently, Shi *et al*. (2002) used partial COI sequences to investigate population structure and interstrain compatibility, with *S. japonicum*, for *O. h. hupensis*. However, in spite of the progress made for the Pomatiopsinae, few DNA-sequence data are available for well characterized species of Triculinae (most taxa studied are only identified to the genus level). Among the Triculinae, partial mt16S, COI and nuclear 18S sequences are available for two named species of *Gammatricula* (Davis & Liu, 1990) (see Davis *et al*., 1998; Wilke *et al*., 2000, 2001). In addition, Attwood & Johnston (2001) provided partial COI sequences for *N. aperta* and *T. bollingi*. Nevertheless, sequence data are still lacking for the majority of *Tricula* and *Neotricula* species, especially those from China. Of the nine pomatiopsid taxa studied here, six had not previously been included in DNA-sequence studies, and none had previously been sampled at the nuclear 28S (large subunit or LSU) rRNA gene. Such new data were expected to help elucidate further the phylogeography of the Triculinae, and also of Asian *Schistosoma*, by contributing to our existing data set.

MATERIALS AND METHODS

Sampling

The sampling area comprised 2 countries and 6 different collecting localities (Fig. 1). Table 1 gives details of sampling sites and dates of collection. The snails were identified on the basis of general form, conchology, radular characters, ecological habit and gross dissection of pallial and reproductive structures. All snails were collected by the first author and were readily identified. *Tricula hortensis* is a recently described species and was subject to more detailed anatomical study by Attwood *et al*. (2003). Fresh samples were fixed (in 100% ethanol) directly in the field. Samples for *N. aperta*, *N. burchi* Davis, Subba Rao & Hoagland, 1986, *T. bollingi* and *T. hortensis* were also taken in 10% neutral formalin to aid identification. It was considered preferable to use field fixed samples to avoid problems of selection in transit and government controls on live imports.

DNA amplification and sequencing

The snails were gently crushed and the body separated from the shell. The gut and digestive gland were removed and DNA extracted from the remainder by standard

Table 1. Snail collecting sites, dates and species for taxa sampled during the present study. The area "Dianchi lake" refers to the associated drainage system rather than to the lake itself. A total of eight specimens was examined for each taxon

Taxon	Collecting site	Date	Co-ordinates
Delavaya dianchiensis	Dianchi lake, Yunnan Province, China	13/04/01	25°04′30″N; 102°42′15″E
Neotricula aperta	Khemmarat, Mekong river, Laos	09/05/00	14°6′30″N; 105°51′45″E
Neotricula burchi	Fang District, Chiang-Mai, Thailand	23/05/01	19°23′30″N; 98°56′00″E
Oncomelania hupensis robertsoni	Mianzhu, Sichuan, PR China	14/04/00	30°04′00″N; 104°08′30″E
Tricula bambooensis	Dianchi lake, Yunnan Province, China	13/04/01	25°04′30″N; 102°42′15″E
Tricula bollingi	Fang District, Chiang-Mai, Thailand	22/03/00	19°38′30″N; 99°05′20″E
Tricula hortensis	Han-Wang, Sichuan, China	12/04/00	30°04′15″N; 104°08′15″E
Tricula ludongbini	Dianchi lake, Yunnan Province, China	13/04/01	25°04′30″N; 102°42′15″E
Tricula xiaolongmenensis	Dianchi lake, Yunnan Province, China	13/04/01	25°04′30″N; 102°42′15″E

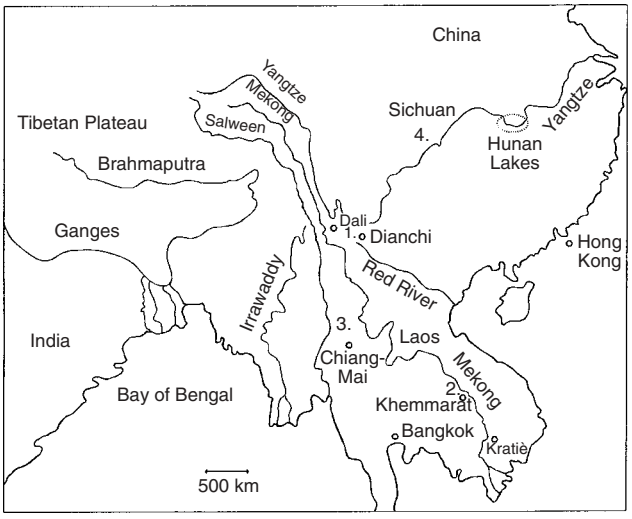

Fig. 1. The main rivers draining eastern Asia and the localities at which samples were taken during the present study. Collecting site 1, Dianchi; 2, Khemmarat; 3, Fang; 4, Mianzhu and Han-Wang (the two sites were only around 15 km apart). Scale approximate.

methods (Winnepenninck, Backeljau & De Wachter, 1993). Selected DNA sequences were amplified by polymerase chain reaction (PCR). Part of the mt16S rRNA gene was amplified using the primers of Palumbi *et al*. (1991); this primer combination amplified a 515 bp region of the 16S gene. Amplification of a section of the 28S rRNA gene was achieved using a specifically designed forward primer (5′-aactcagatcggacgagattac-3′); this was based on an alignment of sequences AY014159 and AY014163, deposited in GenBank by Wade, Mordan & Clarke (2001), for *Potamopyrgus antipodarum* Gray, 1843 (Rissooidea: Hydrobiidae) and *Pomacea* sp. Perry, 1810 (Viviparacea: Ampullariidae), respectively. The forward primer was used in combination with primer LSU4 of Wade & Mordan (2000). The primer combination amplified a 750 bp region of the 28S rRNA gene beginning (at the 5′ end) approximately 65 bp downstream of the 5′ end of the gene (according to the *P. antipodarum* sequence of Wade *et al*., 2001).

The mt16S gene was chosen because it has been used successfully in earlier studies aimed at assessing family and subfamily level relationships among gastropods (e.g. Remigio & Blair, 1997) and among Pomatiopsidae (Wilke *et al*., 2000). The mtDNA loci with their maternal pattern of inheritance, and therefore smaller effective population size, may be expected to evolve more rapidly than nuclear loci and be better suited to reconstructing more recent phylogenetic events. It has been suggested (see Attwood, 2001) that the entire Triculine radiation has taken place within the Pliocene, around 5–1 Ma (million years ago). The 28S rRNA gene was selected because it had proven useful in earlier studies (see Wade & Mordan, 2000) for examining evolutionary relationships at several taxonomic levels within the Gastropoda. It was expected that the nuclear (28S) gene would prove less variable than the mt16S locus; thus maximizing the chance of arriving at a well resolved phylogeny by one or other of the loci.

Total genomic DNA was used as a template for PCR amplification on a Progene thermal cycler (MWG), using standard PCR conditions as described in Clackson, Güssow & Jones (1991). Unincorporated primers and nucleotides were removed from PCR products using the QIAQuick PCR purification kit (QIAGEN). Sequences were determined directly from the PCR templates by thermal-cycle-sequencing using Big Dye fluorescent dye terminators and an ABI 377 automated sequencer (Perkin-Elmer), using procedures recommended by the manufacturers. DNA extracts were not pooled and one DNA sequence represented one snail. Sequences were assembled and aligned using Chromas (McCarthy, 1996) and ClustalX (Thompson, Higgins & Gibson, 1994). DNA sequences for both the forward and reverse strands were aligned and compared to confirm accuracy. Negative controls were run alongside all PCRs, and results from different DNA extractions, PCRs and sequencing reactions (performed at least one week apart) for the same OTUs (operational taxonomic units) were checked for agreement.

Phylogeny reconstruction

Data (as consensus sequences) for the OTUs sampled were grouped together into sets of aligned sequences of equal

length, so that all taxa were represented in each set. Sample sizes (i.e. numbers of snails sampled), upon which the consensus sequences for each OTU were based ranged from 4 to 10 (mode 4). No intrataxon variation was found in any OTU sample. An outgroup sequence, taken from the Genbank (AY314009, T. Wilke, unpublished, 2003), for *P. antipodarum* (Gastropoda: Rissooidea: Hydrobiidae), was added to the 16S data set. An outgroup sequence (GenBank sequence AY014159, Wade *et al*., 2001) for *P. antipodarum*, was also added to the 28S data set. No orthologous 28S sequence was available for any pomatiopsid or hydrobiid taxon and so a member of another rissooidean taxon was chosen.

For each data set a χ^2-test for intertaxon variation in nucleotide frequency was performed using PAUP* v. 4.0b10 (Swofford, 2002). The data were tested for (substitution) saturation using plots of the numbers of transitions and transversions against the genetic distances estimated under the appropriate nucleotide substitution model (following DeSalle *et al*., 1987). The indications of these plots were further evaluated using the entropy-based test of Xia *et al*. (2002) as found in the DAMBE software package of Xia (1999), which provides a statistical test for saturation. The test was chosen because it was thought more likely to detect saturation in the present data, where several closely related species are compared, than say randomization or permutation tests, or the test of Lyons-Weiler, Hoelzer & Tausch (1996).

Phylogenetic analysis was undertaken using maximum likelihood (ML) and Bayesian (MB) methods. The present data showed significant variation in the rate of nucleotide substitution both among sites and among lineages, together with considerable bias among the 6 different types of nucleotide substitution (see Results). In such cases the ML method, making possible a fully optimized model of substitution, is considered more robust than other phylogenetic methods (Nei, 1991). A Bayesian method was also used because the algorithm is more rapid than a heuristic search (with estimation of nodal support), whilst still allowing the specification of a full range of model parameters. In addition, the credibility or nodal support of each clade (and the overall tree) may be readily assessed using Bayesian methods. A maximum parsimony (MP) approach was also used in order to help choose, if necessary, between conflicting phylogenies, resulting from the ML and MB methods. The MP method was implemented using the DAMBE software of Xia (1999).

A suitable model of nucleotide substitution was selected using hierarchical testing of alternative models by mixed χ^2-test, as implemented by Modeltest v. 3.06 (Posada & Crandall, 1998). In this way a general time reversible model (GTR) of nucleotide substitution, accommodating among site rate heterogeneity with $\Gamma = 0.2512$ (the shape of the gamma (G) distribution), was chosen for the 16S data (i.e. GTR + G). The model chosen for the 28S data (again using Modeltest) was GTR + I + G, with a proportion (0.7197) of the sites treated as invariant (I) and $\Gamma = 0.8351$. Under both models a full matrix of rates for the 6 classes of nucleotide substitution was estimated, as were the 4 different nucleotide frequencies. The ML analyses were performed (under the respective substitution model) using PAUP* with random addition sequence (10 replicates) and tree-bisection-reconnection branch swapping options in effect. Nodal support was assessed by bootstrap resampling (5,000 replicates). The Bayesian (MB) search strategy was implemented using the program MrBayes v. 2.01 (Huelsenbeck & Ronquist, 2000) with substitution model parameters again set to the GTR + G and GTR + I + G models (for the 16S and 28S data, respectively) and 4 Markov chains run simultaneously. Log-likelihood scores for the MB trees were plotted against generation number (over 20 000 generations) and the generation number where the log-likelihoods first reached a plateau was noted. Only trees saved after this generation number (i.e. after the point of assumed stationarity) were used to produce the consensus tree. Posterior probabilities were then estimated over 500 000 generations beyond the assumed point of stationarity. The clade probability values generated by MrBayes were used as an indication of nodal support.

LRTs (likelihood ratio tests) were performed in order to compare the maximum likelihoods obtained in the ML analyses with and without a molecular clock enforced; the resulting probability is the probability that one would be incorrect in rejecting the null hypothesis that there is no difference in evolutionary rate among taxa in the data set (Felsenstein, 1988). In order to determine if differences between alternative topologies of trees produced using the maximum likelihood method were significant a Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999; see also Goldman, Anderson & Rodrigo, 2000) was used. The test was performed using PAUP*, with 1000 bootstrap replicates. Statistics relating to polymorphism (see Table 2) were computed using the DNAsp program of Rozas & Rozas (1999). All genetic distances quoted are those calculated under the appropriate substitution model using PAUP*.

Table 2. Statistics relating to each locus used in the phylogenetic analyses. Length of amplicon, discounting primer sequences (L); number of OTUs in the full ingroup taxon set (N); number of haplotypes found in ingroup (Hap); total number of sites excluding those with alignment gaps (T); number of polymorphic sites, with parsimony informative sites in parentheses (PS); significance of the χ^2-test for heterogeneity in the frequencies of the different nucleotides (P_N); nucleotide diversity π, with the Jukes & Cantor (1969) correction ± S.D.

	16S	28S
L (bp)	515	750
N	9	9
Hap	9	8
T	496	732
PS	123 (43)	83 (31)
P_N	0.999	1.000
π_{JC}	0.085 ± 0.018	0.038 ± 0.009

RESULTS

Sequence analysis

The data were submitted to the GenBank under accession numbers AF531542, 531545, 531551, 531554, 531556 and AY207030-AY207042. Table 2 provides basic statistics for the two loci examined. The mt16S data set appeared most informative; 24.8% of the sites were polymorphic and of these 35.0% were parsimony informative (i.e. showed a minimum of two different characters with each present in more than one taxon), the remaining 65.0% were singletons. At the 28S locus only 11.3% of sites were polymorphic and 37.3% of these were parsimony informative. The distribution of polymorphic sites across the 16S sequences was, on casual inspection, quite uniform (i.e. there were no long stretches of conserved sequence) but there was one "hot spot" at 230–370 bp within which there was a concentration of polymorphic sites. In contrast the 28S data showed a conserved region at 239–368 bp and a "hot spot" at 406–724 bp (near the 3′ end of the sequence), with variation quite evenly spaced elsewhere; these observations support the choice of ML model for each locus. Ingroup genetic distances across the 16S data set ranged from 0.0059 (*Tricula ludongbini* Davis & Guo, 1986 to *Tricula bambooensis* Davis & Zheng, 1986) to 0.0953 (*O. h. robertsoni* to *N. aperta*). *T. bambooensis*, *T. hortensis*, *T. ludongbini*, *Tricula xiaolongmenensis* Davis & Guo, 1986 and *Delavaya dianchiensis* Davis & Guo, 1986 showed the smallest genetic distances (both amongst themselves and with the remaining taxa), ranging from 0.0059 (*T. bambooensis* to *T. ludongbini*) to 0.0455 (*T. hortensis* to *T. xiaolongmenensis*). The largest distances were observed when *O. h. robertsoni*, the *Neotricula* spp., *T. bollingi* or *T. hortensis*, were compared with the remaining taxa. In contrast, the smallest genetic distances for the 28S data were seen in comparisons involving *T. bambooensis* and *T. bollingi*, whilst the larger distances were for *O. h. robertsoni*, *N. burchi* and *N. aperta* when these taxa were compared with the remainder of the ingroup. Distances for the 28S data set ranged from 0.0000 (*T. bambooensis* to *T. ludongbini*) to 0.0653 (*O. h. robertsoni* to *N. burchi*). In both data sets *N. burchi* appeared more divergent from the other ingroup taxa than did *N. aperta* although the distances between the two taxa were among the smallest. As would be expected when comparing a nuclear locus with a mt locus, overall variation was greater within the 16S data set (see π_{JC}, Table 2).

T. bambooensis and *T. ludongbini* were homogenetic in the 28S data set but these taxa were distinguished by the 16S sequence data. The collecting sites for *T. bambooensis* and *T. ludongbini* were only 20 km apart and the level of divergence at the 16S locus may reflect intraspecific variation (at least by comparison with levels in other rissooidean groups). However, a subsample of the collection for both these taxa was examined and clear differences in terms of shell, radula (shape and number of cusps), and characters of head/foot, penis and operculum, were found; these differences conformed to the descriptions of the two species provided by Davis *et al*. (1986*a*). These findings suggest that the variation observed at the 16S locus is a result of inter-, rather than intra-, specific variation.

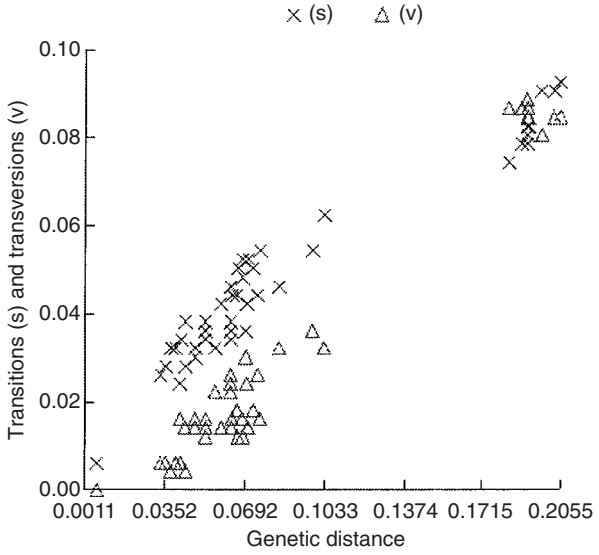

Fig. 2. A plot of the frequencies of transitions (s) and transversions (v) against genetic distance for the mitochondrial 16S rRNA sequence data (ingroup taxa). The plot shows some substitution saturation as the rate of transversions begins to exceed that of transitions at the higher distance values.

LRTs for a molecular clock, on both the 16S and 28S data sets, failed to support the hypothesis that the different lineages had been evolving at the same rate ($P < 0.01$ and 0.05, respectively). No significant heterogeneity in terms of nucleotide frequency was detected in either the 16S or 28S data sets ($P > 0.50$). Quite low levels of saturation were detected at the 28S locus (I_{ss} (0.47) < $I_{ss.c}$ (0.7443) $P = 0.000$; note, a statistically insignificant difference here would imply a poor phylogenetic signal); however, much higher levels of saturation were indicated for the 16S data ($I_{ss.c} < I_{ss}$). Consequently, a plot of both transition and transversion frequency against genetic distance was generated (Fig. 2); this plot shows a slight excess of transversions over transitions at higher D values (indicative of saturation) but the level of saturation implied is not great and its effect on the phylogenetic signal is likely to be minimal, especially as fully optimized models of sequence evolution are used in this study.

Phylogeny reconstruction

Figure 3a shows the MB tree for the 16S data. The ML tree is not shown because its topology is identical to that of the MB tree, and the level of support is greater for all nodes on the MB tree. The topology of the 16S MP tree, which is also not shown here, was the same

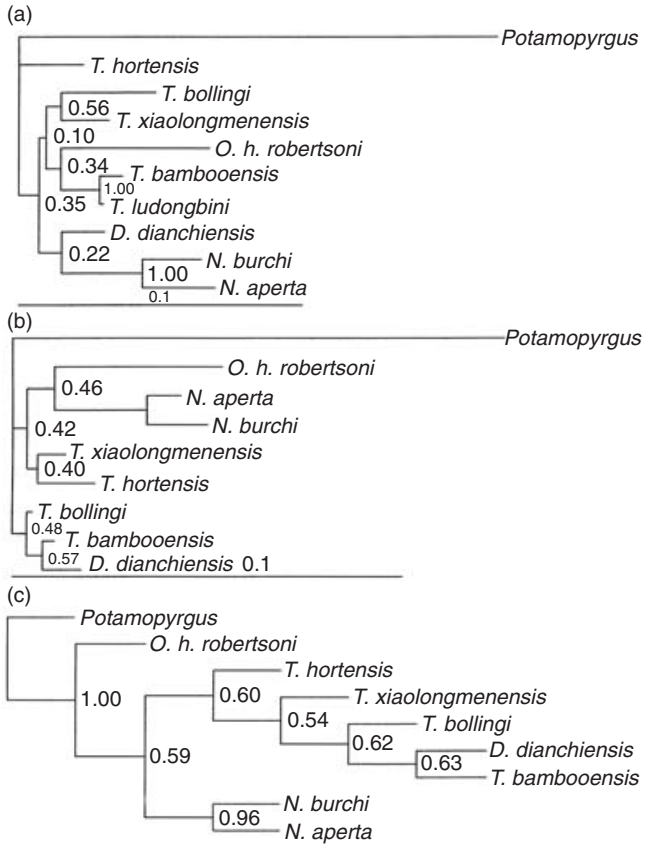

Fig. 3. (a) Phylogenetic tree produced using maximum likelihood and a Bayesian method with the mitochondrial 16S rRNA sequence data. The numbers at each node are clade credibility values computed by MrBayes v. 2.01 (Huelsenbeck, 2000). Outgroup taxon *Potamopyrgus antipodarum*. (b) A phylogenetic tree produced as above but with the 28S rRNA sequence data, outgroup taxon as in (a). (c) A maximum parsimony derived phylogram based on the 28S data. Outgroup taxon as in (a), with bootstrap support values (proportion of 5,000 replicates) assigned to each node.

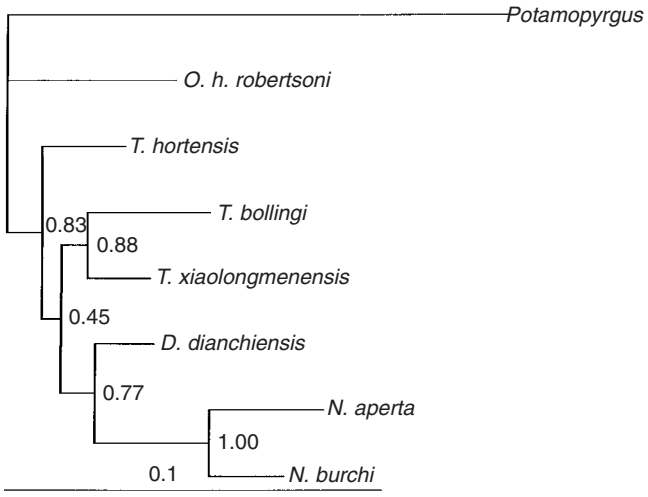

Fig. 4. Phylogenetic tree produced using maximum likelihood and a Bayesian method with the mitochondrial 16S rRNA sequence data and with all but one of the Dianchi lake *Tricula* spp. omitted from the data set. The numbers at each node are clade credibility values. Outgroup taxon *Potamopyrgus antipodarum*.

as that for the MB tree except that the MP tree shows *T. bollingi* as arising from a lineage separate from the Yunnan *Tricula* clade. The 28S MB tree (Fig. 3b) shows the *T. hortensis* clade as branching from that lineage leading to *O. h. robertsoni* and the *Neotricula* clade. Indeed the *T. hortensis*/*T. xiaolongmenensis* clade was the least well supported on both the 28S ML and MB trees. The ML tree for the 28S data differed from the corresponding MB tree only in that the ML tree showed the clade bearing *T. hortensis* and *T. xiaolongmenensis* as branching from the lineage leading to *T. bambooensis*, *T. bollingi* and *Delavaya*. The 28S ML tree is not shown because it showed lower nodal support overall than the MB tree. The main, and most important, difference between the 28S MB tree and the MP tree (Fig. 3b,c) is that the latter supports the monophyly of the Triculinae and Pomatiopsinae; the MB tree does not because it shows the pomatiopsine *O. h. robertsoni* as part of the *Neotricula* clade (i.e. as part of the Triculinae). The 16S MB tree also shows *O. h. robertsoni* as within the Triculinae clade, this time as basal to the *Tricula bambooensis* and *T. ludongbini* lineage. The inclusion of *Oncomelania* in the triculine clade goes against the wealth of morphological characters which distinguish the Pomatiopsinae from the Triculinae, and casts serious doubt on the reliability of the 16S and 28S ML and MB trees. Further, the 16S and 28S MB (and ML) trees disagree as to the positions of *Delavaya*, *T. bollingi*, and *T. hortensis*, as well as that of *O. h. robertsoni*. The levels of support for the nodes joining these taxa to the tree are also low (0.22–0.56 and 0.40–0.48 on the 16S and 28S MB trees respectively). The low levels of sequence divergence observed among the Yunnan *Tricula* were considered to be a possible explanation for the low phylogenetic resolution found here. In order to examine the effect of reducing the number of taxa sampled from the Yunnan lake area, the ML and MB trees for both loci were re-estimated using only *T. xiaolongmenensis* from Yunnan; this taxon was chosen because it was the most geographically isolated of the Dianchi Lake (Yunnan) taxa. Figure 4 shows the 16S MB phylogeny for this reduced taxon set; the 28S MB tree was identical to this tree except that the positions of *Delavaya* and *Oncomelania* were reversed (the new ML trees were identical to their corresponding MB trees but with lower levels of nodal support). The log-likelihood of the 16S ML tree was –1484.28 for the reduced data set, compared with –1542.25 for the full taxon set. An SH test indicated that the likelihood was significantly ($P = 0.012$) improved by adopting the 16S ML tree over that of the 28S ML tree under the 16S data set (there was no significant difference between the two models when compared under the 28S data ($P = 0.150$, reduced data sets compared).

DISCUSSION

Phylogenetic incongruence – a result of a rapid Pleistocene radiation of the Triculinae?

Clearly there are (biologically) important topological differences between the 16S and 28S MB, and ML, trees. The topologies only fully agree in that *N. aperta* and *N. burchi* always appear as congeners (clade credibility values of 1.00 on both trees), and that *Delavaya* and *Neotricula* are derived taxa relative to other triculine species. Comparisons among those trees showing best agreement (the 16S MB/ML/MP and 28S MP trees) and relevant clade credibility values, reveal that the positions of *Delavaya*, *O. h. robertsoni* and *T. hortensis* are not well resolved. The weight of morphological data supporting the monophyly of the Pomatiopsinae and Triculinae, and the result of the SH-test, cast further doubt on the topology of the 28S ML and MB trees (which show *Oncomelania* as derived Triculinae, even after the removal of taxa). An examination of the implications of morphological data, likelihood values and overall nodal support, suggests that the true phylogeny for these taxa may lie somewhere between those depicted by the 16S tree (Fig. 4) and the 28S MP tree (Fig. 3c).

The questions remain as to what factors could have caused the differences between the 16S and 28S MB trees (Fig. 3a,b) and what impact could they have on our future sampling strategies. In the case of the (nuclear) 28S gene, concerted evolution (see Swofford *et al*., 1996) may have inflated levels of divergence between certain recently diverged, and in fact really quite closely related, taxa. In the case of the mt16S gene one could expect a group of recently diverged monophyletic taxa to appear paraphyletic as their original polymorphic lineages are terminated and replaced by variants unique to each taxon (i.e. sorting of ancestral polymorphisms, see Neigel & Avise, 1986); this process can be estimated to take around one million years for an annual snail such as *N. aperta*, which occurs at high population densities (Attwood, 1995).

The differences between the two MB trees in Fig. 3 can only be explained by the confounding molecular evolutionary processes described above if these taxa are relatively recently diverged; the question may now be posed as to what evidence is there to support such a recent divergence. Davis (1979) suggested that the main route of colonization for the early Pomatiopsidae, from north-east India into south-east Asia and China, was the north-west–Burma–Brahmaputra–upper Irrawaddy corridor which opened approximately 18 Ma. The elevation of the Tibetan Plateau (38 Ma) led to the creation of the Salween, Mekong and Yangtze rivers (Fig. 1). In Yunnan, (southern China) the three rivers run close together from their origins in Tibet. The ancient lakes of Burma and Yunnan, which lie between the rivers (for example, Dali lake, Fig. 1) are remnants of bridges between these rivers and contain vestiges of triculine stock as isolated, endemic, populations (Davis *et al*., 1983). The Triculinae comprises three tribes (see Davis *et al*., 1992); these are the Jullieniini, Pachydrobiini and Triculini, with the former having the most derived anatomies and the latter generally with the most conserved. All three tribes are represented in each of the three rivers and this led Davis (1979) to cite northern Yunnan, near the border of the Tibetan Plateau, as the origin of the clade. The mountains of Tibet were of much lower relief during the Pliocene, providing more suitable habitats and routes for the dispersal of triculine snails than today (Attwood, 2001). It is therefore possible that a proto-*Tricula* snail, arriving in Tibet from northern India, may have dispersed across Tibet and Yunnan and into Sichuan and south-east Asia, occupying the highland streams and lakes of the region. The second Tibetan uplift (2.5 Ma, see Xu, 1981) led to harsher conditions in the region, and drainages in other regions such as the upper Yangtze and Mekong rivers, the result of which was to isolate Sichuan *Tricula* (e.g. *T. hortensis*) to the east of the Yunnan mountain ranges away from the ancestors of Yunnanese *Tricula* in the Dali lakes region (see Fig. 5). Lake levels in the Tibet-Yunnan region are likely to have remained high until the cataglacial (20 000-13 00 years ago), when populations of *Tricula* inhabiting the lakes of northern Yunnan would have been fractionated and forced into various new drainages as lake extinction occurred. The evolution of the Triculinae appears to have been driven by the opening up of new habitats along the nascent river systems as these cut their way to the sea, with the more conserved taxa being found closest to Tibet (Davis, 1992). Consequently, it is likely that very little differentiation occurred among the Triculinae until the Pleistocene, when a series of interconnected stable lake environments was exchanged for river, stream and lake environments. During the Pleistocene it appears that those taxa isolated into the remaining lakes have undergone a limited radiation, with little anatomical innovation, leading to quite morphologically similar taxa in near sympatry and occupying similar habitats, Davis (1994) called these radiations 'morphostatic'. Consideration of the palaeogeography of the Tibet–Yunnan region therefore suggests that the Triculinae have undergone a quite recent radiation and that this is a morphostatic radiation. In view of this, the answer to the question posed above is probably yes, the phylogenetic incongruence observed here may well be a result of a rapid Pleistocene radiation of these taxa.

Are triculine groupings monophyletic?

Davis *et al*. (1998) reported that the placement of *Tricula* within the Pomatiopsidae was unresolved by COI sequences, as its position varied with the choice of phylogenetic method and outgroup. Although the former study used only one (unnamed) species of *Tricula*, the present study, based on five species of *Tricula*, has also suffered some incongruence in the placing of this genus relative to higher categories (e.g. Triculini or Pachydrobiini?). The Triculini (the tribe to which *Tricula* and *Delavaya* belong) appear paraphyletic on the

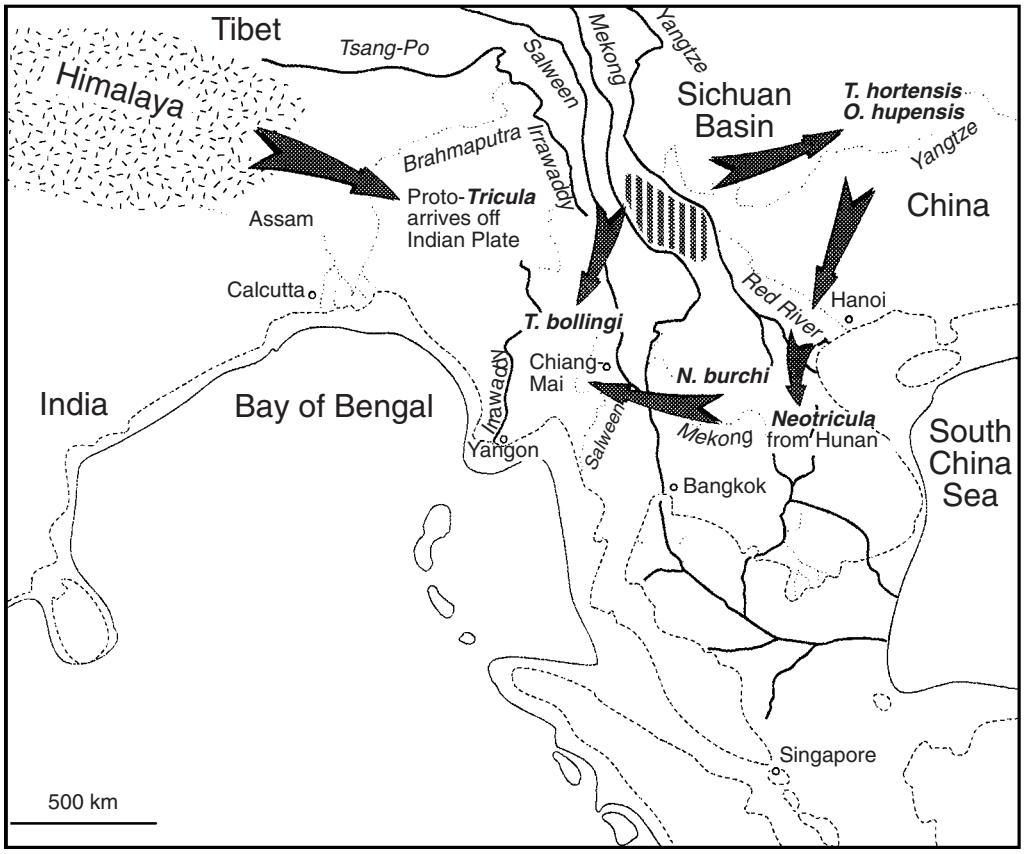

Fig. 5. Pliocene river courses and coastlines in eastern Asia and hypothesised routes of colonization for the Pomatiopsidae shown by arrows, each arrow is followed by the extant taxa believed to be the result of that radiation. The thick solid (i.e. unbroken) lines represent palaeo-river courses, fine broken lines present day river courses. Present day coastlines are shown by thick broken lines and Pliocene coastlines by fine solid lines. The shaded region between the Mekong and Yangtze rivers represents the extended Dali lake region of the Plio-Pleistocene. Scale approximate.

16S MB tree (Fig. 3a) because the clade also includes pachydrobiine taxa (*Neotricula*). Both the 16S and 28S MB trees (Fig. 3a,b) show the Triculinae itself to be paraphyletic, as the clade also includes the pomatiopsine taxon *Oncomelania*; this was the basis for rejection of these trees. The Yunnan *Tricula* sampled in this study represent part of a rapid Plio-Pleistocene morphostatic radiation and may, for reasons discussed above, contribute to phylogenetic noise rather than improve our phylogenies. In order to test this idea, the ML and MB trees were re-estimated using only one species of Yunnan *Tricula*, *T. xiaolongmenensis*. Figure 4 shows the 16S MB tree for the reduced taxon data set. The likelihood of the 16S ML tree was found to be maximized by adopting the smaller data set, although simply reducing the number of taxa (selecting taxa for removal at random) can lead to an increase in likelihood (see Swofford *et al*., 1996). Nevertheless, the greater level of nodal support for the 16S 8-taxon tree (range (0.45, 1.00) mean 0.79), when compared with the 10-taxon tree (range (0.22, 1.00) mean 0.51; compare Figs 3a and 4), and improved congruence between the 16S and 28S trees, suggest that phylogenetic resolution was improved by removing the Dianchi lake taxa and that the Triculinae are indeed monophyletic.

Was the colonization of south-east Asia by *Neotricula* independent of the radiation of *Tricula*?

The present data have confirmed the congeneric status of *N. aperta* and *N. burchi*. The current, and historical, biogeographical deployment of these taxa are more consistent with *N. burchi* being a species of *Tricula*. *T*. *bollingi* is found in north-west Thailand (near Chiang-Mai, see Fig. 1), less than 300 km from the border with Yunnan and interconnected by the Mekong and Ping river drainages. It is assumed that proto-*T. bollingi* arose from taxa of the Yunnan lakes region becoming isolated in the extended Mekong-Ping river (around 1.5 Ma, see Hutchinson, 1989), which ran from northern Yunnan, through Laos, and into Thailand in the region of Chiang-Mai. *N. burchi* is also found near Chiang-Mai and one might expect this taxon was derived from the *T. bollingi* radiation into Thailand, with *N. aperta* evolving from antecedent taxa entering the Mekong river at this point, and diversifying as the river cut eastwards and then southwards into Laos and Cambodia. *Neotricula aperta* is found in the Mekong river (and certain tributaries) of central Laos (close to the border with Vietnam), southern Laos, and Cambodia (from Khemmarat to Kratié, Fig. 1).

However, none of the trees presented here shows a close relationship between *T. bollingi* and *Neotricula*. In addition, the main radiation of *Neotricula* occurs around the Yangtze river in Hunan, which is a region with clear dispersal corridors (along the Yangtze Plain) to Sichuan but not to Yunnan or south-east Asia or to the Mekong river. Furthermore, *Neotricula* is not found in Yunnan or along the Mekong in northern Laos. In view of these factors it seems unlikely that the radiation of *Neotricula* was associated with the Mekong river, indeed the Pliocene Mekong river did not run through the areas of southern Laos and Cambodia, where *N. aperta* is most common today. The findings thus suggest that the Pleistocene phylogeography of *Neotricula* in south-east Asia was indeed independent of that of *Tricula* in that region. The problem is an important one because triculine phylogeographies are being used to reinforce models of the origins and evolution of the *Schistosoma* spp. they transmit (see Davis, 1992; Attwood *et al*., 2002*b*). Attwood (2001) advanced the "Red river hypothesis" to explain how *Neotricula* from Yunnan could have crossed the Annam mountain barrier from Hunan and into Vietnam and Laos during the late Pliocene. The essence of this model is that the Red river (which is the only river to cut through the mountains separating China from south-east Asia today) could have provided a dispersal corridor from Hunan to Vietnam because this river and the Yangtze river once flowed along a common course (Fig. 5). *Neotricula burchi* may then be derived from the *N. aperta* lineage and carried to northern Thailand via the extended Mekong-Loei river which ran westwards from central Laos to north-east Thailand and on to the Ping river and Chiang-Mai (indeed *N. burchi* is found along the northern border of north-east Thailand and Laos). Woodruff *et al*. (1999) suggested that the ancestors of *Oncomelania* may have been dispersed over long distances (and across mountain barriers) on the feet of birds, presumably trapped in mud. However, this mode of dispersal is unlikely to have been a factor in the radiation of the Triculinae, which unlike *Oncomelania*, are not amphibious and do not survive long out of water.

If the origin of the *Neotricula* clade is in Hunan, then *Neotricula* could easily have been derived from taxa of the *T. hortensis* radiation into Sichuan, which would have been in a position to colonize the lakes of Hunan via the Yangtze river drainage. *T. hortensis* is most likely descended from *Tricula* which had crossed the mountains of eastern Yunnan before the Pliocene uplift, and had therefore the opportunity to colonize the Yangtze Plain. In support of this, the trees in Figs 3(a,c) and 4 show *T. hortensis* as basal to the clade bearing *Tricula* and (excepting Fig. 3c) *Neotricula*. These findings fit well with those of Attwood *et al*. (2002*b*) concerning the phylogenetics of Asian *Schistosoma* and historical biogeographical models for the origin and early radiation of the genus in Asia.

The present study has shown that the two *Neotricula* spp. are indeed congeners, that this genus is a derived (i.e. apomorphic) taxon relative to other Triculinae, and that the position of *T. hortensis* is probably basal to the main *Tricula* clade (Figs 3a,c & 4). These findings agree with current historical biogeographical hypotheses for the Triculinae including the Red river hypothesis. In addition, the better supported trees were consistent with the monophyly of the Triculinae. The study has demonstrated levels of variation and phylogenetic incongruence (between nuclear and mt loci) consistent with the hypothesised rapid Plio-Pleistocene radiation of the Triculinae. The work has also shown that the findings of DNA-sequence based studies of the Pomatiopsidae based on single loci and/or single phylogenetic methods will be of little value on their own, and that the Yunnan Triculinae appear to be a group for which sequence based phylogenies should be used to confirm, not contest, those based upon morphological data.

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