GENETIC VARIATION IN NEOTRICULA APERTA, THE INTERMEDIATE SNAIL HOST OF SCHISTOSOMA MEKONGI: ALLOZYME DIFFERENCES REVEAL A GROUP OF SIBLING SPECIES

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ABSTRACT

Neotricula aperta (Temcharoen) is a highly variable pomatiopsid gastropod found in the Mekong River and its tributaries in Thailand. Three races and two other variant phenotypes have been described, originally as Tricula aperta. Samples of the sympatric alpha and gamma races from the Mekong River and of the beta race from the Mun River were characterized at 16 allozyme loci. Highly significant heterozygote deficiencies and differences in allele frequencies between males and females were apparent at many polymorphic loci in each of the three samples. The observed heterozygote deficiencies and sex differences were artifacts produced by the presence of cryptic taxa in each original racial sample. In the Mun River beta race sample, we found two sibling species separated by a significant multilocus genetic distance (D = 0.22). In the Mekong River, the snails representing the alpha and gamma races were found to be referable to two other well differentiated sibling species (D = 0.34), both of which have individuals of “alpha” and “gamma” morphotypes. The Mekong River species pair and the Mun River species pair are very well differentiated from the Mun River species pair (D = 0.74). Formal taxonomic revision of the N. aperta sibling species complex is postponed until topotypic material (N. aperta gamma race) from Laos can be examined. As only the “gamma race” had been shown to transmit Schistosoma mekongi naturally, it remains to be established which of the newly recognized species are epidemiologically significant.

The major Late Tertiary radiation of Triculinae (Prosobranchia: Rissoidae: Pomatiopside) in Southern China and Southeast Asia has resulted in more than 12 genera and 120 species of small freshwater snails (Davis, 1979, pers. comm., 1986; Kang, 1983, 1984a, b, 1986; Liu et al., 1983). Neotricula aperta (Temcharoen) is the best known member of this extraordinary radiation as it is the intermediate host for the human blood fluke, Schistosoma mekongi Voge, Bruckner and Bruce. In this paper we will present evidence, based on multilocus allozyme variation, suggesting that N. aperta actually comprises a group of at least four sibling species.

The species was first described as Lithogyphopsis aperta by Temcharoen (1971) and subsequently placed in the genus Tricula by Davis (1979), and Neotricula by Davis et al. (1986). These are small (2-4 mm shell length), dioecious, aquatic prosobranch snails. In their monograph, Davis et al. (1976) described three races of this species in Thailand and Laos on the basis of shell size, shape, and microsculpture, mantle pigmentation, developmental rates, radular traits,

features of male reproductive anatomy, habitat and distribution. Kitikoon et al. (1981) described the last two traits of the three races in more detail. The alpha and gamma races are found, frequently together, along 300 km of the Mekong River. The beta race is found only in the Mun River (alternatively transliterated as Mood), a tributary of the Mekong in northeast Thailand. Shells size, shell shape, and mantle pigmentation have been the diagnostic characters used in field identification for the sympatric alpha and gamma races. Gamma race snails typically have four large, distinctive pigment spots on their mantles that are absent in alpha and beta race snails. Gamma race snails are also often smaller than sympatric alpha race snails; beta race snails are intermediate in size. In the Mekong River, alpha and gamma race snails occupy the same range of benthic microhabitats: from near shore to river center and also in seasonal pools on exposed rock islands. Beta race snails occur in and near rapids in the Mun River. Snails of all races are found on solid substrata (rocks and sticks) and never on sand, mud or algal strands.

Davis et al. (1976) discussed the possibility that the three races could be reproductively isolated from one another. They suggested that the beta race, with its allopatric distribution and pronounced microhabitat preferences, could be specifically distinct from the Mekong River races. They further speculated that differential rates of growth and maturation could act to isolate the sympatric alpha and gamma races reproductively, and that these two taxa also could have reached full species rank. They concluded, however, that their evidence for significant intraracial variation and for racial intermediacy did not support such conclusions. They argued that the known differences in the reproductive organs, shell size and sculpture, pigmentation, and radular formulae could simply reflect differences in ontogeny and ecology rather than genetically-based evolutionary divergence. They found apparent hybrids (snails with irregular pigment patterns and shell size and shape intermediate between the alpha and gamma races) at one locality and noted that the alleged anatomical differences between these races were somewhat artificial. Similarly, microhabitat preferences overlap broadly (Kitikoon et al., 1981). Thus, no formal subspecific nomenclature was proposed to partition the variation recognized in this species from the outset.

This view of Neotricula aperta as a highly variable species with recognizable ecophenotypic races was subsequently challenged by Kitikoon's reports (1981a, b; 1982a, b; Kitikoon et al., 1981) that the so-called alpha, beta, and gamma "races" of T. aperta are at least different subspecies and may well be different species. One quantitative population study of allozyme variation in this taxon supports the latter conclusion, but in a manner completely unanticipated in our preliminary report (Woodruff et al., 1986b).

MATERIALS AND METHODS

Using distributional, ecological, and morphological criteria to identify snails in the field, samples of Neotricula aperta alpha, beta, and gamma races were collected in northeastern Thailand in May 1984. N. aperta alpha race and gamma race snails were taken from the Mekong River near Ban Bungkhong in the Khemarat District of Ubon Ratchathani Province. Alpha snails were found in pools on a rock island and gamma race snails were taken nearby from rocks cropping out in the main river channel where the water was deeper and the current swifter. In both cases, however, water depths in May were less than 1 m. N. aperta beta race snails were collected in the Mun River near Khao Phra near the Phribun Mangsuan District of Ubon Ratchathani Province. This site is midway between the town of Ubon and the Mun's juncture with the Mekong and about 100 km directly south of the alpha-gamma collection site. Snails were taken from rocks in fast flowing water less than 1 meter deep. In every case, sampling was conducted along less than 10 m of river bottom. Racial identities were confirmed and snails were sexed using a binocular microscope in Bangkok soon after collection. Snails were then frozen at -70°C until electrophoresis was carried out at the University of California, San Diego in 1985. Voucher specimens were deposited in the museum at the Center for Applied Malacology, Mahidol University.

The electrophoretic techniques used are described in general terms elsewhere (Mulvey and Vrijenhoek, 1981; Woodruff et al., 1988). Individual snails were quickly homogenized in less than 0.1 ml (2-3 drops from a standard Pasteur pipette) of grinding solution (0.01 M Tris, 0.001 M EDTA, 0.25 mM NADP; pH 7.0) with a glass rod. The homogenate was centrifuged at 10,000 g for 2 min in a Fisher 225A microcentrifuge, and the supernatant was absorbed onto 3 x 9 mm tabs of Whatman No. 3 chromatography paper which were then inserted into cold 12% Sigma® starch gels (one tab per snail per gel). Electrophoresis was carried out using four different buffer systems at 4°C for 15-18 hr (Table 1). A bromophenol blue marker dye migrated 100-125 mm anodally during this time except in the case of buffer system Tris-Citrate pH 6.8 in which the marker migrated 70-100 mm. Following electrophoresis, 4-5 slices were cut from each gel and each slice was stained for a specific enzyme following standard methods (Shaw and Prasad, 1970; Harris and Hopkinson, 1978). The esterase substrate was alpha-naphthyl acetate; the peptidase substrate was leucyl-alanine.

Electrophoretic conditions for the resolution of 12 enzymes coding for the 16 allozyme loci reported here are described in Table 1. These enzymes were selected on the basis of their interpretable electromorphs from among 30 enzymes tested under ten different electrolyte and pH conditions and are associated with a variety of metabolic pathways. Snails from different samples were run on each gel to facilitate comparisons; isozymes were numbered and alleles assigned mobility values relative to the common electromorph in Neotricula aperta alpha race. In Table 6, alleles are listed in order of their decreasing anodal mobilities; cathodal mobility is indicated by a negative value. For each locus, relative mobilities are those for the first buffer system reported in Table 1. These are reported to two decimal places only where

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GAMMA RACES

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VARIATION IN THE THREE ORIGINAL SAMPLES SORTED INTO ALPHA, BETA AND GAMMA RACES

they cannot be distinguished by a single decimal place approximation. Commonly used enzyme abbreviations are typeset in capital letters to indicate the protein and in italics to indicate the presumed locus.

The mean number of alleles per locus (A), the proportion of loci polymorphic (a locus was considered polymorphic if more than one allele was detected,) individual heterozygosity (by direct count) (h), were calculated for each sample. Allozyme frequencies for the polymorphic loci were tested for their agreement with Hardy–Weinberg expectations for panmixia were detected in mean genetic distance value between beta race and the two Mekong River races was unexpectedly large (D = 0.66 ± 0.24). However, panmixia is an important assumption of Nei’s genetic distance statistics and significant departures from Hardy–Weinberg expectations for panmixia were detected in 62% on the polymorphic loci in these samples (Table 2). In all 18 cases, there was a deficiency of heterozygotes and all but two tests were significant at the 1% (p < 0.01) level.

The above analyses were first performed on the original “racial” samples with sexes pooled and then with sexes separated. As the original samples were found to be highly heterogeneous, it became necessary to repeat the analyses with the snails from each sample site resorted according to individual genotype. The sorting procedure, based on three or more diagnostic loci, is described below.

RESULTS

Table 1. Electrophoretic buffers used for resolution of proteins in Neotricula aperta.

<table>
<thead>
<tr>
<th>Enzyme (E. C. #)</th>
<th>Abbreviation</th>
<th>Buffer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase (3.1.3.2)</td>
<td>ACP</td>
<td>TC 6.8</td>
</tr>
<tr>
<td>Aspartate aminotransferase (2.6.1.1)</td>
<td>AAT</td>
<td>TBE 6.0</td>
</tr>
<tr>
<td>Esterase (3.1.1.1)</td>
<td>EST-1</td>
<td>TBE 6.0</td>
</tr>
<tr>
<td></td>
<td>EST-2</td>
<td>TBE 6.0</td>
</tr>
<tr>
<td></td>
<td>EST-3</td>
<td>TBE 6.0</td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase (1.1.1.8)</td>
<td>GAP</td>
<td>AP 6.0, TC 6.8</td>
</tr>
<tr>
<td>Glucose phosphate isomerase (5.3.1.9)</td>
<td>GPDH</td>
<td>AP 6.0, TC 6.8</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (1.1.1.42)</td>
<td>GPI</td>
<td>TC 6.0, TC 6.8</td>
</tr>
<tr>
<td>Leucine aminopeptidase (3.4.11)</td>
<td>IDH</td>
<td>TC 6.8</td>
</tr>
<tr>
<td>Malate dehydrogenase (1.1.1.37)</td>
<td>LAP (PEP-2)</td>
<td>TC 6.0, TBE 8.0</td>
</tr>
<tr>
<td>Peptidase (3.4.13)</td>
<td>MDH</td>
<td>TC 6.0</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase (1.1.1.44)</td>
<td>PEP-3</td>
<td>TBE 8.0</td>
</tr>
<tr>
<td>Phosphoglucomutase (5.4.2.2)</td>
<td>PEP-4</td>
<td>TBE 8.0</td>
</tr>
<tr>
<td></td>
<td>PGD</td>
<td>AP 6.0</td>
</tr>
<tr>
<td></td>
<td>PGM-1</td>
<td>TC 6.0</td>
</tr>
<tr>
<td></td>
<td>PGM-2</td>
<td>AP 6.0</td>
</tr>
</tbody>
</table>

*AP 6.0: 0.04 M citrate adjusted with N-(3-aminopropyl)-morpholine to pH 6.0; diluted 1:19 for gels and undiluted for electrodes (16 hr., 80 v). TBE 8.0: 0.6 M Tris, 0.065 M borate. 0.02 M EDTA. adjusted to pH 8.0; diluted 1:9 for gels and undiluted for electrodes (16 hr., 100 v). TC 6.0: 0.378 M Tris, 0.165 M citrate, adjusted to pH 6.0; 13.5 ml diluted to 400 ml for gel and undiluted for electrodes (16 hr., 60 v). TC 6.8: 0.188 M Tris, 0.065 M citrate. adjusted to pH 6.8: diluted 1:9 for gels and 1:15 for electrodes (16 hr., 150 v).

For reasons that will become clear below, the allele frequencies at all 15 presumptive loci are not reported here. These data on variation in the original alpha, gamma and beta “racial” samples are presented elsewhere (Staub, 1988).

The ratio of females to males in the original racial samples was 32:36 for alpha race, 34:43 for beta race, and 32:34 for gamma race. In each original sample, the pattern of loci with heterozygote deficiencies was essentially the same within each sex as it was with the sexes pooled. Neither males

Table 2. Number of loci showing a significant deficiency of heterozygotes, as a fraction of the total number of polymorphic loci, before and after resorting Neotricula aperta racial samples by three-locus genotype.**

<table>
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<th>Original Samples</th>
<th>Resorted Samples</th>
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<tr>
<td>alpha race</td>
<td>Meakong River taxon 1</td>
</tr>
<tr>
<td>gamma race</td>
<td>Meakong River taxon 2</td>
</tr>
<tr>
<td>beta race</td>
<td>Mun River taxon 1</td>
</tr>
</tbody>
</table>

*Number of Fisher exact tests significant at 0.01 < p < 0.005 and at p < 0.01 (in parentheses).

**See text and Tables 4 and 5 for full explanation.
nor females contributed more to any sample's overall deficiencies and no single-locus genotype appeared to be sex-linked for any sample. However, allele frequencies were notably different between the sexes at many loci. This too was unexpected as males and females allegedly represent a random sample of each population and sex-linked allozymes are rare (Richardson et al., 1986). Tests of sample independence between male and female subsets revealed significant \( p < 0.05 \) differences in each original sample (Table 3). Although the initial analysis suggested that the Gap-3\(^{a}\) and Gap-4\(^{a}\) alleles were equally abundant in the alpha and gamma races (Staub, 1988), no heterozygotes were observed among 118 animals. Likewise, no Pep-3\(^{a}\)\(^{b}\) heterozygotes were observed among 120 animals. Concordance by specific genotype between these two loci and a third with a marked deficiency of heterozygotes, Gap\(^{b}\) (\( N = 126 \)) was 100\% (Table 4). Similarly, no heterozygotes were observed at three polymorphic loci in the beta race sample: Lap (\( N = 59 \)), Mdh (\( N = 85 \)), and Pep-3 (\( N = 48 \)) and the concordance by genotype between these three loci is also nearly complete (Table 5).

The unexpected differences between sexes and these striking associations among alleles at loci with no heterozygotes suggested that the original sample sorting had been insensitive to the genetic heterogeneity present at each locality. The snails from each original collecting locality were accordingly resorted by three-locus genotype, as identified in Tables 4 and 5, and the analyses were repeated. The original racial designations were abandoned.

**VARIATION IN MEKONG RIVER AND MUN RIVER SAMPLES FOLLOWING REASSORTMENT BY INDIVIDUAL MULTILOCUS GENOTYPE**

The alpha race and gamma race samples were pooled as a Mekong River sample within which we discovered two genetically defined groups of individuals, hereafter called Mekong River taxon 1 and Mekong River taxon 2. Each of these newly recognized taxa includes snails previously referred to both alpha and gamma races. The ratio of alpha race to gamma race snails was 30:34 and 38:32 for taxon 1 and taxon 2 respectively; the ratio of male to female snails in the new taxa was 32:32 and 32:32, respectively.

Similarly, the snails in the Mun River sample originally referred to the beta race are hereafter assigned to two genetically defined groups: Mun River taxon 1 and Mun River taxon 2. The ratio of female to male snails was 3:8 and 31:31 for taxon 1 and taxon 2, respectively. Three snails had apparently intermediate genotypes but were assigned to taxon 2 on the basis of variation at Mdh and Pep-3 only because the Lap\(^{a}\)\(^{b}\) and Lap\(^{a}\)\(^{b}\) electromorphs are too similar for reliable discrimination of all individuals. Four snails could not be assigned because they did not show activity for any of the three diagnostic enzymes.

Allele frequencies for the newly recognized taxa are displayed in Table 6 along with summary genetic variability statistics. The number of polymorphic loci is eight in Mekong River taxon 1 and ten in Mekong River taxon 2. There are striking differences in allele frequencies between these two taxa at Est-1, Pep-4, Pep-1, and Pep-1, as well as at the three loci (Lap, Mdh, and Pep-3) on which the reassortment was based - in other words, at virtually all the polymorphic loci. This observation is quantified by the significant genetic distance value for the two taxa, \( D = 0.34 \pm 0.16 \).

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**Table 3. Number of loci showing a significant difference in allele frequencies between males and females, as a fraction of the total number of polymorphic loci, before and after resorting *Neoceratites aperta* "racial" samples by three-locus genotype.**

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*Number of Fisher exact tests significant at \( g = 0.01 \) and at \( p < 0.05 \) (in parentheses).

**Table 4. Genotypes at three electrophoretic loci for 154 snails originally referred to *Neoceratites aperta* alpha race and *Neoceratites aperta* gamma race, showing Mekong River taxon to which snails of each genotype were assigned.**

<table>
<thead>
<tr>
<th>Locus</th>
<th>(118)</th>
<th>(126)</th>
<th>(120)</th>
<th>No. snails*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gap</td>
<td>Goi</td>
<td>Pep-3</td>
<td>N(_1)</td>
</tr>
<tr>
<td>1</td>
<td>1.4/1.4</td>
<td>1.0/0.0</td>
<td>1.2/1.2</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>1.0/1.0</td>
<td>1.0/0.0</td>
<td>1.0/1.11</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>1.0/1.0</td>
<td>2.0/2.0</td>
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</tr>
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<td>2</td>
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<td>1.1/1.0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1.0/1.0</td>
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<td>1.0/0.0</td>
<td>1</td>
</tr>
</tbody>
</table>

*\( N_1 \) = number of individuals scored at all three loci; \( N_2 \) = number scored at two of the three loci; \( N_3 \) = number scored at one locus.

**Table 5. Number of loci showing a significant difference in allele frequencies between males and females, as a fraction of the total number of polymorphic loci, before and after resorting *Neoceratites aperta* "racial" samples by three-locus genotype.**

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**Table 6. Genotypes at three electrophoretic loci for 154 snails originally referred to *Neoceratites aperta* alpha race and *Neoceratites aperta* gamma race, showing Mekong River taxon to which snails of each genotype were assigned.**

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<td>1.0/0.0</td>
<td>2</td>
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<tr>
<td>2</td>
<td>1.0/1.0</td>
<td>2.0/1.0</td>
<td>1.1/1.0</td>
<td>1</td>
</tr>
<tr>
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<td>1.0/1.0</td>
<td>2.0/1.0</td>
<td>1.0/0.0</td>
<td>1</td>
</tr>
</tbody>
</table>

*Total number of individuals scored at this locus shown in parentheses.*
Among the Mun River taxa, formerly lumped as beta race, the number of polymorphic loci is five in Mun River taxon 1 and six in Mun River taxon 2 (Table 6). There are notable differences in allele frequencies between these two taxa at Acp and Est-3 in addition to the three loci (Lap, Mdh, and Pep-3) used to rescore the original sample — again, at virtually all the polymorphic loci. The genetic distance value for the two Mun River taxa is $D = 0.22 \pm 0.12$.

The newly recognized Mekong River taxa show markedly fewer heterozygote deficiencies than the original alpha race and gamma race samples (Table 2). There are residual deficiencies ($p < 0.05$) at Lap and Pep-3 in Mekong River taxon 1 and at Acp and Pgd-4 in Mekong River taxon 2; two of these four positive tests are significant at the 1% level, those for Acp and Pep-3 in Mekong River taxon 2. In the Mun River taxa, the number of loci showing a deficiency of heterozygotes is also reduced compared to the original beta race sample (Table 2). The sample of Mun River taxon 1 is too small to analyze. Deficiencies remain at Est-1, Lap, Acp, and Pgm-1 in Mun River taxon 2; two of the four positive tests are significant at the 1% level, those for Est-1 and Lap.

Differences in allele frequencies between the sexes were eliminated by the reassortment of alpha and gamma race samples (Table 2). A difference ($0.01 < p < 0.05$) remains at the Acp locus for both Mekong River taxa. The effect of the beta race reassortment on sex differences in allele frequencies cannot be established for Mun River taxon 1 as the sample is too small to analyze ($N = 41$). In Mun River taxon 2, two loci (Acp and Pgd) show minor (and statistically insignificant) sex-related differences. In each of the four newly recognized taxa, the pattern of loci with heterozygote deficiencies is essentially the same within each sex as it had been for the sexes pooled. Neither males nor females contributed more to any sample's overall deficiencies and no single-locus genotype appeared to be sex-linked for any sample.

The genetic distance value between the Mekong River taxa and the Mun River taxa is very significant ($D = 0.074 \pm 0.025$), as depicted in the phenogram (Fig. 1). Table 7 displays genetic distance and identity values for each of the six pairwise comparisons for the four taxa.

**DISCUSSION**

**HETEROZYGOTE DEFICIENCIES AND THEIR INTERPRETATION**

The validity of our conclusion depends on our interpretation of the massive heterozygote deficiencies as evidence for a type of sampling error commonly referred to as the Wahlund effect. However, a discussion of other reasons for heterozygote deficiencies is appropriate here as there are several other possibilities that demand critical consideration. Heterozygote deficiencies across most or all polymorphic enzyme loci have been reported for other mollusc populations, particularly among marine species (Johnson and Black, 1984; McMeekin, 1985; Singh and Green, 1984; Woodruff et al., 1986a; Zouros and Foltz, 1984) and a number of potential causes have been considered. These can be roughly classified into two groups: first, true deficiencies of heterozygotes in natural populations and, second, apparent deficiencies due to sampling error or other experimental error. A true heterozygote deficiency at an enzyme locus could be due to (1) location of the locus on a sex chromosome (Zouros et al., 1980), (2) complete or partial inbreeding (Hedrick and Cockerham, 1986), or (3) selection against heterozygotes, for instance, at a particular developmental stage (Singh and Green, 1984). There are, in addition, a number of ways in which heterozygote deficiency at a locus could be apparent, but not real, due to (4) scoring bias for homozygotes (Ayala et al., 1973), (5) presence of one or more null alleles (Zouros et al., 1980), (6) biased sampling of homozygotes, or (7) the so-called Wahlund effect (Singh and Green, 1984). Biased sampling of homozygotes could be due to (6a) genetic patchiness across a population's habitat at the time of collection, referred to as population subdivision by Zouros et al. (1980), or (6b) differential survival of homozygotes following collection. The Wahlund effect is the result of mixing representations of two (or more) independent gene pools with differing allele frequencies in the same sample. It could be due to (7a) the existence of cryptic (sibling) taxa or (7b) error in field identification and taxonomic separation among already recognized taxa showing similar features. We consider each of these hypotheses in turn.

1. **Real heterozygote deficiencies due to chromosomal constraints.** The mechanism of chromosomal determination of sex in *Neotricula aperta* is not known but was speculated by Kitikoon (1962a) to be an XO-male/XX-female mechanism, based on chromosome pairing data. Such a system would result in an absence of heterozygotes in males at any X-linked locus. We did not find sex-linkage at any locus, either before or after the reassortment (29 and 24 tests, respectively). Moreover, such a system (or any other) does not predict the generalized (multilocus) strong genotypic disequilibrium shown by our data.

2. **Real heterozygote deficiencies due to inbreeding.** Although self-fertilization is not possible in dioecious tricolines, it is possible that partial inbreeding could be contributing to increased homozygosity in these snails. *Neotricula aperta* snails have low vagility and are substrate limited (Davis et al., 1976); they are found packed tightly on solid substrata, often rocks, presumably where the eggs from which they hatched were deposited, and it is possible that sib matings occur with high frequency. The snails fit the profile of re-selected colonists whose patchily distributed habitats are changed annually when the monsoon flood raises river levels dramatically (up to 15 m in the Mekong) (Davis et al., 1976). Female survivorship from the prefooding time of copulation to the postflooding time of egg deposition is low and founder effects could further increase the likelihood of sib matings. As inbreeding affects all loci in a uniform way, the generalized disequilibrium described by our data is consistent with this hypothesis. However, the complete absence of intermediates between the two genetically-defined Mekong River taxa negates this hypothesis.

3. **Real heterozygote deficiencies due to natural selection.**
Constructing reasonable selection hypotheses is difficult because we lack adequate quantitative data on the ecology and behavior of *Neotricula aperta*. These snails have a life span of approximately one year, and one might expect strong selection for competitive ability during the two month period of explosive population growth and high juvenile mortality. Differences in growth rates have, in fact, been noted between "races" and sexes (Davis et al., 1976) and between localities (Kitikoon et al., 1981). Relationships between heterozygosity and growth have been suggested for many other organisms including several marine molluscs (see review by Allendorf and Leary, 1986). Testing this hypothesis, like the previous one, would require continuous ecobehavioral and demographic observations in nature and sampling that is very sensitive to local population structure.

4. Artificial heterozygote deficiencies due to gel scoring errors. We are highly confident of our scoring for the enzymes on which the reassortment of the original "racial" samples was based: GAP, PGI, and PEP-3 in the alpha race and gamma race samples and MDH and PEP-3 in the beta race sample all form clear, distinct bands. On the other hand, some of the residual heterozygote deficiencies could be explained by scoring bias for homozygotes: ACP and PEP-4 in Mekong River taxa 2 and EST-1 and LAP in Mun River taxon 2. ACP electromorphs were diffuse and never clearly double-banded. In the case of PEP-4, there were only slight mobility differences between the five electromorphs, making presumptive heterozygotes difficult to discern and, therefore, possibly underestimated. EST-1 banded faintly and diffusely, never being clearly double-banded. The two LAP electromorphs seen in the Mun River taxa were very close in mobility and it is possible that heterozygotes were not recognized. Even with these minor qualifications scoring bias cannot account for our observations.

5. Artificial heterozygote deficiencies due to null alleles. There was no evidence for a common null allele at any locus in any of the original "racial" samples. The missing data for unscorable snails (Tables 4 and 5) did not assume the random pattern expected for non-lethal null allele homozygotes or codominant null allele heterozygotes but tended to occur together on inferior gels. If null homozygosity was lethal at a locus, this could account for a heterozygote deficiency even if blank spots, due to other causes, appear on gels. However, lethal or sublethal null alleles would have to originate by mutation at unrealistically high rates or have unreasonably high selection coefficients to explain the levels of heterozygote deficiency shown by the loci in our study. It seems highly unlikely that null alleles, lethal or not, could account for the generalized strong genotypic disequilibria shown by our data.

6a. Artificial heterozygote deficiencies due to biased sampling of homozygotes. The possibility that one or more of the original "racial" populations was genetically subdivided in some ecobehavioral way at the time of collection was entertained. If, for instance, a snail's selection of microhabitat is correlated with its growth and growth rate is in turn associated with heterozygosity at one or more loci then it could be possible to simply miss a highly heterogeneous portion of the population if the sampling protocol is not carefully designed. However, the general hypothesis (Zoos et al., 1980) does not require the degree of genetic differentiation seen in our data, and for this reason alone we think it unlikely to account for the pervasive heterozygote deficiencies seen in all three of our original samples. Again, the very high genetic identity of the two Mekong River taxa as we have defined them (Table 4) argues against this less-than-dramatic sort of population structuring.

6b. Artificial heterozygote deficiencies due to differential survival of genotypes following collection. Our snails were maintained in aquaria according to established culture methods (Kitikoon, 1981a) for about one month following collection and then frozen. Artificial selection due to collecting, scoring, and live maintenance procedures is always a concern in this type of study. Although we have no quantitative information on snail mortality during this one month period, it was not excessive and we doubt artificial selection accounts for our observations.

7a. Artificial heterozygote deficiencies due to the unsuspected presence of cryptic taxa in the allegedly homospecific samples. The sampling error known as the Wahlund effect (Wahlund, 1928) is the hypothesis we think best accounts for the heterozygote deficiencies in the original "racial" samples and is, of course, the premise on which our sampling reasortment was made. The complete lack of heterozygotes without concomitant evidence for null alleles or sex-linkage at not one but several loci strongly supports this hypothesis. If the heterozygote deficiencies in the original samples were real, we would not expect such a marked reduction in the pattern
Table 6. Allele frequencies for 16 loci in four resorted samples of Neotricula aperta, with summary statistics of genetic variability.

<table>
<thead>
<tr>
<th>Locus/ Allele</th>
<th>Taxon 1</th>
<th>Taxon 2</th>
<th>Taxon 1</th>
<th>Taxon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mekong River</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As (51)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Acp (53)</td>
<td>0.94</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Est-1 (52)</td>
<td>0.90</td>
<td>0.99</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Est-2 (43)</td>
<td>0.96</td>
<td>0.97</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Est-3 (44)</td>
<td>0.99</td>
<td>0.98</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Est-4 (45)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Gpi (63)</td>
<td>0.99</td>
<td>0.99</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Gpd (51)</td>
<td>0.67</td>
<td>0.72</td>
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<td>1.00</td>
</tr>
<tr>
<td>Pgm-1 (45)</td>
<td>0.88</td>
<td>0.85</td>
<td>0.78</td>
<td>0.85</td>
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<tr>
<td>Pep-3 (57)</td>
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<td>0.29</td>
<td>0.25</td>
<td>0.29</td>
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<tr>
<td>Pep-4 (59)</td>
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<td>0.25</td>
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<td>1.00</td>
</tr>
<tr>
<td>Pgd (51)</td>
<td>0.75</td>
<td>0.75</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm (45)</td>
<td>0.45</td>
<td>0.45</td>
<td>0.75</td>
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<tr>
<td>(continued)</td>
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</tbody>
</table>
following reassortment (Table 2). Instead, we would expect to see random changes among loci. The same sort of reasoning applies to the decrease in the number of loci showing significant differences in allele frequencies between males and females. The virtual absence of these sexual differences in our resorted samples (Table 2) argues that they and not the original racial samples best represent the natural taxa present.

7b. Artificial heterozygote deficiencies due to errors in field identification and the inclusion of several previously unrecognized taxa in allegedly homospecific samples. We discuss this hypothesis last as, if correct, it would seriously compromise our conclusions. Two species found in the Mekong River have been reported to be similar enough to Neotricula sp. in size, shell shape, and mantle pigmentation to confuse collectors (Temcharoen, 1971; Davis et al., 1976). "Mammingalea" conica is closely related to or congeneric with Sicule (sensu latu; Davis, 1979; Kitikoon, 1981b) but so far has only been found at Khong Island, southern Laos (Davis et al., 1976; Kitikoon, 1981). Some 200 river km south of the site where we collected our alpha and gamma race samples. Moreover, it is most often found by sieving sand, a substratum not associated with N. sp. (Davis et al., 1976). It has also been suggested that Pachydrobia benyi could also be confused with N. aspersa since its young have a shell very similar to that of "M" conica (Davis et al., 1976; Upatham et al., 1985). We think that the possibility of a generic misidentification is remote as, in the Mekong River in May, Pachydrobia sp. are typically 2-3 times the size of N. aspersa and associated with soft-bottom microhabitats (Davis, 1979).

We conclude that the observed heterozygote deficiencies were most probably artificial and due to the insensitivity of our field sampling, and field and laboratory sorting to detect the previously unrecognized cryptic taxa coexisting at each site. We accordingly proceed to discuss the significance of the observed genetic distances between these newly discovered taxa.

TAXONOMIC INTERPRETATION OF MULTILocus GENETIC DISTANCES

As shown in Figure 1, we have detected two well-differentiated (D = 0.34) sympatric taxa in the Mekong River. In the Mekong River, we discovered two other well-differentiated (D = 0.22) sympatric taxa. The newly recognized taxa within each river are more closely related to one another than to the taxa in the other river (D = 0.74). These estimates of genetic differentiation are based on 16 loci and a technique appropriate to establishing evolutionary relationships among congeneric taxa (Richardson et al., 1986; Nei, 1987).

At the outset it must be stressed that there is no simple relationship between a Nei's genetic distance (D) value and taxonomic level. Other factors including innate genetic variability, mating system, effective population size and degree of population subdivision will all affect the rate of genetic divergence in a clade. Nevertheless, much can be learned from the vast literature on generic differentiation within and between other well-characterized amphimictic (sexually reproducing, outcrossing and moderately polymorphic) species. For example, Thorpe (1983) reviewed over 7000 comparisons of congeneric populations of plants and animals and found that only 2% of the intraspecific D values exceeded 0.10. In contrast, he found that the average interspecific genetic distance in 900 congenic comparisons was about 0.40 (range: 0.03-1.0). A survey of 23 genera of amphimictic...
molluscs revealed they too typically have intraspecific genetic distances of <0.10 and congeneric interspecific genetic distances in the range 0.20 - 0.50 (Woodruff et al., 1988). We conclude that our estimates of genetic differentiation within the taxon formerly called *Tricula aperta* are of such magnitude that each of the four newly discovered taxa warrant recognition as separate full species. Our only reservation about this recommendation arises from the lack of data on intraspecific variability within each of these sibling species. If, as expected, intraspecific variation is small (D <0.10), and the gap between intraspecific and interspecific genetic distances remains relatively large, then the genetic distance values alone indicate these taxa are evolving separately as different biological species.

There is, of course, nothing new about the use of allozyme electrophoresis to detect sibling species. Bullini (1983) and Ayali (1993) review the successful use of the technique in the detection of sibling species in ascarid worms, plethodontid salamanders, Anopheles mosquitoes and Drosophila. Other examples involve the Asian schistosomes transmitted by snails of the genera *Tricula*, *Robertsiella* and *Oncomelania* (Fletcher et al., 1980; Woodruff et al., 1987; Merlenlender et al., 1987). Studies of allozyme variation used in conjunction with traditional methods have been particularly useful in resolving the evolutionary relationships of taxonomically difficult groups of molluscs (Woodruff and Gould, 1980, 1987; Gould and Woodruff, 1985, 1987; Woodruff et al., 1987b; Klinhom, 1989; Palmor, Gaymon and Woodruff, unpub. data). Davis (1983, 1984) has used electrophoretic data to detect sibling species in other molluscs, but did not include this technique in his early studies of the triculines.

### OTHER EVIDENCE THAT *NEOTRICULA APERTA* COULD BE A COMPOSITE TAXON

Kitikoon (1982a) described extraordinary variation in chromosome number and appearance for each of the so-called races of *Neotricula aperta*. Haploid chromosome numbers ranged from 13 to 17 in alpha race males, from 14 to 17 in beta and gamma race males, and from 16 to 17 in alpha and gamma race females. Diploid chromosome numbers were 29, 31, and 33 in alpha and gamma race males, and 32 and 34 in alpha and gamma race females. Only beta race females showed no variation with 17 haploid and 34 diploid chromosomes. The pairing patterns at prophase I were also variable as were other aspects of the karyotype. This degree of variation within a single species is almost unknown (White, 1973) and suggests that Kitikoon's samples could have been as heterogeneous as our own. A new analysis based on allozymically sorted specimens could provide more coherent results.

Kitikoon's (1982b) electrophoretic study of 5 enzymes in the three races also revealed considerable intraracial variation, but his results cannot be interpreted genetically as he pooled tissues of 40-100 snails to prepare his racial samples. Kitikoon's work was based primarily on field collections made in 1972-4 and in 1979. In addition to recognizing the three so-called races, and noting the occurrence of some populations that did not conform to this classification, he distinguished two additional phenotypes of the gamma race from Sompamit Falls, southern Laos (Kitikoon and Schneider, 1976; Kitikoon et al., 1981). He subsequently referred to the latter as (unnamed) separate species (Kitikoon, 1984). Clearly, both he and his colleagues recognized the complexity of the taxon called *Neotricula aperta*.

### PARASITOLOGICAL IMPLICATIONS

So far only the gamma race has been unequivocally shown to transmit *Schistosome mekongi* naturally (Kitikoon et al., 1973), but the alpha and beta races are susceptible to miracidia infection with subsequent cercarial shedding in the laboratory (Kitikoon, 1981b; Yuen et al., 1984). Several authors have compared rates of snail susceptibility but with inconsistent results (see Kitikoon, 1981b). However, there seems to be general agreement that, in the laboratory, beta race snails are highly susceptible, alpha race snails have low susceptibility, and gamma race snails are intermediate with respect to this trait (Kitikoon, 1981b). As host-parasite compatibility evolves on a very localized geographic basis in nature (Rollinson and Southgate, 1985; Woodruff, 1987), these laboratory experiments tell us rather little about the potential for the spread of human schistosomiasis from the transmission site at Khong Island, Laos. Our findings suggest that the identity of the intermediate host snail must now be reestablished and the epidemiological significance of its sibling species reinvestigated.

### CONCLUSIONS

It is now apparent that there are two discrete taxa present in the Mekong River that do not coincide genetically with the so-called alpha and gamma races of *Neotricula aperta*. Similarly, in the Mun River we discovered two sibling species presently confused under the name of the beta race of *N. aperta*. The genetic distances between these four taxa are large enough for us to conclude that all have reached the rank of full species. The lack of evidence for intermediacy in diagnostic allozyme characters supports this. Formal taxonomic revision must, however, await the confirmation of these patterns by more careful collection in the field and reexamination of the anatomy, morphology and karyotypes of the genetically defined taxa. The type locality of *N. aperta* is...
Khong Island, Laos, and the holotype conforms to the so-called alpha race (Davis et al., 1976). Until snails from this area can be re-collected, it is unlikely that we can resolve the issues raised by this study of genetic variation in Thai animals.

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LITERATURE CITED


