Genetic divergence between two clinostomatid fish endoparasites, inferred from allozyme and RAPD data

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We used allozyme and RAPD-derived data to analyse genetic divergence between *Clinostomum complanatum* and *Neutraclinostomum intermedialis*. Allozyme results showed an average heterozygosity of 4.04% in the former species and 0.7% in the latter, and with extreme divergence between the two species with fixation for alternative alleles in five out of 13 loci screened. Genetic distance and fixation index values between the two species were 0.921 and 0.920 respectively. Results from random amplified polymorphic DNA (RAPD) fingerprinting revealed additional genetic diversity within species (genetic similarities 0.742 and 0.829) and confirmed considerable divergence between the two clinostomatid species (genetic similarity 0.559). The results obtained suggest reproductive isolation between the two species and convincingly nullify earlier attempts to synonymise them.

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Introduction

The taxonomic designations of the subtaxa of the family Clinostomatidae have, in the past, been in somewhat chaotic disarray (Mokgalong 1996). *Clinostomum vanderhorsti* was first described by Ortlepp (1935) as a parasite of fishes and herons. *Clinostomum tilapiae* was reported by Ukoli (1966) from the gill chambers of several *Tilapia* intermediate hosts and experimentally from the egret, *Bubulcus ibis*. Feizullaev & Mirzoeva (1983) synonymised 35 clinostomatid species into one species, *C. complanatum*, the latter including both *C. tilapiae* and *C. vanderhorsti*. Britz, Saayman & Van As (1984) recovered metacercariae from the gill chambers of *Oreochromis mossambicus* from various water bodies in the Northern Province (South Africa) and assigned these parasites to *C. tilapiae* as described by Ukoli 1966. Following the classification system of Feizullaev & Mirzoeva (1983), it would mean that these parasites are no different from *C. complanatum/C. vanderhorsti* usually found on the swim bladder of *Marcusenius macrolepidotus*, notwithstanding some conspicuous morphological differences. Mokgalong (1996) re-examined material used by Britz *et al.* (1984) and came to the conclusion that what these authors described as *C. tilapiae* is in fact *Neutraclinostomum intermedialis*. This was prompted by the location and shape of the testes, the position of the cirrus sac and the genital pore in the intertesticular space, the extent of the vitellaria, the junction of the uterus with the uterine sac at its proximal end and the relative sizes of metacercariae of the two species.

The investigations referred to above are without exception based on morphological studies. Systematists often infer phylogenetic relationships from studies of phenotypic divergence using pre-selected aspects considered 'diagnostic' for the taxon. This can lead to conclusions that are partially subjective and can be subsequently reversed by others who may place greater emphasis on alternative characteristics (Thorpe & Sole-Cava 1994). While we are not discrediting the use of phenotypic characteristics, genetic techniques can form a valuable addition to such studies (Littlewood, Rohde & Clough 1998). For example, the RAPD technique has been proven to be very useful in studies of schistosomes (Kaukas, Neto, Simpson, Southgate & Rollinson 1994; Rollinson & Stothard 1994). In the South African context, biochemical and molecular genetic techniques have permeated studies involving parasites of medical or economic importance, but these techniques are still largely underutilised in taxonomic studies of more mundane species. In the present study, we report on the feasibility of using allozymes and RAPDs to elucidate discords in clinostomatid systematics. We demonstrate that there are significant genetic differences between *C. complanatum* (*C. vanderhorsti*) and the metacercariae found in the gill chambers of *O. mossambicus*, using these contemporaneous approaches.

Materials and methods

Specimens of the two host species, *Oreochromis mossambicus* and *Marcusenius macrolepidotus*, were collected from Tzaneen dam, Northern Province, with the aid of gill nets. We collected metacercariae of *Neutraclinostomum intermedialis* from the gill chamber of *O. mossambicus* and *Clinostomum complanatum* from the body cavity (on the peritoneum of the swim bladder) of *M. macrolepidotus*. Skeletal muscle samples from the two fish host-species were also collected. All samples were placed in liquid nitrogen immediately after collection for transportation and short-term storage.

Allozyme electrophoresis

Before analysis, individual metacercariae were removed from cysts where necessary, placed in 0.2 ml micro-centrifuge tubes and distilled water added at the ratio of approximately 2:1 (H_2O:tissue per volume). After homogenisation samples were loaded onto gels using 3 × 10 mm wicks of sample saturation pads. In all instances, samples of host muscle tissue were loaded on gels next to parasite samples, to screen for

expression of host proteins in parasites. Gels were prepared using 13% starch and standard buffers (Markert & Faulhaber 1965, Ridgway, Sherbourne & Lewis 1970 and Whitt 1970). A current of 50 mA was used to facilitate migration of proteins. Staining and scoring of gene products followed standard techniques, for example Murphy, Sites, Buth & Haufler (1990). All loci scored are based on at least 20 individuals for *C. complanatum* and 30 individuals in the case of *N. intermedialis*. In the absence of an accepted nomenclature system for trematode allozymes, we used the well-known guidelines of Shaklee, Allendorf, Morizot & Whitt (1990). We used average heterozygosity **H** (Nei 1975), proportion of polymorphic loci (**PPL**), average number of alleles per locus (**A**), fixation index $\mathbf{F_{ST}}$ (Wright 1965), and genetic distance **D** (Nei 1972) to quantify genetic diversity and divergence.

RAPDs

Genomic DNA was extracted from whole parasites and host muscle tissue using the Boehringer Mannheim high pure sample preparation kit. To identify usable oligonucleotide primers, a trial study was conducted using parasite DNA and six different 10-mer primers. For amplification, we used a mixture including 0.125 µl DNA polymerase (*Takara* Taq), 2.5 µl buffer (Tris-HCl, pH 8.3, with KCl and $MgCl_2$), 2 µl dNTP's, 1 µl template DNA and 1 µl oligonucleotide primer (OPERON Technologies). Amplification was performed for 45 cycles, each of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C (following Williams, Kubelik, Livak, Rafalski & Tingey 1990). Products of the PCR reaction were studied using 1.5% agarose gels, TAE buffer and ethidium bromide (included in the gel). Two primers giving the best results were selected for further investigation. We then used these two primers to screen 8 to 11 individuals each of *C. complanatum* and *N. intermedialis* for each primer, and also screened DNA from a specimen of host species. In all instances, PCR were performed on two independent reaction mixtures per individual to test reproductivity of fingerprints. We used a Lambda DNA (EcoR I and Hind III digest) molecular weight marker to gauge the size of fragments amplified. The genetic similarity (**GS**) coefficient of Nei & Li (1979) was used to calculate intra- and interspecies variability. Non-reproducable results were not included in the statistical analysis.

Results

Thirteen allozyme loci could be scored woth confidence in clinostomatid metacercariae. Gene expression in parasite tissue appeared to be unaffected by host proteins for all loci screened. Figure 1 shows host and parasite expression for gene products **GPI** (glucose-6-phosphate isomerase). The two parasites displayed disparate sets of bands in five of the 13 loci screened. In the absence of heterozygotes in most instances, it is not possible to declare whether these represent different loci or alternative alleles at the same locus. Considering the close systematic relationship between the species, we followed the latter (more conservative) approach. For **GPI-1**, heterozygotes (+ 100/+ 140) and the slower migrating homozygous genotype (+ 100/+ 100) were found in *Neutraclinostomum intermedialis*, whereas *Clinostomum complanatum* was fixed for an allele clearly similar to the + 140 allele in *N. intermedialis*. Average heterozygosity was moderate within *C. complanatum* (**H** = 4.5%) and much lower (0.7%) in *N. intermedialis*, with corresponding **PPL** values (15.4% and 7.7%). The proportion of polymorphic loci for pooled populations was however high at 61.5%. Genotypic frequencies for **EST-2** and **PGM-1** deviated significantly from expected Hardy-Weinberg equilibrium, due to a shortage of heterozygotes in both cases. The fixation index (0.921) and genetic distance (0.920) confirmed allozyme divergence between *C. complanatum* and *N. intermedialis*.

From six primers originally tested, five provided consistent and reproducible fingerprints. In the interest of economy, only two of these, OPA-08 (5' GTGACGTAGG 3') and OPA-10 (5' GTGATCGCAG 3') were selected for further screening. The other three primers which could probably equally be used were OPA-03 (5' AGTCAGCCAC 3'), OPA-04 (5' AATCGGGCTG 3') and OPA-05 (5' AGGGGTCTTG 3'). Results from RAPD fingerprinting showed considerable variation both within and between species. Fingerprints obtained using OPA-10 and OPA-08 are presented in Figures 2 and 3 (five parasites per species shown in each case). Genetic similarity values (Table 1) showed more variation within *C. companatum* (**GS** = 0.742 ± 0.240) than found in *N. intermedialis* (**GS** = 0.829 ± 0.162). Genetic similarity for pooled populations shows more interspecies variation (**GS** = 0.559 ± 0.245) than **GS** values calculated within any single species.

Figure 1 Glucose-6-phosphate isomerase (GPI) polymorphism. Left to right: **M, M** = *M. macrolepidotus*; **C1-10** = *C complanatum* (10 individuals); **N1-10** = *N. intermedialis* (10 individuals); **O, O** = *O. mossambicus*

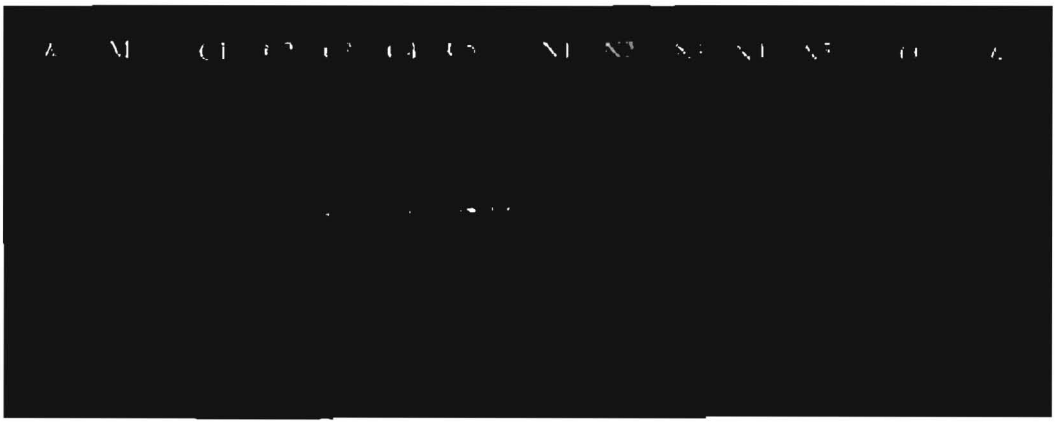

Figure 2 RAPD fingerprints from OPA-10. Left to right: λ = marker; **M** = *M. macrolepidotus* (one individual with repeat); **C1-5** = *C. complanatum* (five individuals, each with repeat); **N1-5** = *N. intermedialis* (five individuals, each with repeat); **O** = *O. mossambicus* (one individual with repeat); λ = marker. Individuals 2 & 3 from *N. intermedialis* considered not reproducible. Note short (furthest migrating band in *N. intermedialis*) fragment clearly differentiating between *N. intermedialis* and C. complanatum

Figure 3 RAPD fingerprints from OPA-08. Left to right: λ = marker; **M** = M. macrolepidotus (one individual, repeat unsuccessful); **C1-5** = *C. complanatum* (five individuals, each with repeat); **N1-5** = *N. intermedialis* (five individuals; each with repeat); **O** = *O. mossambicus* (one individual with repeat); λ = marker. Not reproducible: *M. macrolepidotus, C. complanatum* individual 1 & 4, *N. intermedialis* individuals 1 & 4

Discussion

Allozyme electrophoresis

An important precursor to discussing any systematic results of this study is to establish the influence of host tissue (originating from cysts or in the parasitic alimentary canal) on gene expression in the parasite. Our allozyme results provide credible evidence that protein expression in the clinostomatid species is unaffected by the possible presence of limited quantities of host tissues. Using **GPI** as an example (Figure 1), our results showed two sets of bands scored as separate loci in *Neutraclinostomum intermedialis*, whereas the hosting *Marcusenius macrolepidotus* displayed three bands (most likely two loci with an interlocus band), and with no overlapping mobilities for host and parasite gene products. Similarly, *Clinostomum complanatum* again have two loci for **GPI** and *Oreochromis mossambicus* have three loci, again with no shared bands. Comparable distinctions between host and parasite gene expression were found for all other loci scored.

The allozyme results obtained are characterised by comparatively little allelic polymorphism within species but extreme polymorphism between the two groups. The number of allozyme loci screened (13) is low, but this was unavoidable, considering that individual parasites possess enough substance for two or three gel runs at most. This restriction is common to studies involving small parasites (Gashumba, Baker & Godfrey 1988). Nevertheless, the results obtained leave little doubt that there is considerable allelic divergence between *C. complanatum* and *N. intermedialis*. Intergeneric genetic distance data are relatively scarce for invertebrates (Thorpe & Sole-Cava 1994). Thorpe (1982) concluded that the critical level for genetic identity distinguishing between species and genera appears to be around 0.35 (**D** = 1.05). The **D** value calculated between clinostomatid species from *O. mossambicus* and *M. macrolepidotus* during the present study approaches this figure at **D** = 0.92, which justifies the assignment of the two parasites to separate genera. Scrutiny of allelic frequencies in Table 2 also shows that the two parasitic species were fixed for alternative alleles at five out of 13 loci screened. If

Table 1 Proteins stained for, Enzyme Commission (E.C.) numbers and loci resolved in two Clinostomatid species. Loci marked with * displayed allelic variation between species; # denotes variation within species

Protein	E.C. number	Loci resolved
Esterase	3.1.1-	**Est-1*#**
General (unspecified) protein	–	**PROT-1**
Glucose-6-phosphate isomerase	5.3.1.9	**GPI-1*#**
		GPI-2
L-Lactate dehydrogenase	1.1.1.27	**LDH-1***
		LDH-2*
Malate dehydrogenase	1.1.1.37	**MDH***
Peptidase	3.4-	**PEP-1**
		PEP-2
		PEP-3*
Phosphoglucomutase	2.7.5.1	**PGM-1*#**
		PGM-2
Superoxide dismutase	1.15.1.1	**SOD-1***

these parasites had originated from different (isolated) localities, such differences may have been attributed to geographical variation or generic drift. However, considering that they are occurring sympatrically, such fixation for alternative alleles at five separate loci can only indicate complete reproductive isolation between them, again leaving no doubt that distinct taxa are involved.

Ward (1977) found that invertebrates generally show considerably higher levels of enzyme variability than vertebrates (average **H** of 12.4% compared to 4.6%), confirming earlier predictions by Selander & Kaufman (1973). In the current study, genetic diversity within species differed substantially between *C. complanatum* and *N. intermedialis*. An **H** value of 4.5% was estimated for the former species but much less heterozygosity was found in *N. intermedialis* with an **H** value of only 0.7%. There is little in the life cycles of the two species to account for such a difference. Average infestation of avian hosts with *C. complanatum* and *N. intermedialis* is identical at 12.4 (from Mokgalong 1996). However, *C. complanatum* generally occurs at a higher intensity per fish host than *N. intermedialis* (6.7 *versus* 2.5 – Mokgalong 1996). It could therefore be argued that initial infestation of the Limpopo River system (of which the Tzaneen Dam forms part) with the former would have involved a larger gene pool, if a comparable number of hosts were involved. An investigation of alternative localities where *N. intermedialis* occurs could show whether the lack of genetic diversity in this species is a common (evolutionary) characteristic in the species or a peculiarity of the Tzaneen Dam population, due to its specific history of introduction.

Random amplified polymorphic DNA

As during the allozyme part of this study, it was first necessary to consider the possible influence of host tissue during parasite DNA amplification. In contrast to the allozyme derived results, it is impossible to declare unambiguously that

Table 2 Polymorphic loci, alleles resolved (with relative mobilities), allelic frequencies and indexes of genetic diversity in two Clinostomatid species. Figures below allelic frequencies denotes significance of deviations from expected Hardy-Weinberg equilibrium of genotypes. For all loci listed, significant ($p < 0.001$) differences between allelic frequencies of species were found

		Species	
Locus	Allele	*N. intermedialis* (n = 30)	*C. complanatum* (n = 20)
EST-2	+100	1.00	–
	+200	–	0.10
	+232	–	0.90
GPI-1	+100	0.95	
	+140	0.05	1.00
		$p < 0.815$	
LDH-1	+100	1.00	–
	+1000	–	1.00
LDH-2	+100	1.00	–
	+150	–	1.00
MDH	+100	1.00	–
	+80	–	1.00
PEP-3	+100	1.00	–
	+86	–	1.00
PGM-1	+100	1.00	–
	+164	–	0.75
	+200	–	0.25
			$p < 0.010$
SOD-1	+100	1.00	–
	+73		1.00
Average heterozygosity (**H**).		0.7 (±0.7)%	4.50 (±3.30)%
proportion of polymorphic alleles (**PPL**)		7.7%	15.4%
Number of alleles per locus (**A**)		1.1	1.2
PPL pooled		61.5%	
A pooled		1.38	
Fixation index (F_{ST})		0.921	
Genetic distance (**D**)		0.920	

amplification of parasite DNA is unaffected by host tissue, since our results indicate several shared bands between parasites and hosts, notably when some of the fainter bands were included. This does not necessarily indicate host gene expression in parasite tissue – DNA contains many relatively conservative regions, even across distant taxa. Also, bands with similar mobilty merely reflect similar fragment sizes and could have quite distinct sequences. Shared bands between parasite and host could thus still denote independent amplification rather than coincidental expression of host DNA in the parasite. We should also point out that all cases of shared bands were based on high intensity parasite bands with similar electrophoretic mobility to very faint bands expressed in the host. This does not correlate with the relative quantities of parasite and host DNA present during DNA extraction,

assuming that the parasite's alimentary canal was filled with host tissue. Nevertheless, shared bands call for caution to prevent assignment of host expression to the parasitic genome. Furthermore, some doubts have been raised in recent times concerning the validity of RAPD-derived fingerprints, since reproducibility can be quite problematic (Micheli, Bova, Pascale & D'ambrosio 1994; Dinesh, Lim, Chan & Phang 1996). During the present study, reproducibility was fairly good – 69% for OPA-08 and 75% using OPA-10.

The RAPD fingerprints resolved confirm allozyme-based results in showing clear distinction between the two species, and also revealed considerable variation within species. The primer OPA-10, in particular, proved to be an ideal marker to differentiate between *C. complanatum* and *N. intermedialis*. Reference to Figure 2 shows that there is one band, representing a fragment of approximately 125 kb, that is present in all individuals of *N. intermedialis* screened, even when bands were otherwise different between repeats. By contrast, the fragment was never observed in *C. complanatum* and is absent in host tissue. The oligonucleotide OPA-08 was less informative in differentiating between *C. complanatum* and *N. intermedialis* since there were no fragments unique to a single species. Results from this primer did however contribute to revealing considerable variation within species (Table 3).

Table 3 Genetic similarity within and between *C. complanatum* and *N. intermedialis*

Primer (below)	Within *C. complanatum*	Within *N. intermedialis*	Between species
OPA-10	0.743 ± 0.302	1.000 ± 0.000	0.518 ± 0.031
OPA-08	0.741 ± 0.196	0.780 ± 0.151	0.573 ± 0.283
Pooled	0.742 ± 0.246	0.829 ± 0.162	0.559 ± 0.245

Using the genetic similarity coefficient of Nei & Li (1979) to analyse RAPD results, a value of 1.0 indicates identical DNA fingerprints whereas progressively smaller values (minimum 0) reflect more variation within groups. In the current study, **GS** values showed more variation within *C. complanatum* (**GS** = 0.742 ± 0.246) than found in *N. intermedialis* (**GS** = 0.829 ± 0.162). This endorses the trend suggested by allozyme results, confirming that *C. complanatumn* is the more polymorphic species (at least in the context of Tzaneen Dam). Genetic similarity when the two species were pooled showed considerably more variation (**GS** = 0.559 ± 0.245) than any of the similarity values calculated within single species. This is congruent with our allozyme-based $\mathbf{F_{ST}}$ values and confirms that the greatest component of genetic variation exists interspecific rather than intraspecific.

Conclusion

The results obtained convincingly demonstrate the reliability of allozyme electrophoresis to investigate invertebrate systematics. Findings from RAPD fingerprinting are less conclusive. The oligonucleotide OPR-10 provided a marker which distinguished between species with 100% reliability but unrepeatability of other bands in 25–31% of cases indicates that results from the technique should be treated with caution. The latter technique also revealed possible shared bands between host and parasite, in contrats to allozymes. Nevertheless, the combination of results from the two techniques leaves little doubt that considerable divergence exists between *C. complanatum* and *N. intermedialis*, which warrants their assignment to separate genera. The results also provide good motivation for a more prominant role for biochemical and molecular genetic techniques in local fish parasitic systematic studies.

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