The distribution of *Fasciola hepatica* and *Fasciola gigantica* within southern Tanzania – constraints associated with the intermediate host

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SUMMARY

In East Africa, *Fasciola gigantica* is generally the causative agent of fasciolosis but there have been reports of *F. hepatica* in cattle from highland regions of Kenya, Ethiopia, Uganda and Zaire. The topography of the Southern Highlands of Tanzania provides an environment where the climatic conditions exist for the sustenance of lymnaeid species capable of supporting both *Fasciola hepatica* and *F. gigantica*. Theoretically this would allow interaction between fasciolid species and the possible creation of hybrids. In this report we present molecular data confirming the existence of the snail, *Lymnaea truncatula*, at high altitude on the Kitulo Plateau of the Southern Highlands, Tanzania, along with morphometric and molecular data confirming the presence of *F. hepatica* in the corresponding area. At lower altitudes, where climatic conditions were unfavourable for the existence of *L. truncatula*, the presence of its sister species *L. natalensis* was confirmed by molecular data along with its preferred fasciolid parasite, *F. gigantica*. Analysis based on a 618 bp sequence of the 28S rRNA gene did not reveal the presence of hybrid fasciolids in our fluke samples.

Key words: *Fasciola hepatica*, *Fasciola gigantica*, liver fluke, Lymnaea, snail host.

INTRODUCTION

Fasciolosis, an infection by the liver flukes *Fasciola hepatica* Linn. 1758 or *Fasciola gigantica* Cobbold, 1856, is the major trematode infection of ruminants and a significant zoonosis. The disease has been increasing its range over the past decade (Mas-Coma *et al*. 2005) and it has been proposed that a contributing factor in this increase has been the environmental changes associated with a warming climate (Mitchell, 2002) that has promoted an expansion of populations of the lymnaeid snails that provide the liver fluke with its intermediate host. The potential severity of this situation has been compounded by the selection of anthelmintic-resistant isolates of *F. hepatica* (Wolstenholme *et al*. 2004).

In temperate regions, *F. hepatica* is the more common cause of fasciolosis than *F. gigantica* whereas in tropical regions of Eurasia, the reverse is generally true. This is most likely associated with the occurrence of permissive lymnaeid species. In Europe for example, *F. hepatica* utilizes *Lymnaea*

(*Galba*) *truncatula* (Müller, 1774) as its intermediate host whilst *F. gigantica* in Africa uses *Lymnaea* (*Radix*) *natalensis* (Krauss, 1848). In some locations (Japan, Korea, China, and Iran) molecular and morphometric studies (Itagaki and Tsutsumi, 1998; Agatsuma *et al*. 2000; Huang *et al*. 2004; Ashrafi *et al*. 2006) have shown that intermediate forms of the liver fluke can be found, suggesting that the 2 species may hybridize. Such hybrids might be better adapted to the changing environmental conditions associated with climate change and thus would enjoy a selective advantage (Seehausen, 2004). This potential increase in 'fitness' could be countered by the need to be able to infect and multiply in the local lymnaeid species. The efficiency with which different isolates of *F. hepatica* reproduce in their intermediate host has been shown to vary greatly (Walker *et al*. 2006) and infrapopulations of *F. hepatica* can be genetically very diverse (Walker *et al*. 2007) – in contrast to local populations of lymnaeids which are thought to be largely clonal or have low genetic diversity (Stothard *et al*. 2000; Trouvé *et al*. 2003). It may be assumed that those *F. hepatica*/*F. gigantica* hybrids which may have arisen are likely to be the product of a relatively rare event such that individuals within the founding population will be relatively homogenous,

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lacking populational genetic diversity. The establishment of intermediate/hybrid fasciolids requires the presence of a permissive intermediate host. Whilst *L. natalensis* does not seem to be capable of supporting the asexual multiplicative stages of *F. hepatica* (Boray, 1985), there have been conflicting reports in the literature concerning the ability of *L. truncatula* to serve as an intermediate host for *F. gigantica* (Spithill and Dalton, 1998). More recently Dar and colleagues have demonstrated efficient production of *F. gigantica* cercariae from *L. truncatula* in the laboratory and ascribed the apparent non-permissiveness of the *F. gigantica*/*L. truncatula* combination reported by others to allopatric differences between the parasite and its host (Dar *et al*. 2004).

The topography of the Southern Highlands of Tanzania provides an area in which the interaction between *F. hepatica*, *F. gigantica* and their hosts may be studied. The provinces of Mbeya and Iringa cover an area of approximately 100 000 square kilometres with altitudes varying from 500 to 3000 metres. Fasciolosis is a significant problem (Hammond, 1965; Botcher, 1967; Mahlau, 1970; Ecmovic and Mahlau 1973; Msanga, 1985; Keyyu *et al*. 2005) with prevalences almost universal in some areas. Elsewhere in East Africa, *F. gigantica* is generally the causative helminth but there have been reports of *F. hepatica* in cattle from highland regions of Kenya, Ethiopia, Uganda and Zaire (Ogambo-Ongama, 1969, 1972; Yilma and Malone, 1998; Malone *et al*. 1998). In Ethiopia it has been shown that there is a marked difference in the distribution of *Fasciola* species according to altitude with *F. gigantica* dominating at altitudes below 1800 m whilst *F. hepatica* is limited to altitudes above 1200 m. Much of the data relating to fasciolosis in East Africa was obtained before the introduction of molecular methods capable of distinguishing between *F. hepatica*, *F. gigantica* and possible hybrids (Itagaki *et al*. 1998) or the development of molecular markers for the speciation of lymnaeid snails. In this study we have collected samples of lymnaeid snails and flukes from cattle raised within a range of altitudes in the Southern Highlands of Tanzania. These have been initially identified using morphometric data followed by molecular techniques in order to provide answers to the following questions. What are the intermediate hosts for *Fasciola* spp. at different altitudes? Is *F. hepatica* found at high altitude in East Africa? Is there any evidence of *F. hepatica*/*F. gigantica* hybridization in our samples?

MATERIALS AND METHODS

Snail survey

The freshwater snail survey was carried out between 2001 and 2003 and sample sites were revisited in 2006. The sampling sites (Fig. 1 and Table 1)
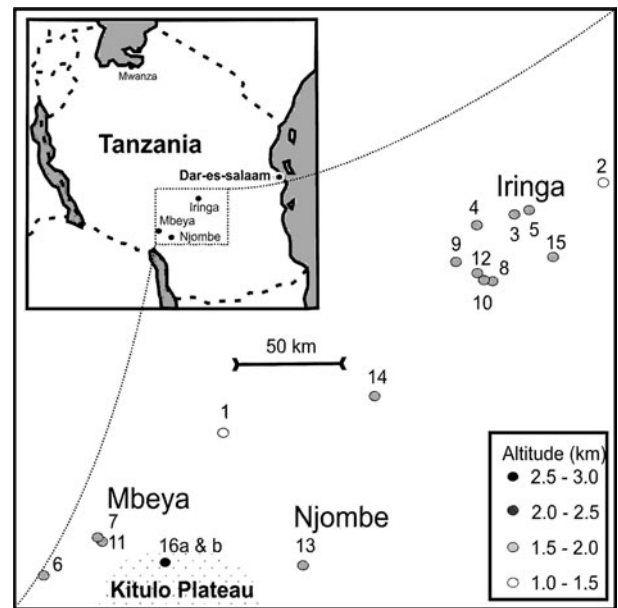

Fig. 1. Map of sampling sites at which lymnaeid snails were found. Locations numbered as in Table 1

were within the Southern Highlands of Tanzania, ranging from Iringa Town, Iringa Region to the Kitulo Plateau of the Mbeya Region. These sample sites range in altitude from 500 m to 2850 m above sea level. Snails were collected from several habitats consisting of streams, small ponds, rivers and swamps. Each habitat was visited on at least 3 occasions: at the beginning of the rainy season (November–December), at the end of the rainy season (May–June), and during the dry season (August–September). In places where snails were found the perimeter of the ponds was sampled at metre intervals and, where possible, the bottoms of shallow ponds were sampled at random points. Between 100 and 200 individuals were collected, and the type of habitat and geo-reference information was recorded. All snails were classified in the field using appropriate keys and field guides (Kristensen, 1987).

Identification of digenean larvae harboured within snails

Snails were screened for trematode infections by placing each into a 10 ml glass beaker with 5 ml of pond water and placed under strong artificial light for between 4 and 6 h to stimulate them to shed cercariae. Digenean larvae species were identified using the cercarial identification key as supplied within the African freshwater snails field guide as described previously by Frandsen and Christensen (1984).

DNA extraction and storage

Snails were initially washed in distilled water and then stored in 99% ethanol. DNA extractions were

performed using a Nucleon® Genomic DNA Extraction Kit (Tepnel Life Sciences) as per the manufacturer's instruction. Extracted DNA was resuspended in 50 μl of Tris-EDTA buffer. Only lymnaeid snails collected in June 2006 were subjected to the later molecular investigation.

Molecular identification of field-collected snail species

Field-based identification of lymnaeid snail species was confirmed using available phylogenetic markers (Stothard *et al.* 2000) based on sequence data of Bargues and Mas-Coma (1997) to amplify 18S rDNA nucleotide sequences coding for the variable V1 and V2 regions within the 18S helical 6 and E10-1 structures. Primers were as used by Stothard *et al.* (2000) except the GC clamp region, placed on the 5′ end for subsequent denaturing gradient gel electrophoresis was removed. Amplification reactions were performed in a total reaction volume of 25 μl containing: 100 mM Tris-HCL, pH 8·8, 1·5 mM MgCl$_2$, 0·2 mM dNTPs, 10 pmols of each primer, 50 ng genomic DNA and 1·0 U *Taq* polymerase (Invitrogen). After optimization, the following thermal cycling conditions were adopted: an initial denaturation of 94 °C for 2 min then 40 cycles of 94 °C for 1 min, 68 °C for 1 min, 72 °C for 1 min, then a final extension of 72 °C for 10 min before cooling to 4 °C. PCR products from 16 to 24 individual snails from each sampling site were cleaned (GenElute™ PCR Clean-up Kit; Sigma) and then commercially sequenced in both directions by Macrogen Inc. Resultant sequences were assembled and aligned against those from 61 other gastropods downloaded from GenBank using Clustal W (Thompson *et al.* 1994) within BioEdit (Hall, 1999). To provide support for the morphological and molecular identification of snail species, phylogenetic analysis was carried out within PAUP (Swofford, 1991) using parsimony. Group support was provided by 1000 bootstrap replicates.

Fasciolid infections within lymnaeid snails

Lymnaeids collected in the field were screened for infection with fasciolids with the aim of ascertaining which species, *F. gigantica* or *F. hepatica*, was present. Markers available in the scientific press were employed to screen snails for *F. hepatica* (Cucher *et al.* 2006) and *F. gigantica* (Mostafa *et al.* 2003; Velusamy *et al.* 2004) infections. Fasciolid species were confirmed by analysis of the 618 bp sequence of the 28S rRNA gene as described by Marcilla *et al.* (2002).

Morphology and size of eggs and adult liver fluke determination from the high altitude zone

Faecal samples were randomly collected from 92 out of 1000 dairy cattle at Kitulo dairy farm. The faecal samples were transported under cool conditions to the laboratory at Sokoine University, Morogoro. At the laboratory the detection of *Fasciola* spp. eggs was carried out using the Malachite green sedimentation technique described previously by Dinnik and Dinnik (1959). The eggs of all positive animals were mixed and 100 eggs were randomly picked with a pipette; their width and length were measured using a calibrated compound microscope. From the same farm, 16 adult flukes were collected from the bile duct of a Friesian bull calf (approx. 12 months old) that had been identified as harbouring mature flukes from analysis of faecal samples, as previously described. The flukes were preserved in 99% ethanol. At the laboratory, the size of the flukes (length and width) was recorded.

Morphology and size of eggs and adult liver fluke determination from the low altitude zone

In the low altitude zone, lengths and widths of a total 100 eggs of liver fluke randomly drawn from a mixture of gallbladder contents of 12 infected cattle slaughtered at Iringa abattoir were measured. In the same low altitude zone lengths and widths of 50 and 48 adult flukes from 10 and 14 cattle slaughtered at Mazizini (Dar es Salaam) and Babati (Manyara) respectively were measured.

Data analysis

Morphological data were analysed by Statistix® windows version 7 statistical software package. Descriptive statistics procedure was used to summarize the data in frequency distribution and histogram charts. T-test procedures were used to compare the sizes of the eggs/adults between the two zones.

Molecular identification of fasciolids using PCR and PCR-RFLP analysis

Fasciolids were detected in snails using PCR primers reported to be species specific (Mostafa *et al.* 2003; Velusamy *et al.* 2004; Cucher *et al.* 2006). Species identification of adult fasciolids collected was undertaken by employing the PCR-RFLP protocol designed by Marcilla and colleagues (2002).

Mitochondrial DNA analysis of adult fasciolids

Using the region of the mitochondrial genome identified as being most informative (Walker *et al.* 2007) we have produced 1250 bp amplicons of representative flukes from each population. These were commercially sequenced in forward and reverse directions and the resulting sequences submitted for analysis using BLAST (Altschul *et al.* 1997) software.

Table 1. *Sampling sites with altitude*

(Numbers in parentheses indicate location on map.)

Habitat Name	Habitat Description	Latitude; Longitude	Altitude (m)
Ruaha-Mbuyuni	Irrigation channel	07°27·96S; 036°29·83E	517
Rujewa District	Stagnant pond (1)*	08°40·64S; 034°26·89E	1090
Kimamba, Darajani, nr Ilula Town, Iringa District	Stream (2)*	07°39·67S; 036°05·27E	1250
Ilula Town stream	Shallow stream	07°40·62S; 036°02·42E	1372
Utige Pond	Shallow pond	08°50·61S; 034°39·92E	1463
Kibebe Farm, Iringa Town	Papyrus swamp	07°48·40S; 035°45·23E	1525
Ipogoro, Iringa Town	Swamp, Little Ruaha River (3)*	07°47·30S; 035°42·10E	1525
Litemela, Kalenga Area	Spillway of water reservoir (4)*	07°49·97S; 035°32·30E	1555
IRDC Farm, Iringa Town	1. Little Ruaha River	07°47·29S; 035°45·59E	1585
	2. Irrigation channel (5)*	07°46·29S; 035°45·59E	
Safisha Stream, Tunduma Town	Stream	09°18·78S; 032°46·35E	1615
Mbimba, nr Mbozi Town	Papyrus swamp (6)*	09°05·16S; 032°57·40E	1615
Kihesa-Kilolo, nr. Iringa Town	Water Reservoir	07°44·09S; 035°43·08E	1615
Mbeya Town	Stream, drinking water source (7)*	08°54·26S; 033°27·61E	1705
Mgama E Stream	Stream (8)*	08°01·28S; 035°35·64E	1707
Ifunda Farm, Iringa Town	Irrigation channel (9)*	07°58·89S; 035°27·21E	1739
Mgama W Stream, nr Ihbumila, Iringa District	Stream (10)*	08°01·20S; 035°35·27E	1737
Salaga River, Mbeya Town	River (11)*	08°53·81S; 033°35·35E	1829
Ibumila, S. Iringa District	Shallow pond (12)*	08°00·97S; 035°33·61E	1829
Kibena Marsh, Njombe	Large, freshwater marshland (13)*	09°12·22S; 034°47·04S	1836
Lake Ngwasi, Mufindi Area	Large, natural lake (14)*	08°31·81S; 035°06·75E	1860
Kilima Pond, Lulanzi Area	Small, shallow pond (15)*	07°57·38S; 035°51·83E	1890
Kitulo LMU, Shamba I, Kitulo Plateau	1. Pond & flushing stream (16a)**	09°05·94S; 033°54·22E	2712
	2. Pond & flushing stream (16b)**	09°06·03S; 033°54·22E	2720
Kitulo LMU, Shamba III, Kitulo Plateau	Pond & flushing stream	09°06·06S; 033°52·67E	2843

* *Lymnaea* (*Radix*) *natalensis* present.
** *Lymnaea* (*Galba*) *truncatula* present.

RESULTS

Distribution of lymnaeids

With regard to altitude, the first lymnaeid snail species (*L. natalensis* by morphology) was found at a location with an altitude of 1090 m having been absent at one lower sample site at 517 m (Table 1). *Lymnaea natalensis* was found at 15 of the 24 sites visited whereas snails identified as *L. truncatula* on morphological characteristics were only found at 2 adjacent sites (2712 m and 2720 m) on the Kitulo Plateau. These ponds were approximately 25 m apart, draining into a shared stream. *Lymnaea natalensis* was not found above an altitude of 1890 m.

Digenean larvae

Both *L. natalensis* and *L. truncatula* shed cercariae which were classified as belonging to the Gymnocephalous group indicating the presence of either fasciolid or paramphistome infections within these snails. *Lymnaea natalensis* was also found to harbour cercariae belonging to Schistosomatidae.

Molecular identification of field-collected snail species

Fig. 2 shows an extract from the phylogenetic analysis illustrating the relationships within the lymnaeids and containing the unknown Tanzanian morphs. The snails from the Kitulo Plateau were clustered with the *L. truncatula* GenBank sequence (Z73985) with bootstrapping support of 99·6%. The Iringa, Mbeya and Njombe samples were clustered with the *L. natalensis* GenBank sequences (AF192272-4) with bootstrapping support of 69%. These results confirm our hypothesis with regard to the existence of *L. truncatula* at Kitulo (GenBank Accession number EU152270) and *L. natalensis* (GenBank Accession number EU152269) at all sites below this altitude.

Identification of fasciolids – morphology

The mean size of the fluke eggs from the low zone was 155 μm (s.e. $\pm 9\cdot56\,\mu$m) in length by 89·6 μm (s.e. $\pm 8\cdot19\,\mu$m) in width with ranges of 130–180 μm whilst that from cattle grazing the Kitulo Plateau was 127 μm (s.e. $\pm 7\cdot38\,\mu$m) by 63·42 μm (s.e. $\pm 4\cdot19\,\mu$m) with ranges of 115–150 μm. Welch's approximate *t*-test gave a value for *t* of 2·318 with 196 degrees of freedom indicating that they were from significantly different populations (*P* = 0·0215). The flukes from the low altitude zone were larger than those from Kitulo and their mean body length to body width
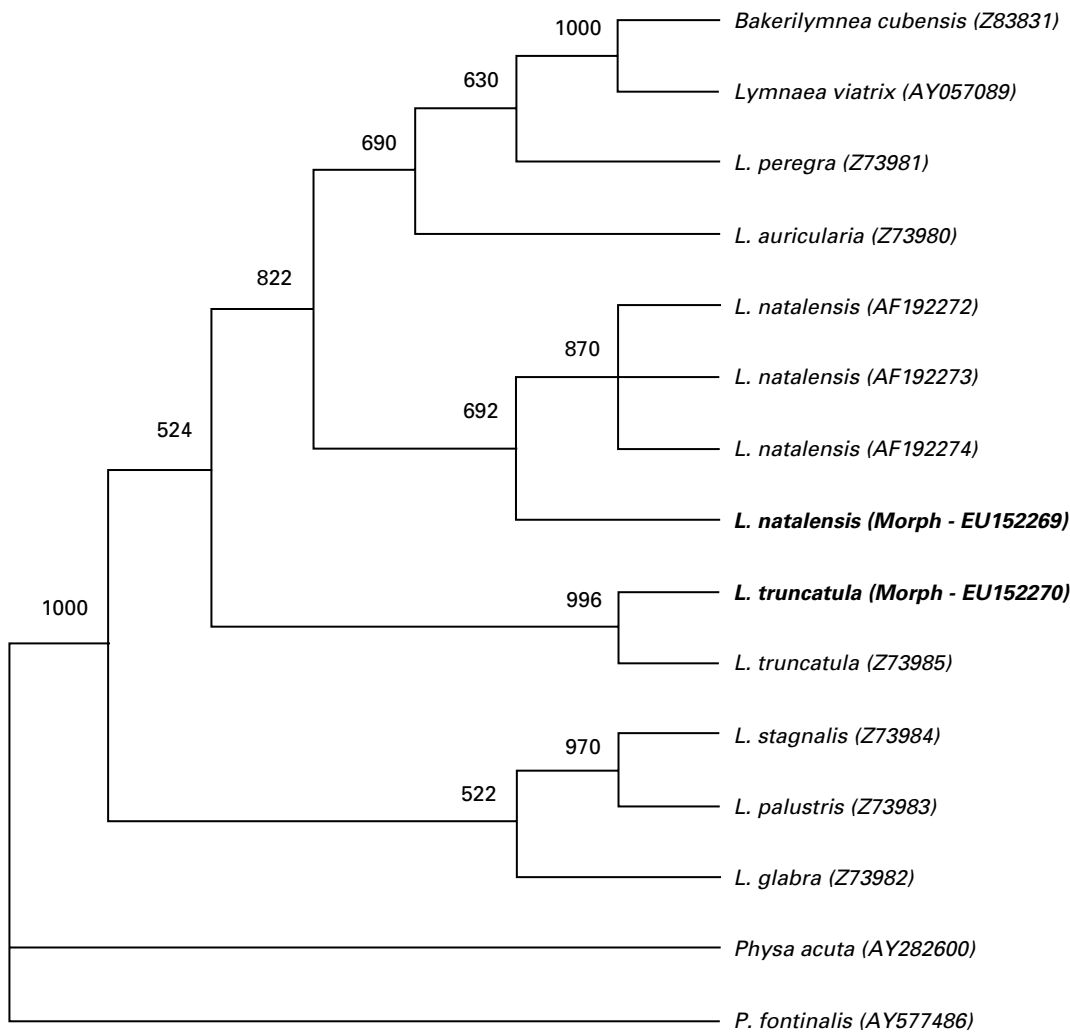
Fig. 2. Parsimony majority rule consensus tree based on 318 bp from the variable V1 and V2 regions within the 18S helical 6 and E10-1 structures of the 18S small subunit. Bootstrap support values based on 1000 replicates are shown at nodes. GenBank identification numbers follow species names. AF192272, *Lymnaea natalensis* from South Africa; AF192273, *L. natalensis* from Madagascar; AF192274, *L. natalensis* from Madagascar; Z73985, *L. truncatula* from Corsica, France.

ratio was 2·85 whereas that for the flukes from Kitulo was 1·59. Statistical analysis of the data using Welch's approximate '*t*'-test gave a value of t of 4·226 ($N = 107$) which gives a probability of $P < 0·0001$ that they were drawn from different populations.

Molecular techniques – fasciolid infections within lymnaeid snails

Using PCR primers to detect the presence of fasciolid infections a representative sample of snails from 4 out of the 5 lymnaeid populations suggested the presence of these trematodes in both *L. truncatula* and *L. natalensis* populations. However, when appropriate controls were employed it was evident that the molecular approach used was not able to discriminate between *F. hepatica* and *F. gigantica* species (results not shown). PCR-RFLP analysis of

28S rDNA from adult fasciolids produced fragments that were diagnostic for *F. hepatica* and *F. gigantica* and indicated that the flukes in all of the infected snails sampled from below 2000 m were *F. gigantica*. The restriction pattern with the PCR products from the snails from the Kitulo plateau was consistent with *F. hepatica* (Fig. 3).

Molecular techniques – BLAST analysis of fasciolid mtDNA

The sequences from the putative *F. hepatica* flukes (Accession number EU282862) gave 97% homology with the appropriate region of the complete mitochondrial sequence of *F. hepatica* (Accession number AF216697) whereas those from the putative *F. gigantica* flukes (Accession numbers EU282859, EU282860, EU282861) gave 90% sequence homology with the appropriate region of the complete
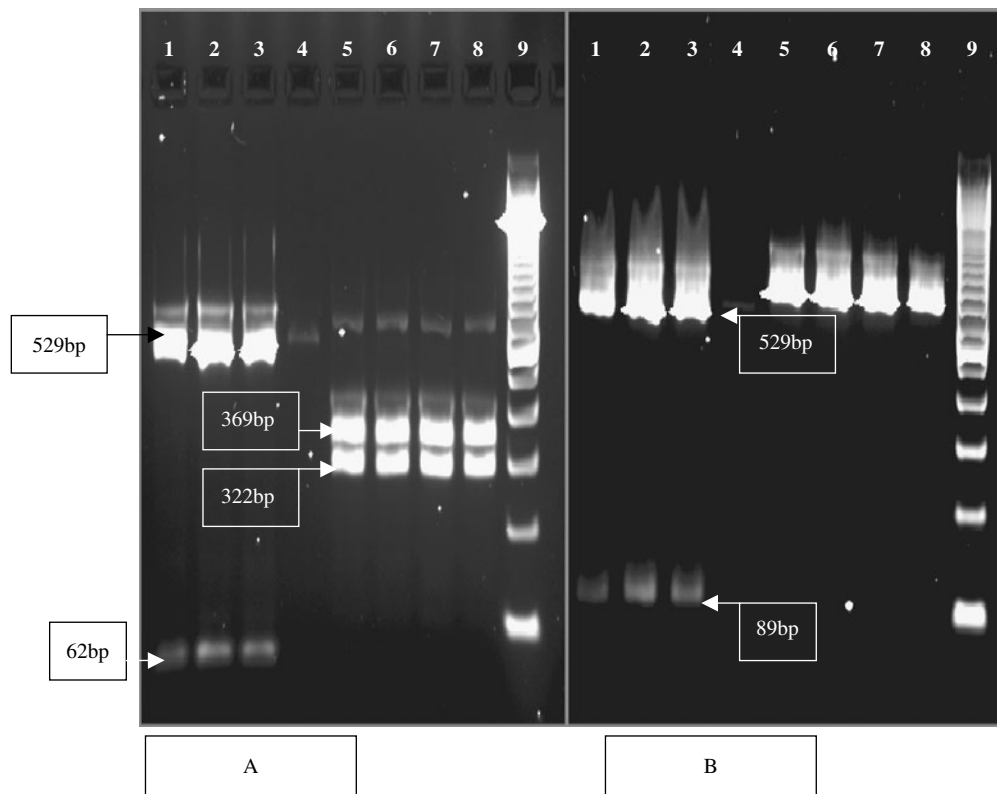
Fig. 3. Identification of fasciolid species by PCR-RFLP analysis of a 618 bp fragment amplified from 28S rRNA gene and digested with Ava*II* (3a – lanes 1–3 *Fasciola hepatica*, lanes 4–8 *F. gigantica*, lane 9 1 Kb DNA ladder) and Dra*II* (3b – lanes 1–3 *F. hepatica*, lanes 4–8 *F. gigantica*, lane 9 1 Kb DNA ladder)) (Marcilla *et al.* 2002).

mitochondrial sequence of *F. hepatica*. (Sequences for *F. gigantica* homologous to this region of its mtDNA are not available in GenBank.) These were the highest scoring homologues in each case.

DISCUSSION

The present findings validate earlier reports that *L. truncatula* exists in the East African tropical highlands including Tanzania (Dinnik and Dinnik, 1959; Kendall, 1965). The occurrence of this temperate snail in tropical highlands may be either the result of inadvertent introduction from other areas or the presence of a relict native population. Migratory birds from temperate countries have been implicated as possible vectors for freshwater pulmonate snails to such tropical regions (Brown, 1994). The Kitulo Plateau provides breeding grounds for several species of migratory birds from temperate regions such as the Blue Swallow (*Hirundo xantholopus* Sundevall, 1850), Pygmy Kingfisher (*Ceyx picta* Boddaert, 1783), and Denham's Bustard (*Neotis denhami* Children and Vigors, 1826). The alternative possibility, that the *L. truncatula* populations of the Kitulo Plateau are an indigenous species, is a question which could be addressed by further sampling and the development and deployment of highly informative phylogenetic markers for this species (Bargues and Mas-Coma, 1997; Stothard *et al.* 2000;

Bargues *et al.* 2001). *Lymnaea natalensis* 18S rDNA sequences were monomorphical across all populations sampled in this study although limited interspecific variation was detected. These changes occurred in the variable V2 region of the predicted RNA secondary structure (E10-1 helix) as reported by Stothard *et al.* (2000) for this species of snail. Likewise, the observed variation at the species level between *L. natalensis* and *L. truncatula* within the nucleotide sites 233 to 253 supports similar findings by other authors (Bargues and Mas-Coma, 1997).

In the present survey, *L. truncatula* was the only lymnaeid species observed to exist on the Kitulo Plateau at an altitude of approximately 3000 m. Such data support previous findings that this snail species is capable of adaptation to many different environments such as the very high altitudes (over 4000 m) of the Bolivian Altiplano (Jabbour-Zahab *et al.* 1997; Mas-Coma *et al.* 2001). The presence of *L. truncatula* on the Kitulo LMU farm further extends the problem of fasciolosis in domestic ruminants of this area where the presence of *L. natalensis* is limited by the extreme environmental conditions. Under both natural and experimental conditions, other lymnaeid snails species may be infected with *F. hepatica* (Soulsby, 1982) but it is widely accepted that variants of *L. truncatula* are the fundamental intermediate hosts (Malone, 1994). Studies have also shown that

L. natalensis is not a good intermediate host for *F. hepatica* (Boray, 1985). Therefore, as in Ethiopia, it would appear logical that the observed widespread distribution of *L. natalensis* in the moderately low altitude areas of the Southern Highlands of Tanzania in this present study, is responsible for the serious problem of ruminant fasciolosis due to *F. gigantica* in this area (Mahlau *et al.* 1970; Wilson, 1995; Makundi, 2001) and that *L. truncatula* is responsible for the recently reported problems with *F. hepatica* at Kitulo LMU farm (Namuba *et al.* 2003).

The origin of the *F. hepatica*-like flukes collected on the Kitulo plateau is of interest. The farm was originally stocked with Australian merino sheep and, more recently, dairy cattle of (ultimately) European origin suggesting that the flukes may have been imported with the sheep or cattle and became established due to the pre-existing presence of *L. truncatula*. We are currently comparing mtDNA sequences from these flukes with those from European specimens of *F. hepatica* in order to determine their provenance.

Although the flukes from the low altitude sample sites were within the range of lengths reported for *F. gigantica*, comparison with morphological data from Ashrafi and colleagues suggested that both the highland and lowland populations found in Tanzania were *F. hepatica*-like, in that the body width to length ratios of the populations were within the ranges quoted for *F. hepatica* in their extensive study (Ashrafi *et al.* 2006). However, PCR amplification of a partial fragment of the 28S ribosomal DNA gene from the flukes and digestion with *Ava* I and *Dra* II gave a more definitive resolution with restriction patterns consistent with the lowland flukes being *F. gigantica*-like and those from the Kitulo plateau being *F. hepatica*-like. We did not observe any RFLP patterns indicative of the presence of hybrid *Fasciola* spp. in either population. The high level of homology seen with the mitochondrial sequences from these flukes confirms their identification. A lower level of homology to the GenBank *F. hepatica* mitochondrial genome was seen with the lowland flukes, the relevant region of the *F. gigantica* mt genome is not present in GenBank.

It has been reported that *F. gigantica* eggs can embryonate and hatch successfully at 17–22 °C (Guralp *et al.* 1964). In this study *F. gigantica* was found at relatively high altitudes (<1800 m) where climatic conditions are within that range and comparable to those experienced by *F. hepatica* in much of Australia, South America and southern Europe. *F. hepatica* was only found above 2700 m where night temperature may drop to −6 °C. There was concordance between the presence of *L. truncatula*/ *F. hepatica*-like flukes and *L. natalensis*/*F. gigantica*-like flukes, suggesting that the determining factor in the distribution of *F. hepatica* and *F. gigantica* is not temperature *per se* but rather the presence of an intermediate host in which each species can reproduce efficiently.

In conclusion, the distribution of *F. hepatica* and *F. gigantica* seen in the Tanzanian Southern Highlands supports the hypothesis that the determining factor in the spread and establishment of either of these species is the pre-existing presence of their permissive intermediate host.

REFERENCES

Agatsuma, T., Arakawa, Y., Iwagami, M., Honzako, Y., Cahyaningsih, U., Kang, S. Y. and Hong, S. J. (2000). Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. *Parasitology International* **49**, 231–238.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Ashrafi, K., Valero, M. A., Panova, M., Periago, M. V., Massoud, J. and Mas-Coma, S. (2006). Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran. *Parasitology International* **55**, 249–260.

Bargues, M. D. and Mas-Coma, S. (1997). Phylogenetic analysis of lymnaeid snails based on 18S rRNA sequences. *Molecular Biology and Evolution* **14**, 569–577.

Bargues, M. D., Vigo, M., Horak, P., Dvorak, J., Patzner, R. A., Pointier, J. P., Jackiewicz, M., Meier-Brook, C. and Mas-Coma, S. (2001). European Lymnaeidae (Mollusca; Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Infection, Genetics and Evolution* **1**, 85–107.

Boray, J. C. (1985). Flukes of domestic animals. In *Parasites, Pests and Predators* (ed. Gaafar, S. M., Howard, W. E. and Marsh, R. E.), pp. 179–218. Elsevier, Amsterdam, Oxford, New York, Tokyo.

Botcher, M. (1967). Studies on the intermediate host of *F. gigantica* in Mwanza region. In *Proceedings of Veterinary Research Conducted at the Central Veterinary Laboratory in Dar es Salaam between 31st July to 2nd August 1967*. pp. 443–445.

Brown, D. S. (1994). *Freshwater Snails of Africa and their Medical Importance*. Taylor and Francis, London.

Cucher, M. A., Carnevale, S., Prepelitchi, L., Labbé, J. H. and Wisnivesky-Coli, C. (2006). PCR diagnosis of *Fasciola hepatica* in field-collected *Lymnaea collumella* and *Lymnaea viatrix* snails. *Veterinary Parasitology* **137**, 74–82.

Dar, Y., Rondelaud, D. and Dreyfuss, G. (2004). Cercarial shedding from *Galba truncatula* infected with *Fasciola gigantica* of distinct geographic origins. *Parasitology Research* **89**, 1432–1955.

Dinnik, J. A. and Dinnik, N. N. (1959). *Symposium on Helminthiasis in Domestic Animals*. *C.C.T.A. Nairobi*, No. 19, 43.

Ecmovic, T. J. and Mahlau, E. A. (1973). Animal health survey of Mbeya region, Tanzania. *Bulletin of Epizootic Diseases of Africa* **21**, 187–192.

Frandsen, F. and Christensen, N. O. (1984). An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. *Acta Tropica* **41**, 181–202.

Guralp, N., Oxcan, C. and Simms, B. T. (1964). *Fasciola gigantica* and fascioliasis in Turkey. *American Journal of Veterinary Research* **25**, 196–210.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Hammond, J. A. (1965). Observations of fascioliasis in Tanganyika. *Bulletin of Epizootic Diseases in Africa* **13**, 55–65.

Huang, W. Y., He, B., Wang, C. R. and Zhu, XQ. (2004). Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. *Veterinary Parasitology* **120**, 75–83.

Itagaki, T. and Tsutsumi, K. (1998). Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA. *International Journal for Parasitology* **28**, 777–781.

Jabbour-Zahab, R., Pointier, J. P., Balzan, C., Khallayoune, K. and Renaud, F. (1997). Phylogeography and genetic divergence of some lymnaeid snails, intermediate hosts of human and animal fasciliasis with special reference to lymnaeids from the Bolivian Altiplano. *Acta Tropica* **64**, 191–203.

Kendall, S. B. (1965). Relationship between species of *Fasciola* and their molluscan hosts. *Advances in Parasitology* **3**, 59–98.

Keyyu, J. D., Kassuku, A. A., Kyvsgaard, N. C. and Monrad, J. (2005). Epidemiology of *Fasciola gigantica* and amphistomes in cattle on traditional, small-scale dairy and large-scale dairy farms in Iringa District, Tanzania. *Tropical Animal Health and Production* **37**, 303–314.

Kristensen, T. K. (1987). *A Field Guide to African Freshwater Snails. 2, East African Freshwater Snails.* 2nd Edn. pp. 1–51. Charlottenlund: Danish Bilharziasis Laboratory, Denmark.

Mahlau, E. A. (1970). Liver fluke survey in Zebu cattle of the Iringa region of Tanzania and the first finding of the small fluke *Dicrocoelium hospes* (Loos, 1907). *Bulletin of Epizootic Diseases of Africa* **18**, 21–28.

Makundi, A. E. (2001). Epidemiology and control of fasciolosis and schistosomosis in the Southern Highlands of Tanzania. Ph.D. thesis. Sokoine University of Agriculture, Tanzania.

Malone, J. B. (1994). The landscape epidemiology of fasciolosis: geographic determinants of the disease risk. In *Immunology, Pathobiology and Control of Fasciolosis* (ed. Boray, J. C.), pp. 65–81. ICOPA VIII, Izmir, MSD AGVET.

Malone, J. B., Gimmes, R., Hansen, J., Yilma, J. M., Slingenberg, J., Snijders, F., Nachtergaele, F. and Ataman, E. (1998). A geographic information system of the potential distribution and abundance of *Fasciola hepatica* and *F. gigantica* in East Africa based on Food and Agriculture Organization databases. *Veterinary Parasitology* **78**, 87–101.

Marcilla, A., Bargues, M. D. and Mas-Coma, S. (2002). A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Molecular and Cellular Probes* **16**, 327–333.

Mas-Coma, S., Bargues, M. D. and Valero, M. A. (2005). Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology* **35**, 1255–1278.

Mas-Coma, S., Funatsu, I. R. and Bargues, M. D. (2001). *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology Research* **87**, 1016–1019.

Mitchell, G. B. (2002). Update on fasciolosis in cattle and sheep. *In Practice* **24**, 378–385.

Mostafa, O. M., Taha, H. A. and Ramadan, G. (2003). Diagnosis of *Fasciola gigantica* in snails using the polymerase chain reaction (PCR) assay. *Journal of the Egyptian Society for Parasitology* **33**, 733–742.

Msanga, J. F. (1985). Prevalence and economic importance of *Fasciola gigantica* and *Stilesia hepatica* in Sukumaland, Tanzania. *Tanzania Veterinary Bulletin* **7**, 9–16.

Namuba, F. V., Makundi, A. E. and Kassuku, A. A. (2003). Studies of fasciolosis in the domestic stock with special reference to the finding of *Fasciola hepatica* for the first time in the southern highland of Tanzania. In *Proceedings of the 23rd Tanzania Veterinary Association Conference, Arusha, 2–4th December 2003.*

Ogambo-Ongama, A. H. (1969). The incidence of *Fasciola hepatica* in Kenya cattle. *Bulletin of Epizootic Diseases of Africa* **17**, 420–431.

Ogambo-Ongoma, A. H. (1972). Fascioliasis survey in Uganda. *Bulletin of Epizootic Diseases of Africa* **20**, 35–41.

Seehausen, O. (2004). Hybridization and adaptive radiation. *Trends in Ecology and Evolution* **19**, 198–207.

Soulsby, E. J. L. (1982). *Helminths, Arthropods and Protozoa Parasites of Domesticated Animals.* Baillere-Tindall. London.

Spithill, T. W. and Dalton, J. P. (1998). Progress in development of liver fluke vaccines. *Parasitology Today* **14**, 224–228.

Stothard, J. R., Bremond, P., Andriamaro, L., Loxton, N. J., Sellin, E. and Rollinson, D. (2000). Molecular characterisation of the freshwater snail *Lymnaea natalensis* (Gastropoda:Lymnaeidae) with an observation of an unusual polymorphism in ribosomal small subunit genes. *Journal of the Zoological Society of London* **252**, 303–315.

Swofford, D. L. (1991). *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.* Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

Trouvé, S., Degen, L., Renaud, F. and Goudet, J. (2003). Evolutionary implications of a high selfing rate in

the freshwater snail *Lymneae truncatula*. *Evolution* **57**, 2303–2314.

Velusamy, R., Singh, B. P. and Raina, O. K. (2004). Detection of *Fasciola gigantica* infection in snails by polymerase chain reaction. *Veterinary Parasitology* **120**, 85–90.

Walker, S. M., Fletcher, H. L., Hoey, E. M., Fairweather, I. and Trudgett, A. (2006). Stage-specific differences in fecundity over the lifecycle of two characterised isolates of the liver fluke, *Fasciola hepatica*. *Parasitology* **133**, 209–216.

Walker, S. M., Prodöhl, P. A., Fletcher, H. L., Hanna, R. E. B., Kantzoura, V., Hoey, E. M. and Trudgett, A. (2007). Evidence for multiple mitochondrial lineages of *Fasciola hepatica* (liver fluke) within infrapopulations from cattle and sheep. *Parasitology Research* **101**, 117–125.

Wilson, R. T. (1995). Animal health and disease control in the Usangu Wetland of Southwestern Tanzania. *Tropical Animal Health and Production* **35**, 47–67.

Wolstenholme, A. J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G. and Sangster, N. C. (2004). Drug resistance in veterinary helminths. *Trends in Parasitology* **20**, 469–476.

Yilma, J. M. and Malone, J. B. (1998). A geographic information system forecast model for strategic control of fasciolosis in Ethiopia. *Veterinary Parasitology* **78**, 103–127.