

ResearchOnline@JCU

This file is part of the following reference:

van Herwerden, Lynne (1998) *The trematode genera Paragonimus and Schistosoma in East Asia: molecular evolution, phylogeny and biogeography*. PhD thesis, James Cook University.

Access to this file is available from:

<http://eprints.jcu.edu.au/24131/>

The author has certified to JCU that they have made a reasonable effort to gain permission and acknowledge the owner of any third party copyright material included in this document. If you believe that this is not the case, please contact ResearchOnline@jcu.edu.au and quote <http://eprints.jcu.edu.au/24131/>

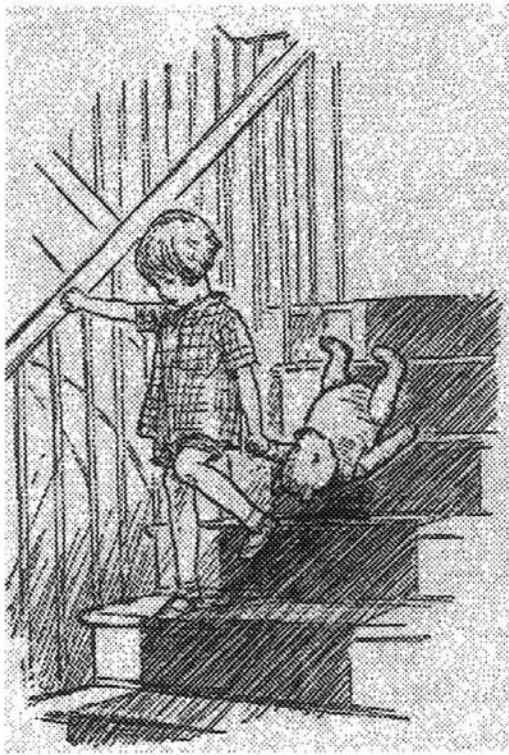
**The trematode genera *Paragonimus* and *Schistosoma* in East Asia :
molecular evolution, phylogeny and biogeography.**

Thesis submitted by

Lynne van Herwerden BSc., BSc. (Med.) Hons., MSc. University of Cape Town.

for the degree of Doctor of Philosophy
in the School of Biological Sciences (Zoology)
James Cook University of North Queensland

Dedication -
For my family



“... bump, bump, bump It is as far as he knows, the only way of coming downstairs, but sometimes he feels that there really is another way, if only he could stop bumping for a moment and think of it. And then he feels that perhaps there isn't.”

taken from *Winnie - the - Pooh*,

- A.A. Milne, illustration by E.H. Shepard

STATEMENT OF ACCESS

I, the undersigned, the author of this thesis, understand that James Cook University of North Queensland will make it available for use within the University Library and, by microfilm or other means, allow access to users in other approved libraries. All users consulting this thesis will have to sign the following statement:

In consulting this thesis I agree not to copy or closely paraphrase it in whole or in part without the written consent of the author; and to make proper written acknowledgment for any assistance which I have obtained from it.

Beyond this, I do not wish to place any restriction on access to this thesis.

(Name)

29-06-1998

(Date)

**STATEMENT ON SOURCES
DECLARATION**

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given.

(Name)

..... 29-06-1998

(Date)

ACKNOWLEDGMENTS

I am indebted to Dr. Takeshi Agatsuma and his colleagues, also to Prof. Don McManus and Dr. Paul Brindley for donating specimens and for their interest in this work. I would like to thank Dr. Robin Gasser for doing SSCP analyses on *Paragonimus* samples for me (Chapter 8) and generally for the interest that he has shown, particularly for valuable comments made during the finalisation of the manuscript from Chapter 8. Dr. Mark Blows gave me a statistical solution to my queries (chapter 6) and Dr. Julian Caley wrote a suitable Basic program and did the statistical analyses for me, (chapter 6). In addition he has contributed substantially to the preparation of the manuscript, based on chapter 6, as a co-author and I gratefully acknowledge his contributions.

I would like to thank many unknown reviewers of work presented here, for their valuable comments during the process of manuscript revision for publication. Thanks to my fellow postgraduate students that have shared in the frustrations and the moments of elation with me, particularly Faye Christidis, Jess Morgan, Dani Tikel, Dave Slaney, Simon Cook, Amanda Brooker and Vicki Hall - thanks for many discussions and pots of coffee, not to mention the copious amounts of chocolate.

My family deserves more gratitude than I can express. Firstly, I wish to thank my parents for providing me with the foundations, upon which I have been able to build my dreams, of which the culmination of this thesis is one. They have provided me with a strong work ethic and are always there when support of any kind is needed, despite the great distance between us. Secondly, Carl, my husband has always had faith in me and has boosted me with his loving support at all times. Finally, my children, Calyn and Leon, deserve a very special thanks for keeping my feet on the ground and providing me with the other half of being.

This thesis would not have been possible without the support and patience of my supervisor, Assoc. Prof. David Blair. Thank you for being enthusiastic and interested in the project and for giving me the freedom to explore.

ABSTRACT

Trematode infections affect one fifth of the worlds' population, with more than 200 million Chinese alone being at risk (World Health Organisation 1997, Hotez *et al.* 1997). Of particular interest to this study are the Asian species of the trematode genera *Paragonimus* and *Schistosoma*, which infect many mammals, including humans. It is becoming clear that within both genera species complexes exist that require further characterisation. Molecular markers have given some insight into the nature of these complexes. In this thesis additional molecular markers are investigated as sources of information about the nature and distribution of the *S. japonicum*, *P. westermani* and *P. ohirai* species complexes. These two genera are of additional interest because they are thought to have shared a similar biogeographic history in Asia, which should be reflected in their molecular evolution.

I have obtained DNA sequences from one nuclear (ITS1) and one mitochondrial (ND1) marker of five *Paragonimus* species (*P. westermani*, *P. miyazakii*, *P. macrorchis*, *P. ohirai* and *P. iloktsuenensis*) present in East Asia, including numerous geographic isolates of the *P. westermani* and *P. ohirai* species complexes. In addition, I have obtained ITS1 sequences of three schistosome species (*S. japonicum*, *S. malayensis* and *S. mekongi*) present in East Asia, which comprise the *S. japonicum* species complex and an African schistosome species (*S. mansoni*) for comparative purposes. I could differentiate all species using ITS1 and ND1 sequence data.

ITS1 is contained within the ribosomal DNA gene cluster, which is repeated in tandem many times. ITS1 does not experience the constraints of coding regions and theoretically undergoes sequence homogenisation within individuals and species. Despite this, intra-individual sequence variation was sometimes greater than inter-species variation, particularly among northeast Asian *P. westermani* isolates. Such variation confounded phylogenetic inferences for this group. There are some interesting aspects relating to processes of concerted evolution generally, when considering the differences within and between the two trematode genera, *Paragonimus* and *Schistosoma*, in this study. Additionally, a transcriptional enhancer motif (TATAAT) is embedded within the repeats of the ITS1 of the Asian schistosomes. The repeats are the cause of size variation and given the improbability of such motifs occurring by chance, I propose that the

abundant, large variants containing multiple copies of repeats may have a role in the stage- or tissue-specific regulation of transcription of the ribosomal genes.

In contrast, the ND1 gene codes for a mitochondrial protein and is therefore functionally constrained. Phylogenetic inferences could be made from ND1 sequence data obtained from *Paragonimus*. However, many clones had to be sequenced per individual to achieve this, as multiple ND1 lineages occurred within individuals of all *Paragonimus* species and strains investigated. Presumed pseudogenes were identified, which may be present in either the nuclear genome or in different types of mitochondria, as *Paragonimus* species have two types of mitochondria which differ structurally. It seems likely that both nuclear pseudogenes and heteroplasmic mitochondrial genes occur, though this hypothesis remains to be tested.

The *Paragonimus westermani* species complex has been further studied, to determine the origins of parthenogenetic triploid forms. Ribosomal DNA-ITS restriction fragments, ND1 sequences and simple sequence repeat (SSRs) “fingerprints” of triploids from China, Korea and Japan all indicate that the triploids are genetically different from one another. I conclude from this that triploids may have arisen more than once independently, possibly by relatively rare matings between diploid and tetraploid *P. westermani* individuals, which occur in sympatry with the triploids in NE China. Alternatively, triploid lineages may have arisen once and diverged subsequently by mutation alone.

Despite their suggested shared biogeographic history, members of the two genera exhibit rather different properties with respect to their ND1 and ITS1 genes. This is likely to be a reflection of their different phylogenetic histories. Molecular techniques have been used successfully to infer phylogenies which could be used to evaluate a previously published biogeographic hypothesis, but only to a limited extent, using the markers. It is clear that the situation for *P. westermani* in particular is complex and requires further investigation. Molecular evolution of these markers has proved to be an interesting component of the study that has brought to light some novel ideas and applications of these markers, not as phylogenetic tools, but as tools to study processes of concerted evolution in the nuclear genome and mitochondrial evolution.

CONTENTS

Title.....	i
Dedication	ii
Statement of access.....	iii
Declaration.....	iv
Acknowledgments.....	v
Abstract.....	vi
Table of contents.....	viii
List of tables.....	xii
List of figures.....	xiv
List of publications arising from this study.....	xvii
Conference proceedings.....	xviii
Departmental conferences.....	xviii
Chapter 1. General Introduction.....	1
1.1 Background.....	1
1.2 Thesis structure.....	2
Chapter 2. Literature Review.....	5
2.1 Background - why study these trematodes?.....	5
2.1.1 The genus <i>Paragonimus</i>	5
2.1.2 The genus <i>Schistosoma</i>	8
2.2 Asian biogeography and the Davis Hypothesis.....	10
2.3 Predictions of Davis' Hypothesis	16
2.4 Why use molecular techniques?.....	17
2.4.1 Molecules versus morphology.....	17
2.4.2 Which genome - nuclear or mitochondrial?.....	18
2.4.2a Nuclear DNA.....	19
2.4.2b Mitochondrial DNA.....	21
2.5 Sequence alignment.....	22

2.6 Genetic distance estimates.....	23
2.7 Phylogeny reconstruction procedures.....	25
2.7.1 Quantitative distance methods.....	25
2.7.2 Qualitative character-state methods.....	26
2.7.3 Evaluating phylogenetic inferences.....	28
Chapter 3. General materials and methods	
3.1 Sample material preparation.....	31
3.2 DNA extraction procedures.....	32
3.3 DNA amplification and PCR primers.....	32
3.4 Cloning.....	34
3.5 Sequencing.....	34
3.6 Sequence alignment.....	36
3.7 Phylogenetic analysis.....	36
Chapter 4. Intra- and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the <i>Schistosoma japonicum</i> species complex	
4.1 Abstract.....	37
4.2 Introduction.....	37
4.3 Materials and Methods.....	39
4.3.1 DNA extraction, PCR, cloning and sequencing.....	39
4.3.2 Genomic digests and Southern blots.....	39
4.4 Results and Discussion.....	40
Chapter 5. Intra - and inter- individual variation in ITS1 of <i>Paragonimus westermani</i> (Trematoda: Digenea) and related species	
5.1 Abstract.....	49
5.2 Introduction.....	49
5.3 Materials and Methods.....	50
5.4 Results & Discussion.....	52
5.4.1 Variation in repeat sequences.....	52
5.4.2 Variation in “post-repeat” sequences.....	52

5.4.3 Relative abundance of ITS1 size variants.....	55
5.4.4 ITS1 variants: implications for processes of concerted evolution.....	55

Chapter 6. Multiple repeats in ITS1 variants of some trematodes (Digenea) contain a putative enhancer.

6.1 Abstract.....	58
6.2 Introduction.....	59
6.3 Materials and Methods.....	61
6.4 Results & Discussion.....	63

Chapter 7. Intra - and inter- individual variation in ITS1 of *Paragonimus* and *Schistosoma*: phylogenetic inferences

7.1 Abstract.....	69
7.2 Introduction.....	70
7.3 Materials and Methods.....	71
7.3.1 DNA extraction and related methods.....	71
7.3.2 Phylogenetic analysis.....	71
7.4 Results & Discussion.....	72
7.4.1 Phylogenetic analysis of <i>Paragonimus</i> species.....	72
7.4.2 Phylogenetic analysis of <i>Schistosoma</i> species.....	73
7.5 Conclusions.....	84

Chapter 8 Multiple variants of NADH Dehydrogenase subunit 1 (ND1) genes in some trematodes.

8.1 Abstract	87
8.2 Introduction	87
8.3 Materials and Methods	88
8.3.1 ND1 amplification, cloning and sequencing	88
8.4 Results & Discussion.....	89
8.4.1 Multiple forms of ND1	89
8.4.2 Phylogenetic analysis	90
8.4.3 Molecular variation summarised.....	97

8.4.4 Pseudogenes and <i>numt</i> DNA	98
8.4.5 Pseudogenes and heteroplasmy	99
8.5 Conclusions	103

**Chapter 9 Clonal diversity in populations of a parthenogenetic trematode,
Paragonimus westermani.**

9.1 Abstract.....	104
9.2 Introduction.....	104
9.3 Materials and Methods.....	106
9.3.1. DNA extraction.....	106
9.3.2. Genomic digests and Southern blots.....	107
9.3.2a rDNA variants	107
9.3.2b Fingerprinting of SSRs.....	107
9.3.3. ND1 amplification, cloning and sequencing	108
9.4 Results.....	108
9.4.1 rDNA-ITS variation.....	108
9.4.2. ND1 sequences	108
9.4.3. SSR fingerprints.....	109
9.5 Discussion.....	112
9.5.1 rDNA RFLPs	112
9.5.2 ND1 sequences	115
9.5.3 Fingerprinting data	115
9.6 Conclusions	115

Chapter 10 General Discussion

10.1.1 Aims	116
10.1.2 Phylogenetic hypotheses	116
10.1.3 Biogeographic hypotheses	117
10.1.4 Proposed single clonal origin of triploid <i>P. westermani</i>	119
10.2 Molecular evolution	120
10.2.1a ITS1 variation in trematodes and implications for processes of concerted evolution	120

10.2.1b Putative regulation of rDNA transcription by ITS1 in trematodes	121
10.2.2a ND1 pseudogenes and presumed numt DNA in trematodes	121
10.2.2b ND1 pseudogenes and heteroplasmy in trematodes	121
10.3 Concluding comments	122
References	123

LIST OF TABLES

Chapter 1. General Introduction.

Chapter 2. Literature Review.

Table 2.1 Asian schistosomes, their transmitting snail hosts and geographic locations	15
Table 2.2 Critical values of the g_1 statistic.	29

Chapter 3. General Materials and Methods

Table 3.1 Sample material used in this study	33
Table 3.2 PCR primers used in this study	35

Chapter 4. Intra- and inter-specific variation in nuclear ribosomal Internal transcribed spacer 1 of the *Schistosoma japonicum* species complex.

Table 4.1 % Pairwise differences of repeat α sequences in ITS1 clones in three Asian schistosomes.	42
---	----

Chapter 5. Intra - and inter- individual variation in ITS1 of *Paragonimus westermani* (Trematoda: Digenea) and related species.

Table 5.1 <i>Paragonimus</i> species investigated, indicating geographic origin, whether complete or "post-repeat" only ITS1 sequences were obtained and whether Southern blot (Sb) data is available.	51
--	----

Chapter 6. Multiple ITS1 variants and putative transcriptional enhancers in Trematodes (Digenea).

Table 6.1. Platyhelminth ITS sequences compared in this study, for the presence of “promoter” motifs.	62
Table 6.2 The occurrence of promoter motifs in ITS1 of trematode species and a cestode.....	64

Chapter 7 Intra - and inter- individual variation in ITS1 of two trematode (Digenea) genera, *Paragonimus* and *Schistosoma*: phylogenetic inferences.

Table 7.1: <i>Paragonimus</i> species indicating geographic origin of clones sequenced and Genbank numbers for variants.....	75
Table 7.2: Asian <i>Schistosoma</i> species investigated here	76

Chapter 8 Multiple lineages of the mitochondrial gene NADH Dehydrogenase subunit 1 (ND1) in some trematodes.

Table 8.1 Trematode ND1 clones sequences utilised in this study	90
Table 8.2: Pairwise differences in amino acids (above the diagonal) and nucleotides (below the diagonal) for different types of ND1.....	95

Chapter 9 Clonal diversity in parthenogenetic triploid *Paragonimus westermani*.

Table 9.1 <i>Paragonimus westermani</i> isolates investigated, indicating which data was obtained for particular isolates	106
Table 9.2 Pairwise differences among ND1 sequences of diploid and triploid <i>P. westermani</i> in East Asia	109
Table 9.3 Comparison of fingerprint bands of three triploid isolates of <i>P. westermani</i> , using SSRs	112

Chapter 10 General Discussion

LIST OF FIGURES

Chapter 1. General Introduction.

Chapter 2. Literature Review.

Fig. 2.1 Phylogenetic tree of species of <i>Paragonimus</i> based on CO I sequence data.	6
Fig. 2.2 Dorsal view of an adult <i>P. westermani</i>	7
Fig. 2.3 Asian distribution of parasite genera	8
Fig. 2.4 Paired adult <i>S. japonicum</i>	9
Fig. 2.5 Phylogeny of <i>Schistosoma</i> and <i>Paragonimus</i> transmitting snail hosts	12
Fig. 2.6 Map of the Asian rivers down which snails speciated	13
Fig. 2.7 Congruent phylogenies of transmitting snails and a) <i>Schistosoma</i> and b) <i>Paragonimus</i> species, based on COI and ITS2 sequence data.....	16
Fig. 2.8 Ribosomal DNA unit, illustrating position of ITS1 and ITS2	20

Chapter 3. General Materials and Methods

Fig. 3.1 Diagram of rDNA indicating position, direction and sequence of PCR primers used to amplify and sequence ITS1	34
--	----

Chapter 4. Intra- and inter-specific variation in nuclear ribosomal Internal transcribed spacer 1 of the *Schistosoma japonicum* species complex.

Fig. 4.1 ITS1 PCR products of <i>S. japonicum</i>	43
Fig. 4.2 a) Diagram based on Southern blots (b) of restriction digested <i>S. japonicum</i> genomic DNA, probed with the ITS1 “post-repeat” fragment from <i>S.</i> <i>japonicum</i>	44
Fig. 4.3. Alignment of ITS1 repeats from Asian schistosomes.....	47

Chapter 5 Intra - and inter- individual variation in ITS1 of *Paragonimus westermani* (Trematoda: Digenea) and related species

Figure 5.1: Diagram of sequenced ITS1 variants, indicating intra-individual differences between clones	53
Figure 5.2: Alignment of ITS1 repeat sequences of 12 clones from <i>Paragonimus</i> species	54

Figure 5.3: Diagram and autoradiograph of Southern blot of restriction digested <i>Paragonimus</i> species	56
--	----

Chapter 6. Multiple ITS1 variants and putative transcriptional enhancers in Trematodes (Digenea).

Chapter 7. Intra- and inter-individual variation in ITS1 of two trematode (Digenea) genera, *Paragonimus* and *Schistosoma*: implications for phylogenetic studies.

Fig. 7.1 Sequence alignment of post-repeat sequences from <i>Paragonimus</i> species.....	77
Fig. 7.2 Strict consensus tree of ITS1 sequences from 16 individuals of <i>Paragonimus</i> species.....	81
Fig. 7.3 Sequence alignment of post-repeat sequences from <i>Schistosoma</i> species.....	82
Fig. 7.4 MP consensus tree of ITS1 sequences from the Asian <i>Schistosoma japonicum</i> species complex	85

Chapter 8. Multiple lineages of NADH Dehydrogenase subunit 1 (ND1) genes in some trematodes.

Fig. 8.1 Partial ND1 sequence alignment of representative clones of each ND1 type detected	91
Fig. 8.2 Partial ND1 amino acid sequence alignment	94
Fig. 8.3a 70% Majority rule consensus tree of ND1 sequences from <i>Paragonimus</i> and other trematode genera	101
Fig. 8.3b Summary phylogenetic tree of data presented in Fig. 2a	102

Chapter 9 Clonal diversity in parthenogenetic triploid *Paragonimus westermani*.

Figure 9.1: Autoradiograph of Southern blot of <i>Paragonimus</i> species probed with a “post-repeat” ITS1 fragment.	111
---	-----

Figure 9.2: Fingerprints of *P. westermanni* triploids from China, Korea and Japan based on a) (ATGT)₇ probed *Alu* I fingerprints; b) (ATGT)₇ probed *Hinf* I fingerprints; c) (ATT)₁₀ probed *Alu* I fingerprints; d) (ATT)₁₀ probed *Hinf* I fingerprints.....114

LIST OF PUBLICATIONS

The following papers have been produced from material within this thesis.

- 1) D. Blair, L. van Herwerden, H. Hirai, S. Habe, M. Hirata, K. Lai, S. Upatham, T. Agatsuma (1997) Relationships between *Schistosoma malayensis* and other Asian schistosomes deduced from DNA sequences. *Molecular & Biochemical Parasitology* **85**: 259 - 263.
- 2) L. van Herwerden, D. Blair & T. Agatsuma (1998a) Intra- and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. *Parasitology* **116**: 311-317.
- 3) L. van Herwerden, D. Blair & T. Agatsuma (1998b) Intra - and inter-individual variation in ITS1 of *Paragonimus westermani* (Trematoda: Digenea) and related species: implications for phylogenetic studies. (In press) *Molecular Phylogenetics and Evolution*.
- 4) L. van Herwerden, D. Blair & T. Agatsuma (1998c) Clonal diversity in parthenogenetic triploid *Paragonimus westermani*. (submitted) *International Journal of Parasitology*
- 5) L. van Herwerden, J. Caley and D. Blair (1998) Multiple ITS1 variants and putative transcriptional enhancers in trematodes (Digenea). *Nucleic Acids Research* (in preparation)
- 6) L. van Herwerden & D. Blair. Multiple lineages of NADH Dehydrogenase subunit 1 (ND1) genes in some trematodes. (submitted) *Journal of Molecular Evolution*.

CONFERENCE PROCEEDINGS

1. L. van Herwerden, D. Blair, T. Watanobe & T. Agatsuma. (1996). Molecular phylogenetic studies of the *Paragonimus westermani* species complex. *PASEAN: [Parasites in South East Asia Now.]*The Australian Society for Parasitology and The Parasite Control Association of Indonesia. Bali, Indonesia, September 1996.
2. L. van Herwerden and D. Blair, (1997). Comparison of nuclear and mitochondrial sequences for phylogenetic studies of human lung flukes. *Genetics Society of Australia, (GSA) Perth, Western Australia, September 1997.*
3. L. van Herwerden and D. Blair, (1998) Multiple lineages of NADH Dehydrogenase subunit 1 (ND1) genes in some trematodes. *Genetics Society of Australia, Sydney, New South Wales, July 1998.*

DEPARTMENTAL CONFERENCES

1. L. van Herwerden (1996). ITS sequences as a tool in phylogenetic studies of some trematodes. *ZONQ: Zoology in North Queensland*, James Cook University, Townsville, North Queensland, April 1996.