Investigating genetic population substructure of an Australian reptile tick, *Bothriocroton hydrosauri*, using highly polymorphic microsatellite markers

Jaro Guzinski

Bsc (Hons)

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School of Biological Sciences, Faculty of Science and Engineering, Flinders University.

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Abstract

Despite long-term study, the mechanism explaining the parapatric distribution of two Australian reptile ticks species, *Bothriocroton hydrosauri* and *Amblyomma limbatum*, is not understood. This project aimed to use molecular genetic data to investigate aspects of the population biology of these two tick species, such as population structure and dispersal, to gain further insights into the cause and maintenance of this parapatric boundary. I developed and subsequently tested for Mendelian inheritance a suite of *B. hydrosauri* and *A. limbatum* species-specific microsatellites markers. Pedigree analysis showed one *B. hydrosauri* locus and all of the *A. limbatum* loci to be inherited in a non-Mendelian manner. Thus I could not investigate *A. limbatum* population structure and focused solely on *B. hydrosauri*.

The first part of this study tested predictions of a model formulated to explain *B. hydrosauri* transmission dynamics. The "ripple" model, based on detailed ecological and behavioural data on *B. hydrosauri* and *Tiliqua rugosa*, *B. hydrosauri*'s most common host, predicts higher relatedness among larvae than among nymphs or adults on a host, and significant spatial autocorrelation in larvae extending further than for the later life stages. The model also predicts that adult ticks are likely to encounter related partners and that this will generate inbreeding within the population. I tested those predictions using nine microsatellite loci on a sample of 848 ticks (464 larvae, 140 nymphs and 244 adults) collected from 98 *T. rugosa* hosts at the northern edge of *B. hydrosauri*'s distribution range. My data did support all of the predictions of the "ripple" model and indicated that the dynamics of transmission among hosts play an important role in parasite population structure.

The second part of this project focused on investigating the population genetic structure of *B. hydrosauri* at the edge of its geographic range and testing the predictions of a population model derived to explain *B. hydrosauri*'s parapatric boundary with *A. limbatum*. The "ridge and trough" model suggested the tick population was organised spatially into a series of "ridges" where tick density was high and "troughs" where it was low. Genetically, the expectation was to find clusters of more closely related individuals associated with the ridges. Cluster analysis of microsatellite allele frequencies and analysis of molecular variance of mitochondrial haplotype frequencies revealed the presence of four genetic clusters within a sample of 244 *B. hydrosauri* adults. As the highly genetically divergent

clusters had overlapping distributions, and in some cases were syntopic, the genetic population structure predicted for these ticks by the "ridge and trough" model was not observed. Several explanations were considered for the observed *B. hydrosauri* genetic population structure, but syntopy of the clusters suggested that assortative mating is the most likely. I speculated that the clusters have formed in allopatry, when the environment was extremely heterogeneous, such that the ticks (and their hosts) were confined to isolated patches of high-quality habitat. Given sufficient time, this could have resulted in reproductive incompatibility between ticks occupying different patches. The population structure I uncovered indicates subsequent secondary recontact of divergent groups.

Although my study allowed for a better understanding of *B. hydrosauri* biology and population structure, the reasons for the parapatric distributions of *B. hydrosauri* and *A. limbatum* are still unclear. Further research should focus on investigating the population genetic structure of *A. limbatum* at the edge of its range, as well as on performing a larger-scale study of *B. hydrosauri* population genetic structure and a more detailed investigation of the applicability of the "ridge and trough" model to this tick species. Moreover, it will be useful to inspect the population structure of both these species within the centers of their ranges and compare these findings with population structure found at the edge of the range.

Publications and Presentations

The thesis is based on the following articles:

Journal publications

Chapter 2:

Guzinski J, Saint KM, Gardner MG, Donnellan SC, Bull CM (2008) Development of microsatellite markers and analysis of their inheritance in the Australian reptile tick, *Bothriocroton hydrosauri*. *Molecular Ecology Resources*, **8**, 443-445.

Chapter 3:

Guzinski J, Bull CM, Donnellan SC, Gardner MG (2009) Molecular genetic data provide support for a model of transmission dynamics in an Australian reptile tick *Bothriocroton hydrosauri*. *Molecular Ecology*, **18**, 227-234.

Chapter 4:

Guzinski J, Bull CM, Donnellan SC and Gardner MG (in preparation) The Australian reptile tick, *Bothriocroton hydrosauri*, exhibits marked genetic population substructure at the edge of its distribution range.

Conference publications

Guzinski J, S Donnellan and Bull CM (2008) Population genetics analysis reveals substructuring within a population of an Australian reptile tick *Bothriocroton hydrosauri*. Talk at the Genetics Society of AustralAsia 55th Annual Meeting, Adelaide, Australia.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

JARO GUZINSKI
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Chapter 1 – Literature Review

Parasites

Organisms are classified as parasitic if in order to survive they have to feed on another living organism, potentially causing it harm but usually not killing it. A stricter definition of parasitism includes only organisms that live in or on their host for a significant portion of their life. The exact number of parasitic species is unknown but it is likely to be substantial, given that practically all free-living animals are known to be infested with at least one parasite species (Poulin and Morand 2000). Parasitism of metazoan species on other metazoan species is believed to have evolved independently at least 60 times (Poulin and Morand 2000) and around a fifth of all known protozoan species are parasitic thus parasitism is regarded as one of the most common lifestyles among eukaryotic organisms (Poulin and Morand 2004). Parasites belong to a very diverse range of phyla and hence vary substantially in their morphology, physiology and natural history, including such parameters as the life cycle, the number of life stages, the mating system or the mode of reproduction. However, the one thing that all parasitic life forms have in common is the need to find and invade a host.

Factors influencing parasite population structure

Given the huge diversity of parasites it is not surprising that these organisms exhibit many varied patterns of population genetic structure. Population genetic structure of parasites is governed by several factors that can be assigned to three broad categories. Firstly, the physiological, biological and ecological characteristics (life history traits/life history strategy) of a parasitic species itself, such as fecundity, the range of hosts it infests, the mating system, the life-cycle and the number of life stages, will have an influence on the population genetic structure of that species (Nadler 1995, Huyse *et al*. 2005). Secondly, since the free-living stages of many parasite species have low dispersal capability and some parasite species completely lack the free-living stages (Nadler 1995), parasite population genetic structure may to a very large degree be dependent on the ecology, social overlap, density and especially the vagility (movement patterns) of the host species (Criscione *et al*.

2005). For example, a study in northern Spain on the population dynamics of the Lyme disease tick *Ixodes ricinus* concluded tick distribution to be highly affected by host movement and not only by abiotic factors such as vegetation and weather. Thus dispersal of ticks is a function of how the hosts perceive the habitat, and the habitat's permeability to host movement (Estrada-Peña 2003). A study of a three-host tick *Ambloymma dissimile* in Venezuela also provided a very clear example of how the ecology of the host may affect the population structure of a parasite. This tick species infests toads (*Bufo marinus*), snakes (*Boa constrictor*) and lizards (*Iguana iguana*) and its populations were found to be subdivided into small breeding groups or demes, sizes of which varied according to the host species (Lampo *et al*. 1998). Toad and snake tick breeding groups comprised ticks on a single individual host (thus indicating that mating between ticks from different hosts is limited), whereas lizard tick breeding groups may include ticks from several individual hosts. These results are consistent with the behaviour of these three host species, with toads and snakes generally being solitary and lizards forming larger aggregations (Lampo *et al*. 1998). The population structure and dispersal of the seabird tick *Ixodes uriae* was also found to be host-dependent. A study using microsatellite markers concluded that ticks infesting black-legged kittiwakes had structured populations showing patterns of isolation-by-distance, whereas ticks infesting Atlantic puffins exhibited only weak population structure, even at the largest scale considered (McCoy *et al*. 2003). Indeed, puffins are known to disperse further distances than kittiwakes, which are more closely associated with their natal site (McCoy *et al*. 2003). The population structure of parasites with a complex life cycle (those that are transmitted via an intermediate host) has also been shown to be heavily influenced by host ecology and vagility. For instance, three freshwater trematode species that infest only aquatic hosts are much more subdivided than another trematode species from the same area that infests, apart from aquatic hosts, also a highly mobile terrestrial host (Criscione and Blouin 2004). Lastly, the population genetic structure of parasites could be influenced by the external (i.e. off-host) environmental conditions (Nadler 1995, Huyse *et al*. 2005). Many parasite species have to detach from their host at some stage of their life cycle (for example to moult or to lay eggs) but they are only able to survive off-host for a limited period of time due to an increased exposure to predators and to temperature and humidity. If the environmental conditions are especially severe, then the parasite population genetic structure will be dictated to an

even greater degree by the host movement patterns. The relative importance of the above described factors in shaping the population genetic structure of parasites will thus vary depending on the situation (Huyse *et al*. 2005). Ultimately their combined effect will dictate the dynamics of transmission of a parasitic species, i.e. the extent of mixing amongst individuals (offspring) originating from disparate broods, which will determine such parameters as the effective population size, inbreeding and the relative influences of gene flow and genetic drift and thus the population genetic structure itself (Nadler 1995, Criscione *et al*. 2005, Huyse *et al*. 2005).

Models of parasite population structure

The classic prediction of Price (1977, 1980) states that an infrapopulation, defined as all parasites of a given species within or on an individual host at a particular time (Bush *et al*. 1997), should be considered as the most relevant unit of parasite evolution. Price (1977, 1980) argued that parasites exhibit low connectivity between disparate infrapopulations, and hence this would lead to high levels of subdivision in parasite populations with limited gene flow among populations and low genetic diversity within populations. If parasite populations comprised only individuals infesting a single host, this could result in such populations having a small effective population size, leading to random genetic drift (sampling variance) acting as the predominant evolutionary force, potentially resulting in an eventual erosion of within population genetic diversity (reduction in heterozygosity) that could be compounded due to inbreeding (Nadler 1995, Criscione *et al*. 2005). Thus the disparate infrapopulations would be highly differentiated due to randomizing effect of drift on allele frequencies (Nadler 1995).

Parasite species that are likely to fit Price's predictions of population structure are those that continually reinfest the same host each generation (such as for example the phytophagous insects that Price studied that can have many recurrent generations on a single host plant) or whose offspring are transmitted as a clump from host to host over several generations (Criscione *et al*. 2005). Clumped transmission of siblings onto a definitive host, which occurs for example in some tick species, may lead to biparental inbreeding and hence a heterozygote deficit (Nadler 1995). Another example of a parasite with substantial infrapopulation differentiation are pocket gopher lice, as gene flow among the disparate louse infrapopulations is

almost exclusively dependent upon inter-host contact, which in gophers is relatively rare as it is primarily limited to mating encounters and rearing of young (Nadler *et al*. 1990).

Price's model may be less relevant for other parasites as many species emit their offspring into the external environment or infest highly mobile and/or sociable hosts, which creates opportunities for mixing amongst individuals originating from different broods and thus increases the connectivity between traditional infrapopulations (Criscione *et al*. 2005). For example several host individuals may share the same refuge site and the parasites could detach there, moult to the next life stage and wait for the next host individual to use the refuge. Therefore, in this case, the effective population size of a parasite population would be substantial and hence the within population genetic diversity would be high, with low levels of inbreeding (Criscione *et al*. 2005). For example the allozyme and mtDNA diversity of animal macroparasites such as helminths and arthropods has been reported to be as high or even higher than that of some free-living animals (Criscione and Blouin 2004). Thus the genetic diversity (heterozygosity) of parasite populations will vary, depending on the level of mixing amongst individuals originating from different broods (dynamics of transmission), which itself will depend on the ecology of the parasite and the host and the severity of the external environment and various combinations of these factors.

Investigating parasite population structure

The population biology of parasites is often very difficult, if not impossible, to study via direct observation due to these organisms' small size, location (for example if inside a host), biology and behaviour (de Meeûs *et al*. 2007). Thus, such aspects of parasite biology as the ecology, reproductive modes and/or strategies, dispersal and population structure and sizes can in most cases be assessed only through indirect methods (de Meeûs *et al*. 2007). These methods are based on the use of highly variable molecular markers and subsequent analysis of polymorphism within and between (in some cases predefined) groups of individuals, under an assumption that the observed distribution of genetic variation reflects ecologically relevant population parameters such as those mentioned above (de Meeûs *et al*. 2007). One of the most popular molecular markers are microsatellites, which over the last 15 years

have been very widely applied in the field of population genetics allowing researchers to elucidate the population structure of a very diverse array of organisms, including many parasite species.

Microsatellites

Microsatellite loci, which are also known as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), have been found to be commonly present throughout all the eukaryotic and prokaryotic genomes that have been studied so far (Metzgar *et al*. 2002). They comprise of multiple 1 to 6 base pair (bp) tandem repeat sequences called motifs, such as for example $(AC)_n$, $(AAG)_n$ or $(CCCT)_n$. These loci can often be highly polymorphic due to an addition or a deletion of a single or multiple repeats, which makes them a very useful tool for molecular ecology studies (Li *et al*. 2002). Furthermore, microsatellites are codominant, allowing both homozygous and heterozygous genotypes to be distinguished and are relatively easy and cheap to implement and score.

For these reasons these genetic markers are very widely applied in the fields of population and conservation genetics, as analysis of the multi-locus microsatellite genotypes of the sampled individuals enables researchers to elucidate both the current and historical population structure of their study organism. Crucial to our interpretation of the observed genetic variation amongst the sampled individuals has been the development of various computing packages that inform the user of such population parameters as levels of inbreeding, dispersal, divergence between two populations or whether individuals belong to disparate genetic clusters. Importantly, these programs statistically estimate the likelihood of these events. A vital assumption of the population genetics studies that use microsatellites is that these markers are selectively neutral, randomly distributed throughout the genome and unlinked (Nielsen *et al*. 2006). Thus the observed patterns of allele frequency variation between populations and amongst individuals within a population have almost always been explained in terms of gene flow (migration), genetic drift and to a lesser extent mutation but the potential role of selection has been ignored (Nielsen *et al*. 2006).

However, a large body of evidence indicates that this approach is not universally correct, as in many cases microsatellite repeat length plays a crucial role

in the expression of various genes and microsatellite distribution throughout the genome is non-random, especially since microsatellites are not just present in the non-coding regions of the genome, but in the coding ones as well (Zane *et al*. 2002).

Another controversial issue regarding microsatellites is their evolution - the mutation process, which results in the change in the number of repeats over time, otherwise known as length polymorphism, which is poorly understood (Zane *et al*. 2002). Several different models and mechanisms have been proposed to explain how microsatellites arise and mutate.

This part of the literature review will focus on these two issues, starting with a brief description of what is currently known about the processes involved in the evolution (expansion and contraction) of microsatellites.

Microsatellite polymorphism

Microsatellites are highly polymorphic because they are very highly mutable (10^{-6} to 10^{-2} mutation events per locus per gamete per generation (Schlötterer 2000)), 3 to 4 orders of magnitude higher when compared to point mutation rates at coding gene loci. Mutations at the microsatellite loci can either decrease or increase the number of repeats, either by a single repeat, or by multiple repeats. A strongly favoured mechanism of microsatellite evolution is via slippage events during DNA replication (Schlötterer and Tautz 1992). Another mechanism that has been proposed involves recombination between DNA strands (Harding *et al*. 1992). Mutation rates of microsatellite loci can be affected by a multitude of factors, which include the repeated motif, allele size, chromosome position, GC content in the flanking region, cell division (i.e. mitotic or meiotic), sex and the organism's genome (i.e. other mutations, especially in DNA repair factors) (Li *et al*. 2002).

MisMatch Repair System

Mispairing errors caused by DNA slippage during DNA replication are first edited through 3' to 5' exonucleolytic proofreading by DNA polymerase and subsequently the post-replicative mismatch repair (MMR) corrects any remaining errors, thus maintaining genomic stability (Aquilina and Bignami 2001). As the efficiency of proofreading decreases as sequence length increases, MMR is the predominant

mechanism acting to remove frameshift intermediates (loops of extrahelical bases) in long sequences – for example microsatellite tandem repeats (Gragg *et al*. 2002). Indeed, experiments have shown that loss of MMR may lead to expansion and contraction of the microsatellite repeats, thus causing polymorphism (Aquilina and Bignami 2001). If the extrahelical loops are not removed this could result in frameshift mutations, which may potentially cause the loss of protein function and subsequently a mutator phenotype. As MMR is also responsible for repairing base:base mismatches, disruption of this pathway may lead to an increased frequency of the spontaneous transition and transversion mutations (Aquilina and Bignami 2001). The effectiveness of the MMR system depends on such factors as genomic location of the mismatch, DNA surrounding the mismatch, the presence of strand-recognition signals, methylation state of the sequence and so on (Li *et al*. 2002). Ironically, the MMR pathway may be inactivated due to frameshift mutations of the $T_{(n)}$ tandem repeats that are located within the coding regions of both the major and minor MMR genes (Li *et al*. 2004).

Role of replication slippage in microsatellite polymorphism

Replication slippage occurs during DNA replication when the replicating DNA strand dissociates and subsequently reassociates in a misaligned fashion (out of register realignment) but this fault is not repaired by the MMR system (Ellegren 2004). This process has been observed in the genomes of prokaryotic and eukaryotic organisms and is thought to play a significant role in microsatellite evolution (Schlötterer and Tautz 1992, Viguera *et al*. 2001).

A model for the slippage process (between direct repeats) that is based on experimental observations is as follows: as the polymerase replicates a direct repeat, it stops and drops off the synthesized strand. Such a nascent (daughter) strand dissociates from the template strand, and realigns with another direct repeat (if this direct repeat is a different length to the template direct repeat this will result in polymorphism). After the polymerase reanneals to the DNA, replication resumes (Viguera *et al*. 2001). Pausing of DNA polymerase within a direct repeat is necessary for slippage replication to take place (Viguera e*t al*. 2001). Various experiments have indicated that secondary structures (such as hairpins, triplexes and tetraplexes) often act as preferential pausing sites for DNA polymerase (Viguera *et al*. 2001). Such

structures have been reported to often form within microsatellite sequences, which are highly unstable (for example CTG / CAG or CGG / CCG repeats in yeast forming hairpin-like structures) (Li *et al*. 2002). MMR efficiency has been shown to be especially important for microsatellite slippage rates, as when MMR genes are inactivated, there is an increase in the rate of microsatellite instability (MSI) events (Li *et al*. 2004).

Replication slippage may cause either an addition (which is favored, at least up to a certain allele length) or a deletion of usually a single repeat unit (single step mutation) depending on whether the extrahelical loop forms on the daughter or the template strand, respectively (Buschiazzo and Gemmell 2006). Multi-step mutations are rarer, but they have been reported to occur (Buschiazzo and Gemmell 2006). This type of mutation is called a dynamic mutation and in humans it may lead to several diseases (especially expansions of triplet repeats causing neurodegenerative diseases) (Viguera *et al*. 2001). Factors that may have an influence on slippage (apart from the functionality of the MMR system) include the polymerase type, single-stranded DNA-binding protein (SSB) and the specific point mutations in the polymerase exonuclease domain (Viguera *et al*. 2001).

Influence of point mutations

Recent studies have indicated that the length of the microsatellite repeat arrays (very rarely, if ever, exceeding 50 repeats) is controlled by a balance between two separate events: replication slippage, which favors microsatellite expansions, and point mutations, which break down long arrays into two or more shorter ones (Li *et al*. 2004, Buschiazzo and Gemmell 2006). If the relative frequencies of these two events change, this could have an influence on the number of long repeat arrays present in the genome, and thus it has been proposed that the higher relative rate of slippage may give rise to longer microsatellites (Kruglyak *et al*. 1998). Some of the factors which could influence the relative rates of slippage replication and point mutations include changes in the efficiency of MMR and proofreading during DNA replication, as well as “other potential differences in genome structure and organization between species” (Li *et al*. 2004). However, some studies have concluded that the process of accumulation of interruptions within an array (due to point mutations) itself is insufficient to explain the existence of an array length constraint but the widely

observed (for example in humans) exponential increase of the rate of contractions (due to slippage) as the array length increases may be responsible (Buschiazzo and Gemmell 2006). It is unknown as yet why especially long repeats could be selected against in such a fashion.

Recombination

Recombination has been proposed to play a role in the instability of tandem repeat arrays either via unequal crossing over (reciprocal transfer of genetic information) or more likely via gene conversion (non-reciprocal transfer of genetic information) (Richard and Paques 2000). Tandem repeats could potentially be rearranged due to unequal crossover between sister chromatids, which would lead to a simultaneous expansion of one repeat and contraction of the other. Depending on the motif, the non-reciprocal exchange mechanism may result either in unidirectional (expansion or contraction) rearrangement of the tandem repeats or bidirectional rearrangement (expansion of one repeat and contraction of the other) (Li *et al*. 2002). This effect may be associated either with meiosis or mitosis, although at different rates (Li *et al*. 2002).

Experiments have demonstrated that the rate of intra-allelic rearrangement increases with array size and that intra-allelic duplication events usually cluster within homogenous segments of alleles (Li *et al*. 2002). Thus, recombination has been proposed to be chiefly associated with large-scale contractions and expansions in the repeat array (involving gain or loss of more than just one or several repeats) (Richard and Paques 2000). However, there are major doubts whether recombination has a major influence on microsatellite variability [for example as two types of *Escherichia coli* strains, those with and without functional recombination system, both exhibited very similar microsatellite mutation rates (Schlötterer 2000)], as it does on the variability of minisatellites, a class of tandem repeat arrays comprising motifs of up to 100 bp (Richard and Paques 2000, Ellegren 2004).

Some experiments have indicated that an interaction of replication slippage and recombination takes place in mismatched (heteroduplex) DNA regions, which could affect microsatellite stability (Richard and Paques 2000, Li *et al*. 2002). Heteroduplex DNA regions undergo replication-dependent correction thus a slippage

mechanism could also work in recombination tracts involving microsatellite arrays (Li *et al*. 2002).

Microsatellite mutation models

Even though it is not certain what mechanism is responsible for the very high rate of mutations at microsatellite loci that results in microsatellite variation, it appears almost certain that very many other factors play a role in microsatellite polymorphism. These include nature of microsatellite (allele length, motif length, nucleotide composition), position of microsatellite in the genome (coding or non-coding, flanking sequence variation, local mutation rate), biology of the individual (age, sex, environment) and selective influences (taxon-specific features such as mode of reproduction, metabolic rate or sociality) (Ellegren 2000, Buschiazzo and Gemmell 2006). Thus most likely the dynamics of microsatellite evolution differ between taxons, individuals and chromosomes. Despite this discrepancy several general microsatellite mutation models have been proposed and some have been widely applied, as the genetic distance measures (such as Wright's F_{ST} or Slatkin's R_{ST}) that are commonly used to estimate the connectivity and patterns of gene flow among populations assume particular mutation models (Balloux and Lugon-Moulin 2002).

Microsatellite neutrality

Microsatellites are deemed to be neutral genetic markers and thus it is generally assumed that the specific allele frequencies detected within groups of the sampled individuals are such exclusively due to the relative influences of gene flow and genetic drift. Once these parameters have been estimated, based on the observed multi-locus microsatellite genotypes, such characteristics of the studied organism as levels of dispersal and mating patterns can be inferred. This information may then be used for example to plan a conservation strategy to save an endangered species. However, as an increasing body of evidence strongly indicates that at least some microsatellite loci have an influence on the function and expression of various genes (and variation in the number of repeats may lead to an altered phenotype), the danger

is that differences in allele frequencies may be due to divergent selection, and not restricted gene flow.

Eukaryotic microsatellites have been proposed to be evolving in a generally neutral manner (Schlötterer 2000), randomly or almost randomly distributed throughout the euchromatic genome (Schlötterer 2000) but especially in positions where they are least likely to have a functional effect (i.e. distribution bias towards the non-coding regions of the genome) (Metzgar *et al*. 2000). It has been argued that presence of microsatellites in genes would not be advantageous due to their very high mutability. If a certain microsatellite allele represents an evolutionary optimum at a particular locus (gene), all the new alleles produced at that locus will be less favorable than the current state (unless the evolutionary optimum itself changes). Thus microsatellites would not be expected to be present in the coding (predominantly those under selection) regions of the genome (Schlötterer 2000).

Because in eukaryotic genomes, microsatellites have been found to be present at frequencies many orders of magnitude higher than if they simply occurred by chance (Metzgar *et al*. 2002), and some microsatellites have been detected in such regions of the genome as the protein-coding regions, UTRs (untranslated regions) and also introns (Li *et al*. 2004), this indicates that at least some loci are non-randomly distributed throughout the genome and possibly subject to selection (Li *et al*. 2002).

Influence of microsatellites on gene expression and evolution

Microsatellites are present in all regions of the genome, both coding and non-coding. An increasingly substantial body of evidence indicates that microsatellites in the coding regions have a strong and direct effect on expression of certain genes (microsatellite length variation can lead to gain or loss of gene function due to frameshift mutations) and hence on the phenotype of an organism. Due to their very high mutability, and hence variable length, microsatellites can act as a prolific source of quantitative and qualitative variation, with minimal genetic load (Kashi and King 2006).

Length variation of microsatellites present in the 5’-UTRs can influence gene expression by affecting transcription and translation. Expansion of microsatellites in the 3'-UTRs has been noted to result in transcription slippage and expanded mRNA,

which may disrupt various cellular functions (Li *et al*. 2004). Microsatellite loci present in the introns can have an influence on gene transcription, mRNA splicing or mRNA's export to the cytoplasm. Heterochromatin-mediated gene silencing can be caused by alterations in triplet microsatellites that are located in the UTRs or the introns (Li *et al*. 2004). Thus, all these genotypic changes caused by expansions or contractions of the microsatellites may eventually lead to phenotypic changes (Li *et al*. 2004).

Presence of microsatellites in the transcribed regions has been proposed to provide means of adaptation to a new or a highly variable environment and hence microsatellites have been termed evolutionary "tuning knobs" (Trifonov 2003). Importantly, higher number of repeats would result in finer tuning, as the higher the number of repeats, the weaker the influence of any individual repeat (Trifonov 2003). Of course in this case the sequence of the genes themselves remains unchanged, it is only the repeats in the microsatellite array that either expand or contract, thus changing the expression pattern of the gene. Another important factor is that such a change in the gene expression pattern is immediate, i.e. the organism's response to the environmental change is instant. If such changes in microsatellite length take place in the gonads, the next generation of individuals will inherit any resulting gene expression changes (Trifonov 2003). Such changes in the number of repeats can eventually lead to an adaptation to a particular environment of a whole population, but only after a selection process spanning several sexual generations (Trifonov 2003). Hence, the changes in the tandem repeat tuners are under selective pressure that lead to either expansion or contraction of a certain tandem repeat motif, until desired levels of gene expression have been achieved, which relax the impact of the environmental stress on an organism (Trifonov 2003).

In many eukaryotes, nontriplet (especially mononucleodite) repeats are present in high amounts in the minor genes of the mismatch repair system, hence repeat number variation in these genes could control them, effectively modulating mutation rates over evolutionary time, with the possibility of increased levels of mutation in a changed environment (Kashi and King 2006). In prokaryotic genomes, microsatellites have been found to provide adaptive functional variability (Metzgar *et al*. 2002).

Study species

Ticks are bloodsucking ectoparasites, which together with mites, belong to the order Acarina (Oliver 1989). There are 899 currently recognised species of ticks (Order *Ixodida*), 713 of which are classified as ixodid, or hard-shelled ticks (Barker and Murrell 2004). The majority of them are three-host parasites, but in some species this has been reduced (Oliver 1989). All ixodid ticks undergo three life history stages: larvae, nymphs and adults (Oliver 1989).

Bothriocroton hydrosauri is an ixodid tick species that infests large varanid, agamid and scincid lizards and snakes throughout southern Australia (Smyth 1973) (Fig 1a). Its range extends over temperate to semi-arid climates (Bull and Smyth 1973). *Bothriocroton hydrosauri* is a three-host parasite and each of its life-history stages infests the same range of hosts, with all three life-history stages often being present on the same host (Bull 1978a). Throughout South Australia its most common host is a large skink - the sleepy lizard *Tiliqua rugosa* (Smyth 1973) (Fig 1b). Classification of *B. hydrosauri* has recently been updated based on the sequence data and it is no longer assigned to the genus *Aponomma*, which has been annulled (Klompen *et al*. 2002). The ecology of *B. hydrosauri* is very well understood as for the last 25 years various aspects of its biology have been studied both in the laboratory and also in the field at a study site near Mt Mary, a small town located in the semiarid mid-north of South Australia (34°06' S; 139°26' E).

Life cycle

Bothriocroton hydrosauri ticks always mate on-host. After attaching, usually in the axillae of the forelegs or in the ears of the host, females feed for several days and then emit an air-borne excitant pheromone, which induces males to detach and search for sexually receptive females on the same host (Andrews and Bull 1981, Andrews *et al*. 1986). If not “activated” in this manner, male ticks remain attached for many months, feeding sparingly and waiting for mating opportunities (Andrews and Bull 1980).

The courtship and mating has been divided into six phases (Andrews and Bull 1980). After the male has approached the female, he touches her dorsal surface with alternating forelegs. Subsequently, he climbs onto her (the second phase). After the

male has placed all his legs on the female's dorsal surface and raised his body, he proceeds to turn and face the same direction as his partner (Andrews and Bull 1980). Next, he moves forward so that his capitulum is lying over her scutal shield. As he does that, the male's legs wrap around the edges of the female's body, and his ventral surface touches her dorsal surface (Andrews and Bull 1980). The third phase begins, as the male starts to reverse his position 180°, so that he is still on top of the female, but now facing the opposite direction. The male may turn to the left or to the right (Andrews and Bull 1980). In this position, the male's last three pairs of legs are wrapped around his partner's body, posterior to her last pair of legs. Moreover, in this position, the male's capitulum and the first pair of legs extend beyond the posterior end of the female. The next phase of the courtship begins as the female lifts her body, using her hind legs, until she is at 70° to the surface. This prompts the male to climb over the end of the female and turn his body 180°, this time in the vertical plane, until he is on his back behind the female so that only his capitulum remains under her ventral surface (Andrews and Bull 1980). In the second to last phase, the male attempts to get his capitulum in contact with the female's genital aperture. He does it by moving his legs along the perimeter of the female and pulling himself forward under her body. Once he gets into position, the male's first pair of legs rests on the anterior part of his partner's ventral surface, and his last three pairs of legs are clasped around the female's body, posterior to her last pair of legs (Andrews and Bull 1980). In this phase, as well as in the next and last phase, the female's body remains upright. Copulation is the culmination of the courtship. After the male's capitulum has been turned to point vertically at the female genital aperture, the hypostome is pushed in and two pualps are sprayed onto female's ventral surface (Andrews and Bull 1980). The two ticks may remain in this position for 1.5 to 2 hours, as the male transfers the spermatophore to the female genital aperture (Andrews and Bull 1980). After the copulation has been completed, the males have been observed to go through the whole courtship sequence in reverse. After they reach female's dorsal surface, they move away looking for another female (Andrews and Bull 1980). Mated females are not approached by subsequent males (Andrews and Bull 1980). They engorge (usually less than 15 days), detach, produce a large batch of eggs (1500 to 2000) and die (Chilton and Bull 1991). Mating success is not high as Andrews and Bull (1980) reported that only 33% of on-host partners had reached the final copulatory position. Females, which had not successfully mated

with one male, did try to attract other males. *Bothriocroton hydrosauri* ticks are able to mate throughout the whole year as they do not require a high host body temperature to initiate mating activity (Chilton and Andrews 1988) although the process takes longer during the cooler autumn and winter months as then females engorge at a slower rate (Bull and Burzacott 1994).

While off-host, ixodid ticks are under a much greater risk than on-host due to exposure to predators, mainly ants (Bull *et al*. 1988), and the environmental conditions. *Bothriocroton hydrosauri* ticks are especially poorly resistant to dehydration (Bull and Smyth 1973), hence it is crucial that their off-host habitat has a stable microclimate and is not exposed to extreme temperature regimes or low humidity. Ticks tend to detach in lizard refuge sites (Bull 1978b) and once they have detached they remain there, in the leaf litter, rather than dispersing large distances (Petney *et al*. 1983, Petney and Bull 1984). Lizards usually shelter overnight or during the hottest part of the day under bushes found within their home range. Detached *B. hydrosauri* females often select a position close to the ground surface low down in the litter, most likely to minimise direct exposure to sun and thus avoid desiccation (Chilton and Bull 1993a). The pre-oviposition period may last more than a month, but it declines with increasing temperature so that at 30 °C it lasts less than 20 days (Chilton and Bull 1994). Oviposition occurs over a period of around 40 days, though the majority of eggs are laid between days 3 and 11 of the oviposition period (Chilton and Bull 1993b). *Bothriocroton hydrosauri* eggs develop faster at higher temperatures, such that larvae hatch after 25 days at 30 °C but after around 70 days at 20 °C (Chilton and Bull 1994). After hatching, the larvae aggregate [via an aggregation pheromone (Petney and Bull 1981)] at the soil/litter interface (Chilton and Bull 1993a) and wait for a host to attach to. The host has to be present very close to the larvae as they are unable to detect a host that is further than 20 cm away (Belan and Bull 1991). Unfed larvae of *B. hydrosauri* are especially susceptible to desiccation and they die after less than 9 days at temperatures higher than 20 °C and relative humidity lower than 35%, but are able to survive more than 200 days at 13 °C and 80-85% relative humidity (Chilton and Bull 1993c). It is likely that all surviving larvae belonging to a particular brood will attach to the same host, the host that is the first to be in contact with them after the larvae have hatched. After attaching to a host, larvae feed until engorgement, which on average takes 30 days (at 21 °C and 50-55% relative humidity) (Chilton 1989). Successful engorgement of

larvae is density-dependent, so that the probability of successful engorgement decreases with increasing number of larvae infesting a host (Tyre *et al*. 2003). When they are fully engorged, larvae will detach the next time a host enters a refuge site. Fed and detached *B. hydrosauri* larvae moult to nymphs at temperatures as low as 13 °C but at higher temperatures the pre-moult period is markedly shorter (Chilton *et al*. 2000). At 25 °C, which is a temperature ticks are likely to experience in lizard refuge sites during the spring and summer months, the pre-moult period may last 15 to 24 days (Bull *et al*. 1977). Unfed nymphs can survive up to 26 days at 34 °C and 0% relative humidity (Chilton and Bull 1993c), and are more resistant to desiccation than larvae. Nymphs aggregate (Petney and Bull 1981) low down in the litter (Chilton and Bull 1993a) and attach to a host only when one virtually brushes against them, as experiments show that they stay within the aggregation even if a lizard is as close as 50 cm away (Petney *et al*. 1983). The average *B. hydrosauri* nymph engorgement time at 21 °C and 50-55% relative humidity is 23 days (Chilton 1989). Eventually, the fully engorged nymphs detach in a lizard refuge site and moult [faster at higher temperatures (Chilton *et al*. 2000)] into adult ticks (1:1 sex ratio). These can survive in the leaf litter up to 100 days (Chilton 1989) and will eventually attach to a definitive host, the host on which mating takes place (Andrews and Bull 1980). *Bothriocroton hydrosauri* ticks can go through one complete life cycle within 18 months (Bull and Sharrad 1980).

Parapatric boundary

Bothriocroton hydrosauri has allopatric distributions with two other ixodid reptile tick species *Amblyomma limbatum* and *Amblyomma albolimbatum*, with only very little or no overlap between their ranges (Smyth 1973). The range of *A. limbatum* spans much of northern and central Australia, *B. hydrosauri* is present in the south-eastern part of Australia, whereas *A. albolimbatum* in south-western part of Australia (Bull *et al*. 1981) (Fig. 1c). Smyth (1973) specifically noted the ecological parapatric boundary between *B. hydrosauri* and *A. limbatum* near Mt Mary, on which this part of review will focus.

The zone of parapatry between these two ticks may be very abrupt, with one species completely replacing the other over a distance of between 100 m and 5 km (Smyth 1973, Bull *et al*. 1981). For around the last 25 years, various factors that may

A

B

C

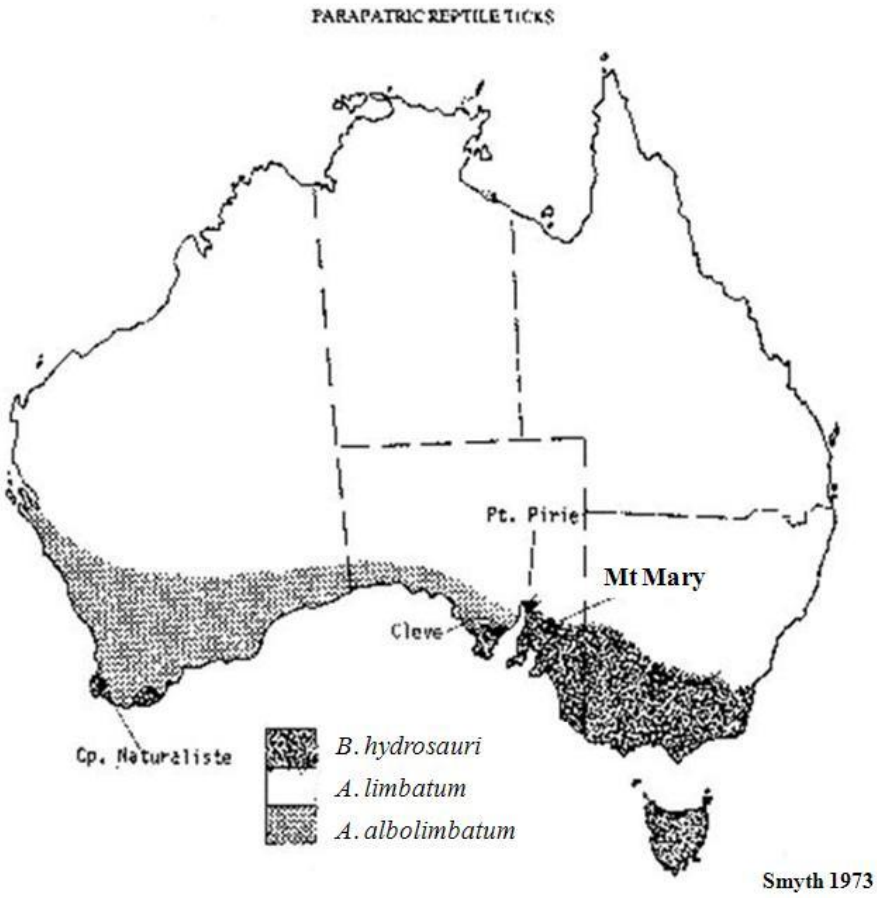


Figure 1. A. Adult *Bothriocroton hydrosauri* male. **B.** An angry *Tiliqua rugosa*, main host of *Bothriocroton hydrosauri* and *Ambloymma limbatum*. **C.** The allopatric distributions of three Australian reptile tick species: *Bothriocroton hydrosauri*, *Amblyomma limbatum* and *Amblyomma albolimbatum*.

explain the distributions of these two tick species have been studied in great detail but so far no definite reason that can adequately explain all attributes of this parapatry, and especially what prevents further overlap, has been determined. Host distribution or inter-specific competition for hosts might be obvious factors but they were quickly discounted after it had been observed that the sleepy lizard *Tiliqua rugosa* [main host for both these species in South Australia (Smyth 1973)] occurs with similar abundance and dispersion on either side of the boundary (Bull *et al*. 1981). Several alternative hypotheses are discussed below.

Factors causing the boundary: Tick adaptations to different temperature regimes

The range of *B. hydrosauri* extends over cooler and more moist climate, whereas *A. limbatum* occupies a much drier habitat. Hence *B. hydrosauri* is a mesic-adapated species whereas *A. limbatum* a xeric-adapted species, illustrated for example by lower rates of water loss in *A. limbatum* (Bull and Smyth 1973). These two species of ticks differ in their response to annual temperature variation, which has been proposed to play a role in the formation of the parapatric boundary and prevention of expansion of one species into the range of the other.

Studies have shown that *B. hydrosauri* and *A. limbatum* ticks differ in their ability to cope with various temperature regimes. Factors that are most important for tick survival while off-host are duration of the pre-moult period and the tick's ability to withstand desiccation. Hence those ticks that can undergo moulting the quickest should have the highest survival capabilities, especially in arid or dry environments (Chilton *et al*. 2000). Studies that investigated the moulting success and the duration of the pre-moult period of engorged larvae and nymphs of these two tick species under different temperature and relative humidity regimes, have found that at lower temperatures (13 °C and 18 °C) *B. hydrosauri* larvae had higher moulting success than *A. limbatum* larvae, whereas at 34 °C, *A. limbatum* larvae were more successful (Chilton *et al*. 2000). Relative humidity only affected the pre-moult period of *B. hydrosauri* engorged larvae at one temperature, whereas *A. limbatum* larvae were affected by changes in relative humidity at a range of different temperatures though this difference was generally only 1 or 2 days at any one temperature (Chilton *et al*. 2000). Pre-moult periods of nymphs of either species did not appear to be significantly affected by variation in relative humidity. For the larvae of both species,

as the temperature increased, there was a significant decrease in the pre-moult period. However, the moulting times of the two species responded to the temperature changes in different ways, i.e. *A. limbatum* larvae took less time to moult than *B. hydrosauri* larvae at temperatures higher than 21 °C, but longer to moult at temperatures less than 21 °C (Chilton *et al*. 2000). Similar trends were observed for the nymphs of both species, with the exception that pre-moult periods for nymphs at different temperatures were longer than those of larvae (but nymphs appeared to able to moult over a wider range of temperature and humidity conditions) (Chilton *et al*. 2000). Thus ticks of both species were only able to moult within certain temperature ranges - that of *B. hydrosauri* including lower temperatures than that of *A. limbatum*. Humidity appeared to be important only at the upper and lower temperatures of a particular tick species' range, as at these "extreme" temperatures, fewer ticks were able to moult at 0% relative humidity (Chilton *et al*. 2000).

Both these tick species attach to hosts more frequently during the warmer months than in the cooler months. During the winter months, the late attaching *B. hydrosauri* females may continue to mate and engorge, although at a slower rate, whereas *A. limbatum* females completely cease development over winter (Bull and Burzacott 1994). Laboratory studies have shown that *A. limbatum* females require significantly higher host body temperatures to induce mating compared to *B. hydrosauri* females (Chilton and Andrews 1988). Such low tolerance to cool temperatures means that *A. limbatum* ticks have only a limited period of time to develop from juveniles, mate, engorge, and lay eggs. Moreover, experiments have shown that *A. limbatum*'s development is delayed in the cooler climate. *Amblyomma limbatum* females that had developed from larvae on the "away" side of the boundary (Flinders University, Adelaide – long way south) were found to be attaching to hosts later than were *B. hydrosauri* females, whose development from larvae started at roughly the same time (Bull and Burzacott 1994). Hence this possibly could explain why *A. limbatum* range does not extended further south than it currently does (as periods of warm weather might not be long enough there for the ticks to complete their life cycle), i.e. into the *B. hydrosauri* side of the boundary, or at least be a part of the reason behind that phenomenon (Bull and Burzacott 1994).

In summary, *A. limbatum* females are better adapted to warmer temperatures as they take less time to lay eggs and egg development times are shorter compared to *B. hydrosauri* at warmer temperatures (Klomp and Bull 1987). *Bothriocroton*

hydrosauri on the other hand are better adapted to cooler temperatures, since these ticks can initiate mating at cooler temperatures and moreover, *B. hydrosauri* larvae can survive for longer periods at lower temperatures (Chilton and Bull 1993c), and also eggs develop at a faster rate than those of *A. limbatum* (Chilton and Bull 1994). Thus different adaptations of these two tick species to different temperature regimes could play a role in their allopatric distribution. These differences may influence broad distribution patterns of the two ticks, but are unlikely to explain the abrupt boundary.

Factors causing the boundary: Ecotone change

The boundary is associated with an ecotone change. *Amblyomma limbatum* occupies the low woodland consisting of black oak (*Casuarina cristata*), the blue bush (*Mariaeana sedifolia*) and occasional sandalwood trees (*Myoporum platycarpum*) that occurs north of the boundary, whereas the environment of *B. hydrosauri* comprises mallee scrub, mainly *Eucalyptus gracilis* and *E. oleosa*, present south of boundary (Bull *et al.* 1981). The close association between the tick species and their habitat is demonstrated by the Burra Creek flood plain. It flows through low woodland but has mallee scrub in its bed (a mallee tongue about 1.5 km wide) where *B. hydrosauri* ticks can be found, whereas around them, in the woodland, the *A. limbatum* ticks are present (Bull *et al*. 1981). There is a narrow overlap zone between the two tick species, which can be as narrow as just a 100 m, up to around 5 km (Bull *et al*. 1981). *Amblyomma limbatum* was less often found in the mallee scrub than *B. hydrosauri* in the low woodland habitat (Bull *et al*. 1981). In the overlap zone, sleepy lizards with both species of ticks were sometimes found (Bull *et al*. 1981).

A change in ecotone could play an important role in maintaining the parapatric distributions of these two tick species due to the requirement of the ticks to survive off the host and a different ability of each of the two tick species to do so. While on host, the ticks are relatively unaffected by the climate or the environment since they can hide beneath the scales of the lizard they infest. Moreover, if the weather becomes very hot, the lizard will seek shade and hence the ticks will be protected from desiccation. Thus the nature of the soil and leaf litter, where the ticks wait for the host, can be paramount for their survival. This has been confirmed by studies that demonstrated engorged *B. hydrosauri* and *A. limbatum* larvae and

nymphs prefer to move away from bare ground and to stay within the surface leaf litter (Petney and Bull 1984). Ticks especially seek out environments that provide warmth, but also protection from excessive heat and humidity (Chilton *et al*. 2000). On the south of the boundary (*B. hydrosauri* range) it was noticed that the mallee scrub habitat is characterized by ridges with shallow patches of brown solonized soil (containing kunkar - spongy limestone), whereas on the north side of the boundary (*A. limbatum* range) there is a change in drainage lines and there is no kunkar in clays and slits (Bull *et al*. 1981). However, more importantly, there is a change in leaf litter. The mallee scrub litter consists of relatively wide leaves and has a layered structure, whereas the woodland litter mainly consists of twig-like *Casuarina* branchlets and thus has a more open structure (Bull *et al*. 1981). Experiments have shown that even though the soil from both sides of the boundary does not differ in its water-holding ability, the litter does, as the eucalypt leaves hold water longer (Bull *et al*. 1981). This then is especially important as *A. limbatum* individuals have been demonstrated to be able to tolerate high temperature and desiccation for longer periods than can *B. hydrosauri* (Bull and Smyth 1973, Klomp and Bull 1987).

When individuals from the two species of ticks were placed on the "away" side (and also "home" side as a control) of the boundary, there was low mortality during the winter months, but for both samples there was still higher survival on their "home" side of the boundary. During warmer summer months there was high mortality of ticks placed both "home" and "away", but *B. hydrosauri* was more strongly affected than *A. limbatum*, especially on the "away" side of boundary (Bull *et al*. 1981). In fact, during the summer period, *A. limbatum* mortality was equal on the "home" and "away" sides of the boundary (Bull *et al*. 1981). Moreover, *B. hydrosauri* mortality rates on the "away" side of the boundary were not significantly higher compared to mortality rates on the "home" side of the boundary (Bull *et al*. 1981). Another experiment has shown that both species of ticks can go through at least one life cycle on the "away" side of the boundary (Bull *et al*. 1981). Hence, based on these results it is difficult to conclude why *A. limbatum* does not disperse further south, especially if the environmental conditions there appear to be less harsh (i.e. less arid). Moreover, since *B. hydrosauri* survival rates on the "away" side of the boundary were only slightly lower than on its "home" side, a change in the ecotone does not appear to be responsible for the maintenance of this parapatric boundary (Bull *et al*. 1981).

Factors causing the boundary: Rainfall levels

Change in rainfall levels is also associated with the parapatric boundary at the Mt Mary site, as the ecotone runs along the 250 mm rainfall isohyet (generally higher levels of rainfall over *B. hydrosauri* range) (Bull *et al*. 1981). However, the change in rainfall levels is very gradual and moreover, variable between years on one side of the boundary. Thus the between-year variation in rainfall levels at a site on one side of the boundary can be as high or higher than variation in rainfall levels between sites on either side of the boundary within a year (Bull *et al*. 1981). Therefore, it would be presumptuous to infer that the rainfall levels are a major influence on the distribution of these two tick species. However, as will be described in more detail later on, in above-average rainfall years, *B. hydrosauri*'s range has expanded northwards, implying that rainfall levels may have an influence on the distribution of these two tick species and especially that *B. hydrosauri* has been prevented from expanding its range due to excessively dry conditions (Bull and Burzacott 2001).

Factors causing the boundary: Predation

Predators of the two tick species have been proposed to have an influence on the maintenance of the boundary, especially if they are distributed such that they are more abundant on the side of the boundary occupied by the tick that is more resistant to the predatory attack, thus preventing the expansion of the more susceptible species (Chilton and Bull 1996).

The major predators of these two tick species are ants (Bull *et al*. 1988). Distribution of ants has been shown to be affected by environmental variation; hence it is possible that the ecotone change at this parapatric boundary may cause ants to be present in greater numbers on a particular side of the boundary (Chilton and Bull 1996). Studies have shown that ticks are most likely to be attacked by predators during warmer months when the ants are most active, and also when the levels of tick engorgement and detachment are the greatest (Bull *et al*. 1988). While the ticks are off the host, they stay in the leaf litter, hence are exposed to predators (Petney and Bull 1984). However, it was determined that variation in the distribution of the predatory ants (major genera investigated were *Crematogaster*, *Iridomyrmex* and *Pheidole*) was not associated in any way with the distribution of the two tick species

and hence the distribution of these predators does not seem to have an effect on the maintenance of the boundary (Chilton and Bull 1996).

There is some evidence that one of these two species of ticks is more susceptible to predatory attack than the other. While waiting to attach to a host, *A. limbatum* tends to move higher up in the litter than *B. hydrosauri* and hence might be more exposed to a potential predatory attack (Klomp and Bull 1987, Dawes-Gromadzki and Bull 1997b). However, on the basis of detailed laboratory experiments, it has been concluded that *A. limbatum* is more resistant to a predatory ant attack than is *B. hydrosauri* (Dawes-Gromadzki and Bull 1997a). Laboratory experiments have shown that the ants took longer to handle *A. limbatum* and that all tested life-stages (fed or unfed larvae, nymphs or adults) of this tick species had higher survival rates than their *B. hydrosauri* equivalents (Dawes-Gromadzki and Bull 1997a). *Amblyomma limbatum* females have a shorter mean period of oviposition than *B. hydrosauri* females (12.9 days versus 19.6 days at 22 °C) (Chilton and Bull 1994) and thus tend to be exposed to predatory attacks for a shorter period of time (Dawes-Gromadzki and Bull 1997a). Moreover, it has been reported that ants are more effective tick-predators in the blue-bush litter (*A. limbatum* environment) than in the mallee litter (*B. hydrosauri* environment) (Dawes-Gromadzki and Bull 1997b). Thus the fact that *B. hydrosauri* is more susceptible to ant predation and that ants are more effective predators over *A. limbatum* range may explain why *B. hydrosauri* is unable to expand further north but not why *A. limbatum* does not spread south.

Factors causing the boundary: Interspecific competition

As *B. hydrosauri* and *A. limbatum* ticks tend to attach to the same sites on their hosts and hence exhibit substantial overlap in the feeding sites they occupy (Andrews and Petney 1981, Bull *et al*. 1989), it has been suggested that interspecific competition for feeding sites may be preventing further overlap of the distributions of the two species and thus be an important factor in the maintenance of the boundary (Bull *et al*. 1981, Bull *et al*. 1989). However, both field observations and laboratory experiments have strongly refuted this hypothesis. Firstly, the observed levels of infestation per host were extremely low for both species, with very many hosts infested with just one or two individuals or not infested at all (Bull *et al*. 1989). Thus

it appears that the resource that the ticks were proposed to be competing for is very abundant and not in short supply. Moreover, there was no evidence of any interactions in which one of the species inhibited the attachment or feeding of the other (Bull *et al*. 1989). Secondly, laboratory experiments in which hosts were infested with almost artificially high numbers of nymphs and larvae either of both species or just one species (infestation levels of several hundred – numbers which have been observed in the field, though on very rare occasions) did not detect any significant difference in the feeding success between the single species and mixed species treatments (Bull *et al*. 1989). Thus there was no evidence of interspecific competition being stronger than intraspecific competition. Therefore, the parapatric boundary is very unlikely to be maintained by the competition for on-host feeding sites between *B. hydrosauri* and *A. limbatum* and it does not seem to be even a contributing factor.

Factors causing the boundary: Reproductive interference

The parapatric boundary may be maintained by reproductive interference. Even though studies have shown that these two tick species cannot mate with each other, and hence infertile hybrids (which would likely prevent the colonization of either species across the boundary) do not form (Bull *et al* 1981, Andrews 1982), individuals of one tick species may interfere with the mating success of the other. If mating interference occurs, the colonizers would most likely be greatly outnumbered by the resident species and hence the invading species would have low chances of establishment across the boundary.

Mating of these ticks is quite a chancy affair, as an adult female has to attach onto the same host as an adult male, which is made difficult by the patchy distribution of the hosts (Andrews and Bull 1980). *Amblyomma limbatum* females, similarly to *B. hydrosauri* females, mate once, detach, produce a large batch of eggs (1500 to 2000) and die, whereas males can mate multiple times (Andrews and Bull 1980). *B. hydrosauri* females have to feed for 5 to 10 days before they can mate (Andrews and Bull 1981). To attract males, fed females (of both species) emit an air-borne pheromone, quite likely to be 2,6 dichlorophenol (Andrews *et al*. 1986). This excitant causes the males to detach and start moving randomly around the host (Andrews and Bull 1982a). These signals are species-specific (Andrews 1982) and

experiments have shown that the males must have fed before they can respond to this signal (Andrews and Bull 1981). Any male coming into a close proximity of the female will detect another female-emitted air-borne pheromone, a non-species specific attractant (Andrews and Bull 1982a, Bull and Andrews 1984). When the males receive this signal, they will cease their random searches and start moving straight towards the female (Andrews and Bull 1982a). To be able to respond to this signal, the males do not need to have fed (Andrews and Bull 1981). A courtship signal will then initiate courtship activity after contact (Andrews and Bull 1982a).

Although mating of *B. hydrosauri* with *A. limbatum* is not known to occur, the colonizing (and hence outnumbered) species may not be allowed to propagate due to interspecific signal jamming (Andrews *et al*. 1982). This reproductive interaction was noticed when males were not responding to the excitant pheromone when both conspecific and non-conspecific females were present on the same host, despite the fact that they did start moving around when only conspecific females were present (Andrews *et al*. 1982). Thus it was concluded that the two sets of signals, from two sets of females, despite being regarded as species-specific, were sufficiently structurally similar to confuse the males who were not able to receive the signals correctly.

Further reproductive interference could be caused due to the attractant signal being non-species-specific, which could for example lead to a local *A. limbatum* male to attempt mating with an invading *B. hydrosauri* female. When this occurs the female does not respond to his courting and does not lift her body, but instead keeps it pressed to the lizard (Andrews 1982). Even if a non-conspecific male does manage to mount a female, mating will not take place (i.e. the male will not be able to transfer the spermatophore) due to the fact that he will not be able to properly align the capitulum against the female's genital pore (Andrews 1982). This is because the two tick species differ in size and body dimensions. Thus male-female leg arrangement (vital in phases 5 and 6 of courtship), which is unique for each species, would be incompatible in an interspecific mating (Andrews 1982). However, in such a situation the male might not be deterred but instead remain at the side of the female, hoping to mate with her. Potentially this could reduce the already slim chances of such a female mating a conspecific male to almost nil, hence making it impossible for that species to establish itself across the boundary. This behavior is known as the "satyr effect" and it has been suggested to prevent the coexistence of

ticks in Africa (Ribeiro and Speilman 1986) and mosquitoes in the Bahamas (Ribeiro 1988). Since *B. hydrosauri* males are more likely to encounter and block off *A. limbatum* females, than *A. limbatum* males encountering and blocking off *B. hydrosauri* females (as *B. hydrosauri* males move more about the host than do *A. limbatum* males, after detecting conspecific female excitant pheromones), *A. limbatum* females are more likely to be affected by the satyr effect (Bull and Burzacott 1994). As a delayed mating of *A. limbatum* females has been shown to result in them having significantly lower egg viability, satyr effect can have a profound influence on the reproductive fitness of the species (Chilton *et al*. 1993). Even though mating delays did not affect egg viability of *B. hydrosauri* females (Chilton *et al*. 1992), larvae that hatch later could be disadvantaged due to potentially being exposed for a shorter period of time to conditions most suitable for their development (Bull and Burzacott 1994).

To further investigate the effect of reproductive interference on maintaining the parapatric boundary, the two species of ticks were kept in pens, together with a certain number of sleepy lizards, and the performance of females in those pens was monitored over a period of 28 months. As a control, performance of females was also monitored when they were kept in pens only with individuals of their species. The percentage of attached females that have engorged and mated was not reduced or altered by the presence of heterospecifics (Bull and Burzacott 1994). No attempts at interspecific mating or male blockage of heterospecific females were observed even though both types of interactions have been reported in the laboratory studies (Bull and Burzacott 1994). These results do not provide any support for the hypothesis that reproductive interference stops females from mating on hosts with heterospecifics attached. Therefore, reproductive interference is unlikely to play a role in maintaining this abrupt parapatric boundary. Another hypothesis that has previously been suggested is that reproductive interference between the two tick species could cause delayed mating in either one or both species (Chilton *et al*. 1993). Again, results from the above described experiments reject this hypothesis. Engorgement times of females in mixed and single species pens did not differ significantly (Bull and Burzacott 1994). This was true for both the females attached during the warmer months of the year, when both species of ticks engorge rapidly and the females attached during the cooler winter months, when *B. hydrosauri* engorges slowly, but *A. limbatum*'s development virtually stops (Bull and Burzacott 1994).

Factors causing the boundary: Host / parasite co-evolution

Another possible reason behind the boundary and the inability of these two tick species to survive on the "away" side of the boundary may be the relationship between the host and the parasite and their co-evolution. The host (most often the sleepy lizard) may have developed various defenses to protect itself from infestation. Hence on the south side of the boundary such defenses would have been developed against *B. hydrosauri* and on the north side against *A. limbatum*. However, as the hosts were evolving to develop defenses against the parasites, so the parasites were evolving resistance to those defenses. It is possible that the sleepy lizard's defense against *B. hydrosauri* infestation is different to that against *A. limbatum*. Therefore, *B. hydrosauri* would only be able to infest lizards from the south side of the boundary and *A. limbatum* only those from the north side of the boundary, as they would not have sufficient resistance (to the lizard's defenses) to infest lizards from the opposite side of the boundary (Bull *et al*. 1981). For this hypothesis to be correct, the genetic make-up of the lizards from the opposite sides of the boundary would have to be different (specifically in the relevant genes) (Bull *et al*. 1981), which has not been tested as yet.

Factors causing the boundary: Microparasites

Another possible explanation for the parapatric boundary could be the indirect role that the haemogregarine blood parasite *Hemolivia mariae* plays. It has been found in some lizards on the *A. limbatum* side of the boundary, but rarely on the *B. hydrosauri* side (Smallridge and Bull 1999). This parasite is transferred into lizards (its primary host) much more effectively by *A. limbatum* than by *B. hydrosauri* (Smallridge and Bull 1999). It is possible that the *B. hydrosauri* ticks, which crossed into the "away" side of the boundary, were not able to persist there, as they ingested *H. mariae* with their blood meal, which negatively affected them (Bull and Burzacott 2001). Hence this could be another potential barrier to the spread of *B. hydrosauri* (although no negative effect on *B. hydrosauri* has as yet been shown). No equivalent blood parasite, associated only with *B. hydrosauri* has been detected, therefore this speculation does not explain why *A. limbatum* does not spread further south (Bull and Burzacott 2001).

Ridge and trough model

Many reasons have been put forward to explain why the abrupt parapatric boundary between *B. hydrosauri* and *A. limbatum* has formed and is maintained but it does not appear to be due to just a single cause. Experiments have shown that the reproductive interference between the two species, interspecific competition, predators, ecotone change, the difference in ambient temperatures between the two habitats or differences in physiology between the two tick species cannot explain the boundary alone but a combination of all or some of these factors could be important.

Several models have been proposed to explain why and how parapatry between two species forms. A model formulated to explain this parapatry is the heterogeneous environment model, applicable if the habitat is non-homogeneous and consists of ecological troughs and ridges (Bull 1991, Bull and Possingham 1995). These are not topogeographical features, but rather sectors of the environment in which an organism has high fitness (ridges) or low fitness (troughs) (Bull 1991). Higher numbers of individual would be expected to persist at the ridges than at the troughs. A parapatric boundary could be maintained at a site, which consists of a mosaic of habitats, some of them more favorable to an organism than others.

The two tick species have an uneven distribution at the Mt Mary study site. The demographic data (average number of ticks per host) showed that ticks of both species are distributed such that areas of high tick density (inferred to be ridges) are interspersed with areas of low tick density (inferred to be troughs) (Bull and Possingham 1995). The troughs may have formed as a result of reduced host density, reduced cover for ticks and hence an increased risk of desiccation or an increased level of predation (Bull and Possingham 1995). However it has not as yet been defined why some areas are less suitable for tick survival than others. In the troughs, the populations are maintained via dispersal of individuals from the ridges and such local populations may periodically die out due to poor environmental conditions if replenishment of the population with new arrivals does not exceed mortality (Bull and Possingham 1995). The model states that if there is a good year and a trough population has increased sufficiently, then it is possible for it to colonize the next ridge. Newly established ridge populations may be in danger of declining, or even becoming extinct, even if the conditions are favorable, if they are not constantly

replenished by individuals dispersing from nearby trough populations (Bull and Possingham 1995).

The model states that for the boundary to be maintained the ridges have to be further apart than the dispersal distance of either tick species (Bull and Possingham 1995). As these ticks disperse passively via host movement (Petney *et al*. 1983), at most 600 m per generation, and usually less than 200 m (Bull 1987), and the demographic study indicated the distance between ridges to be over 1 km, these data agree with the model (Bull and Possingham 1995). If the overlap zone between two species occurred in a density trough, as is the case at the Mt Mary parapatric boundary, and if the two species were involved in an interaction, even a very weak interspecific competition that would lead to a decline of one of those species, this would cause the boundary to be maintained (Bull 1991, Bull and Possingham 1995). Such a decline, even if only very slight, could cause the trough population of the affected species to decrease markedly over time, be unable to disperse and hence be unable to colonize the next ridge, or sustain a population there (Bull 1991, Bull and Possingham 1995). Therefore, in a heterogenous habitat such an interaction could prevent the spread of a species that has lower fitness in the poor regions (i.e. troughs) of the environment (Bull and Possingham 1995).

Expansion of the boundary to the north

During the initial 15 years of study of this parapatry the boundary remained stable, but recently (from 1992) it has been noted that the range of *B. hydrosauri* has expanded into the range of *A. limbatum,* which has caused the boundary to shift some 1 or 2 km north (Bull and Burzacott 2001). This expansion of the boundary has been associated with an increase in annual rainfall levels, as it has been noticed that the higher the rainfall level of the previous year, the further into the *A. limbatum* territory did the boundary advance. Increased rainfall levels have presumably allowed *B. hydrosauri,* which prefers a cool and wet environment, to colonize the usually more arid *A. limbatum* environment (Bull and Burzacott 2001). In these wetter years density of the *B. hydrosauri* ticks on the “away” side of the boundary increased, whereas concurrently *A. limbatum* density on the “home” side of the boundary decreased (Bull and Burzacott 2001). Whereas it appears fairly obvious why *B. hydrosauri* has increased in density on the “away” side of the boundary during and

after the wetter years, it is more difficult to surmise why *A. limbatum* should decrease in density during those years of high rainfall on the "home" side of the boundary. Higher rainfall levels should actually benefit this tick species, as even though it is more resistant to desiccation than *B. hydrosauri*, it is not immune to it and will also die in dry conditions (Chilton and Bull 1993c). Moreover, this decrease would not appear to be due to interspecific competition and an increase in the *B. hydrosauri* density, as these data were collected beyond the extent of the moving boundary. A likely reason is that *A. limbatum* is less resistant to lower temperatures (Chilton *et al* 2000). Alternatively, these changed environmental conditions may have caused an increase in the levels of some *A. limbatum* predator, the host behaviour might have somehow been affected or even the *A. limbatum* blood parasites might also play some role (Bull and Burzacott 2001). However, the change in the boundary's position may have been caused by some other environmental factors or changes rather than an increase in the rainfall levels, as "the actual advances and retreats of the boundary were not correlated with the rainfall the previous year" (Bull and Burzacott 2001).

If it is assumed that the boundary shift occurred due to the increased rainfall levels, two models have been proposed to explain this shift. One model proposes that the boundary has gradually advanced into the *A. limbatum* territory due to the high rainfall levels over several years. Each year, wet conditions allowed *B. hydrosauri* to advance further and further into the "away" side of the boundary. However, if the high rainfall years were to stop, it is predicted that eventually the boundary would have reverted back to its original location (Bull and Burzacott 2001). Another model is based on the ridge and trough model (Bull and Possingham 1995). It proposes that the advancement of the boundary was not a gradual event, but rather was due to one single critical event (e.g. increased rainfall levels), which allowed *B. hydrosauri* ticks to overcome a trough, which previously prevented this tick species from expanding into the "away" side of the boundary (Bull and Burzacott 2001). However, once beyond the trough, the population of these ticks would become self-sustaining and would expand (under most environmental conditions, which possibly could explain the observed lack of correlation between rainfall and year to year boundary movement) until encountering another trough. Thus this model predicts that the boundary will remain stable and will not retreat, even following the years without excessive rainfall (Bull and Burzacott 2001). Only in the event of successive low

rainfall years could this advanced population become reduced and eventually die out, especially if new recruits would be unable to supplement it. The second model appears to be more realistic, especially since no abrupt climate changes over less than a kilometer of the boundary have been observed that could explain the sharp boundary edge and hence a more plausible explanation is that a density trough is the reason for the lack of tick dispersal (Bull and Burzacott 2001).

The parapatric boundary between the two tick species is maintained where these two species meet at a density trough and the centre of the boundary will shift due to variance in the proportions of each species on either side of the density trough (Bull and Burzacott 2001). "The boundary runs along a gradient of environmental conditions and a change in the balance of relative fitness along the gradient, which resulted in one species increasing in density and the other decreasing in density, has led to the boundary shifting along a gradient" (Bull and Burzacott 2001). The fact that as the environmental conditions got wetter *B. hydrosauri* was able to persist on the "away" side of the boundary indicates that the vegetational ecotone change and a difference in soil and litter types that occurs across the boundary is not the sole reason why the boundary has formed (Bull and Burzacott 2001). Thus, a most likely reason for the maintenance of this boundary appears to be due to climatic factors, as a substantial change in the climate has caused a significant change in the boundary dynamics, i.e. "an adjustment of the ecological balance in the interaction between the two tick species" (Bull and Burzacott 2001).

Host

Sleepy lizards (*Tiliqua rugosa*) are large [adults snout-vent length (SVL) of over 30 cm (Bull 1987), weighing 600 to 900 g (Bull *et al*. 1991)] lizards belonging to the Scincidae family (Bull 1987). They are omnivorous, feeding mainly on flowers, but also occasionally on berries and snails (Dubas and Bull 1991) and long-lived [the maximum age has not been defined, but it is suspected to be around 50 years (Bull 1995)]. They are widely distributed throughout southern Australia (Bull 1995). A population of sleepy lizards living in the Mt Mary area of South Australia has been studied in detail for approximately the last 30 years.

Home range

Sleepy lizards occupy stable, non-circular (Bull 1994) home ranges, living in generally the same approximately 4 ha area from season to season (Bull and Freake 1999). Even over a four-year period, the home range centre of an individual sleepy lizard is unlikely to shift further than the span of the home range (i.e. no more than approximately 280 m) (Bull and Freake 1999). Only very rarely were the lizards found to have ventured a significant distance (i.e. approximately 2 km) from their home range (Bull 1987). Male and female lizards' home ranges do not differ in size (Dubas 1987). Home range of one *T. rugosa* lizard often overlaps with several other lizards (Bull 1994). It has recently been reported that the home range of *T. rugosa* comprises an exclusive core area (where neighbouring lizards usually do not venture into with the exception of the resident lizard's partner during the mating season) and a sally zone around that core area, which overlaps with sally zones of other lizard home ranges (Kerr and Bull 2006a). However lizards use the sally zone part of their home range infrequently (Kerr and Bull 2006a). Resident lizards have not been observed to exhibit any territorial behaviors towards the other lizards (Bull 1987, Satrawaha and Bull 1981).

Sleepy lizard environment (at the Mt Mary study site) consists of mallee scrub, chenopod bushland and open grassland (Petney and Bull 1984). It is not well understood how these lizards are able to recognize the boundaries of their home ranges from year to year. Two possibilities that have been put forward are that the lizards can "memorize" some distinct visual cues present within their home range or they use olfactory or chemical cues (Bull and Freake 1999). Experimental evidence indicates that it is the former rather than the latter. These reptiles have been found to be able to distinguish between visual signals of different shapes and degrees of brightness and to associate certain visual cues with the presence of shelter (Zuri and Bull 2000a). Since lizards whose olfactory system was not functional (due to treatment with $ZnSO_4$) were still able to maintain their home ranges and come back to their home range from a distance of up to 800 m, olfactory cues do not seem to play an important role in home range maintenance (Zuri and Bull 2000b).

The reason why recognizing their home range from season to season may be a problem for these lizards is that they are active for only some 4 months of the year, from August to December (Bull 1987). This is the spring season in Australia. From

early December the summer season starts and the temperatures become too hot for the lizards and there is also a lack of food as the plants the lizards feed on dry out. Hence during that period they spend most of the time in shelters such as rabbit or wombat burrows (Kerr *et al*. 2003, Kerr and Bull 2006b). The lizards become highly dependent on the fat reserves stored in their tails to survive that period, and if it is prolonged, for example because of a drought, many of them will die. Sleepy lizards start becoming more active from around March / April, when the weather starts cooling down. They come out of their burrows early each morning and bask in the sun for a few hours to warm before they start feeding. With the onset of winter (around May or July) come rain and cold temperatures, causing the lizards to stay in their burrows. They use fat reserves stored in their tails to survive that period before again becoming active the next spring.

Mating patterns

Sleepy lizard mating season starts in early September and lasts for about 6 to 8 weeks until early November (Bull *et al*. 1998). Sleepy lizards have been found to be monogamous, which is very unexpected and rare for lizards (Bull 2000). At the beginning of the mating season, the majority of the lizards which were together in the previous season(s) reform their bond (How and Bull 2002). Between years, 74% of paired females and 67% of paired males retain the same partner, for up to 7 successive years (now up to at least 21 years for some pairs), whereas within a year 90% of females and over 70% of males were found with the same partner (Bull 1990). The bond between the two partners appears to be very strong, as lizards have been observed to stay in close contact for up to two days with the body of their dead partner (Bull 2000, Kerr and Bull 2001). The paired lizards spend lots of time together, over the period of 8 weeks, but they can also be found apart (Bull 2000). When observed in a pair, the two lizards either follow one another (usually male following the female), rest in a refuge site, bask or feed, although most often just the female lizard was seen feeding, with the male nearby (Bull 2000). They almost always stay close to each other or in actual physical contact (Bull *et al*. 1993a).

After a separation of several days (or 8 months, i.e. between seasons), the two lizards relocate each other via several mechanisms. For example, one of the partners (usually the male) can follow the exact trail of his partner (tongue flicking the ground

as he walks) due to an odor trail left by his partner, the chemicals secreted either from her skin or the cloacal gland (Bull *et al*. 1993a). The odor has been found to persist for up to an hour (Bull *et al*. 1993a). Another way in which two lizards can recognize and find each other is via an airborne pheromone that possibly is the same chemical as the odor trail (Bull *et al*. 1993a). Also, when one partner is missing, the other will walk around and check out the refuge sites, in which the two lizards have previously spent time together (Bull *et al*. 1993a).

Male sleepy lizards have larger and broader heads, as well as larger jaws, than females of the equivalent size (Bull and Pamula 1996). The most likely reason for that is that males with wider heads are able to win more male-male combats and hence are better able to defend their partners. When males fight, they bite each other's heads and try to flip one another over (Bull and Pamula 1996). Even though such fights have only rarely been observed, they must occur as males can have quite significant scale damage to the head. Fights most likely take place amongst younger, smaller males, which are trying to access unpaired females and to form permanent bonds with them (Bull 1990). Even though monogamy is the typical social system for this species, polygamy does occasionally take place (Bull 2000). Social monogamy does not necessarily mean sexual monogamy (Bull *et al*. 1998). In one study, which used microsatellites to determine parentage of young lizards, 19% of females were found to have mated with a male that was not their partner (Bull *et al*. 1998). A survey of the Mt Mary population showed that within the 8 weeks of a mating season, 10% of encountered females were found with different males on at least two different occasions (Bull 1988).

Mating takes place from around late October through to mid November, and between 1 and 3 live young are born in March or early April, after about 5 month gestation period (Bull *et al*. 1993b). The pairs separate after mating (Bull 1988) and neither the mother nor the father look after the juveniles (Bull 2000). However, the mother can discriminate between her own offspring and other juveniles, most likely through vomeronasal olfaction (Bull *et al*. 1994), even after several months of separation (Main and Bull 1996). Moreover, young lizards are able to recognize their mothers (Main and Bull 1996). The young lizards are "permitted" to stay within their mother's home range during their first year, but usually disperse in the subsequent years (Bull and Baghurst 1998). Kin recognition and dispersal of juveniles before

they reach sexual maturity may possibly be used as a way of avoiding inbreeding (Bull and Cooper 1999).

Sleepy lizards take 3 to 5 years to reach sexual maturity at an SVL of at least 28 cm (Bull 1995). Mortality of the juveniles is high (61% to 86%), especially in the first year, as they only have a short period after they are born to feed and to prepare for the upcoming winter (Bull 1987). In contrast, adult lizards have relatively low mortality (Bull 1987). Sleepy lizards currently do not have any natural predators at the Mt Mary study site, with the only danger occasionally coming from feral foxes or dogs as well as from mortality due to road traffic.

Monogamy

The reasons for monogamy in sleepy lizards (within a season as well as between seasons) are not very well understood but one of the more obvious, caring for the offspring, is not one of them (Bull 2000). A simple explanation may be that due to their large territory, males simply would not be able to defend a larger number of females; hence they invest their energy in guarding just one female. The evidence that supports this hypothesis is the sexual dimorphism of males having bigger heads than females and the occasionally observed fights between males (Bull 2000). However, why would males guard their females for around 8 weeks, if they only become sexually receptive in the last week? Moreover, the pairs do not stay together all the time during the mating season, and when they are separated, no single males have been observed to try to mate with the temporarily "unguarded" females (Bull 2000). Also, when a pair was separated, females actively sought out their male partners (as well as vice versa), which does not fit the male-guarding hypothesis (Bull *et al*. 1993a).

Another hypothesis, which would explain why pairs form so early before females become sexually receptive, is that males constantly keep checking if the female is ready for mating, and hence spend most of their time with her. A slight expansion of this hypothesis is that male presence and attention is actually required to cause females to become sexually receptive, the so called "priming" hypothesis (Bull 2000). This hypothesis and the male guarding hypothesis do not have to be mutually exclusive, as when the female finally becomes sexually receptive the male

might have to guard her against other males, so that all his work and attention does not go to waste (Bull 2000).

Another set of hypothesis looks at monogamy from the female perspective, i.e. what advantage she might have from staying with the same partner over the 8 week period. Firstly, the male's function might be to guard the female from predators, or at least to warn her when danger approaches (Bull 2000). Often, when females were observed eating, their male partners were nearby but were not feeding, possibly on a look out for possible danger. Moreover, it was quite common for paired females to be found not feeding when it was evident (from food in her mouth) that she was doing so just a few moments ago. This therefore suggests that the male has warned the female of the scientist's approach (Bull 2000). Secondly, females might use males to spread their tick load around (Bull 2000). Females infested with a substantial number of ticks could have reduced fitness. Lastly, the male's role would be to stop other males from harassing the female, so that she could spend as much time as possible on feeding and gaining enough weight so that both she and the embryos will be able to survive the coming summer (Bull 2000). Moreover, if females "feel" that they are not ready for mating in a particular year, for example due to low food reserves, they might not become sexually receptive.

The reason for pair fidelity across years is equally puzzling. One hypothesis states that familiar partners are more likely to mate successfully than unfamiliar ones. Familiar partners could be more efficient at feeding or avoiding predators (Bull 2000). Moreover, females could possibly become sexually receptive sooner if the "priming" male is a familiar one (Bull 2000). Thus mating could potentially take place sooner, hence young being born sooner and having more time to feed and gain weight before the onset of winter (Bull 2000). Another hypothesis states that pairs form between individuals that are least genetically related to each other, which reduces inbreeding (Bull 2000). It has been shown, using microsatellites, that paired females were significantly less related to their partners than to other males overlapping their home range (Bull and Cooper 1999). However, it is not known how the females recognize which potential partners are least related to them (Bull 2000). Lastly, long term monogamy may possibly prevent spread of parasites and diseases (Bull 2000). If the lizard's current partner is not infected, there would be no reason for it to change partners, especially since there is a possibility that other potential partners in the population may be infected.

Tick infestation

The two tick species (*A. limbatum* and *B. hydrosauri*) that infest sleepy lizards at the Mt Mary study site may reduce their host's fitness if the levels of infestation are sufficiently high. This however seems to be quite a rare occurrence as during an 8 year study the highest within-year average number of *B. hydrosauri* ticks per host was 20.56, followed by 14.15 within another year (Bull and Burzacott 1993). During that same study period the highest within-year average number of *A. limbatum* ticks per host was 14.10. Just 32 out of the 3416 hosts captured during that period had more than 100 *B. hydrosauri* or *A. limbatum* ticks on them (Bull and Burzacott 1993).

An experiment, in which lizards were infested with an unnaturally high number of ticks, showed that such lizards had reduced activity compared to lightly infested lizards. They had significantly lower average home ranges, were found basking more often and having significantly lower daily movement rates (Main and Bull 2000). In the laboratory, highly infested lizards had slower sprint speeds and lower endurance levels compared to the uninfested controls (Main and Bull 2000). Reduced activity may result in reduced fitness, as such lizards would not be able to escape predators as efficiently or hold large home ranges, which would be significant if there is a food shortage (Main and Bull 2000). These results contrast sharply with conclusions made from another study (Bull and Burzacott 1993), which concluded that the lizards with the highest tick loads were the biggest, with the largest home ranges and were more likely to have a partner than lizards with lesser tick loads. The home ranges of such successful individuals would likely contain well-protected refuges, frequented by the lizards. Since ticks awaiting in those refuges would also be advantaged and able to persist longer due to the sheltered microclimate, the lizards visiting them would be more likely to be infested with a high number of ticks than less fit lizards occupying home ranges with poorer refuge sites (Bull and Burzacott 1993). Sleepy lizards must attain a balance between the cost of parasite infections and benefits of occupying a high quality habitat (good refuge sites, enhancing their survivability, especially during the hot summer months), which may have an influence on *T. rugosa* population structure (Main and Bull 2000).

The movement patterns of the sleepy lizards will have a strong influence on *B. hydrosauri* and *A. limbatum* population structure as these ticks are almost

exclusively dispersed passively via host movement. As the distance a tick disperses is the distance between the position of its host when the tick attached and the host's position when the tick detached and almost all attachments / detachments take place in lizard refuge sites, the pattern of usage of refuge sites by the lizards is likely to have a major influence on the distribution and population dynamics of these ticks. *Tiliqua rugosa* use of refuge sites is season-dependent (Kerr *et al*. 2003). During early spring they use a large number of individual bushes, each of them infrequently (Kerr *et al*. 2003). An increase in ambient temperature (late spring / early summer) prompts them to choose larger, dome-shaped, densely canopied bushes and also to use rabbit and wombat burrows (Kerr *et al*. 2003). As such refuges are relatively scarce, often a number of lizards can be found occupying the same refuge (Kerr and Bull 2006b).

Blood parasite

The two tick species (and especially *A. limbatum*) transmit a blood parasite *Hemolivia mariae*, which infects the sleepy lizards (Smallridge and Bull 2000). The life cycle of this microparasite is as follows: when the ticks engorge on infected lizard blood, they themselves become infected. Next, *H. mariae* reproduces within the tick gut. The lizards become infected with the parasite when they ingest infected ticks. In the lizard, the parasites undergo further division and subsequently invade red blood cells, in which they can persist for up to a year (Smallridge and Bull 2000). Research has found that female lizards infected with this blood parasite were not affected by the presence or the intensity of the infection, whereas infected males declined in body condition (Smallridge and Bull 2000). Possible reasons for this may be that the infection increases the males' rate of metabolism or affects the blood cells so that they carry lower amounts of oxygen (Smallridge and Bull 2000). Alternatively, it is possible that males whose body condition has worsened for some independent reason were more susceptible to infection (Smallridge and Bull 2000). For example they may have suffered malnutrition and hence not be able to mount a successful immune response against the infection (Smallridge and Bull 2000). Male lizards' body condition may especially worsen during the mating season when they do not feed very much and are under constant stress to "guard" the female. Moreover, during the mating season their testosterone levels rise, which may

decrease their body condition and make them more susceptible to parasite infections (Smallridge and Bull 2000).

Project aims

The main purpose of this study was to investigate the genetic population structure of *B. hydrosauri* and *A. limbatum* sampled either side of the zone of parapatry at Mt Mary to better understand the formation and maintenance of this boundary. Microsatellites were the molecular marker of choice for this study. Since there were no available *B. hydrosauri* or *A. limbatum* specific microsatellite primers, they were developed during the course of this project. As the suite of microsatellite loci developed for *A. limbatum* was unsuitable for a population genetics study (see *Chapter 2*), elucidating *B. hydrosauri* population structure became the sole focus of the project.

Aim 1: Specifically I investigated the dynamics of transmission of the different life stages of *B. hydrosauri* ticks among hosts. The predictions of the 'ripple' model, proposed to describe this process, were tested with the multilocus microsatellite genotypes of the sampled individuals. The predictions were that there will be higher relatedness among ticks on individual hosts than in the overall population, and on individual hosts relatedness will be higher among larvae than among nymphs or adults, that spatial genetic structuring within the tick population will extend further for larvae than for the later life stages, and that there will be detectable inbreeding within the tick population.

Aim 2: Furthermore, I used the microsatellite loci in a landscape genetics approach to detect genetically distinct groupings of ticks. The aim of this part of the project was to test the predictions of genetic structure arising from the ridge and trough model, and to explore other patterns of genetic structuring of tick populations. The prediction that was tested was the presence of several non-overlapping *B. hydrosauri* genetic clusters, representative of ridge populations, with trough areas acting as barriers to gene flow.

Since population structure of parasites has not been as popular a study topic amongst population geneticists as that of the free-living organisms a lot still remains to be discovered. Thus a more general aim of this study to gaining further insight as to why *B. hydrosauri* and *A. limbatum* are allopatrically distributed was to contribute

to our understanding of parasite population structure and ecology and enhance our knowledge of this unique class of organisms.

Chapter 2 - Development of microsatellite markers in two Australian reptile tick species and inheritance analysis

Introduction

Bothriocroton hydrosauri and *Amblyomma limbatum* are allopatrically distributed species of Australian reptile ticks. *Bothriocroton hydrosauri* occupies the wetter south-eastern part of the continent and *A. limbatum* the arid zone to the north (Smyth 1973). The parapatric distributional boundary between these tick species has been studied in detail close to Mt Mary, South Australia (34°06' S; 139°26' E) for the last 25 years. The most common host for the two tick species at the study site is the large scincid lizard *Tiliqua rugosa*, which is similarly abundant on either side of the boundary (Bull *et al*. 1981). The boundary is very abrupt, so that on some transects there is a very narrow overlap zone between the two tick species, between 100m and 5km (Bull *et al*. 1981). Several mechanisms have been postulated to explain this parapatry, including ecotonal change, interspecific competition, predation, mating interference and indirect interactions but none of them satisfactorily explain such a narrow overlap zone (Bull and Burzacott 2001). Some aspects of the tick population biology that are not known presently may provide further insights into the cause and maintenance of the parapatric boundary. In particular these include the population structure and dispersal patterns of the ticks, especially how these might vary between the different life stages. Here I describe the isolation, characteristics and inheritance of 10 *B. hydrosauri* and 10 *A. limbatum* polymorphic microsatellite markers that can be used to analyse population structure and dispersal.

Materials and Methods

Microsatellite marker isolation and primer design

Microsatellites were isolated following an enrichment protocol (Gardner *et al*. 2008). DNA was extracted following the DNeasy Tissue kit (QIAGEN) protocol from whole bodies of four male *B. hydrosauri* ticks (DNA yield: about 95 μg) and one *A. limbatum* male tick (DNA yield: 16.5 μg) that were collected from *T. rugosa* hosts at the Mt Mary study site. The extracted tick DNA was amplified with host lizard

specific nuclear (G426/G416 – amplifies β globin intron 2 region) and mitochondrial (Forstner *et al*. 1995) PCR primers in order to check if the tick DNA extract was contaminated with host DNA due to the presence of host blood in the tick's gut.

The two tick DNA aliquots were separately digested with *Rsa*I and *Bst*UI, in the presence of *Xmn*I (New England Biolabs). Immediately after the digestion the cut DNA fragments were ligated with double stranded adapters (SNX linkers, S475 and S476). Subsequently, the fragments were PCR amplified with the adapter specific primers and separated on a 1.5% agarose gel. 300 to 1000 bp fragments were size-selected and extracted from the gel matrix using a column (MO BIO GelSpin UltraClean DNA purification kit) after which the *Rsa*I and *Bst*UI digested fragments were pooled. Next the size-selected DNA was enriched for AAAG, AAAC or AC motif containing fragments. DNA was hybridized to the 3' biotinylated repeat oligos [$(AAAG)_6$; $(AAAC)_6$; $(AC)_{11}$ - three separate reactions] using an Eppendorf Mastercycler, with an initial denaturation at 95 °C for 5 min, after which the temperature quickly ramps down to 70 °C and steps down 0.2 °C every 5 sec for 99 cycles. There was a pause at 50 °C for 10 min, after which temperature ramped down 0.5 °C every 5 sec for 20 cycles. Streptavidin MagneSphere® Paramagnetic particles (Promega) were used to isolate the fragments to which the AAAG, AAAC or AC biotinylated probes were bound. The enriched solution was removed from the beads and cleaned (MoBio column), with enriched fragments eluted in 25 μl of 10 mM Tris (pH 8.0). The cleaned up, enriched fragments were PCR amplified with S475 primers, purified and used for the second round of the enrichment process, performed as described above. The purified, double enriched DNA was ligated into TOPOTA (Promega TA Cloning Kit, Invitrogen) vectors, and subsequently One Shot Mach1 TIR (Promega TA Cloning Kit, Invitrogen) *Escherichia coli* cells were transformed with the plasmid. The colonies were incubated overnight with ampicillin selection. Inserts containing clones were screened using a general hybridization process (Sambrook *et al*. 1989) with the appropriate biotinylated probe [$(AAAG)_6$; $(AAAC)_6$; $(AC)_{11}$] and an alkaline phosphatase/streptavidin colourimetric reaction (Boehringer Mannheim) (Jordan *et al*. 2002). Subsequently minipreps were prepared from the colonies containing inserts with the wanted repeat type using the Wizard® Plus SV Minipreps DNA Purification System kit (Promega). The eluted plasmid DNA was then amplified with T7 promoter and M13 reverse plasmid vector primers and sequenced on both strands using the ABI Prism Big Dye cycle sequencing kit in ABI

3700 sequencers (Applied Biosystems). OLIGO 4.0 (National Bioscience, Inc.) was used to design PCR primer pairs complementary to the flanking regions of microsatellites that had at least five repeat motifs. Primers were designed to amplify 20 unique *B. hydrosauri* and 16 unique *A. limbatum* microsatellite loci.

DNA extracted from 30 adult ticks of both species collected at Mt Mary was amplified in the order to determine whether the loci were polymorphic. Amplifications were done with fluorescent M13 tagged primers or using fluorescent dUTPs and products were separated on a Gelscan acrylamide gel system (Corbett). Subsequently ten *B. hydrosauri* and six *A. limbatum* loci were dropped due to monomorphism or a high incidence of non-specific bands. The scorable polymorphic *B. hydrosauri* and *A. limbatum* loci are listed in Table 1a and Table 1b, respectively.

PCR conditions - Bothriocroton hydrosauri

Each forward *B. hydrosauri*-specific primer was 5' labeled with a permanent fluorescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) (Table 1a). PCR-amplifications were performed for two multiplexes, a triplex (set 1) and a pentaplex (set 4), and two single locus reactions (sets 2 and 3) (see Table 1a). Lack of dimer formation between primers present in the same multiplex reaction was estimated using the software AUTODIMER (Vallone and Butler 2004).

PCR amplifications were performed in 10-µl reaction volumes in the Eppendorf Mastercycler thermal cycler. Each reaction contained 2x reaction buffer (Applied Biosystems) and 2.5 mM $MgCl_2$ (set 1) or 1x reaction buffer (Applied Biosystems) and 2.5 mM $MgCl_2$ (set 3) or 2x Eppendorf reaction buffer (including 2.5 mM $MgCl_2$) (set 4) or 1x Eppendorf reaction buffer (including 2.5 mM $MgCl_2$) (set 2), 0.2 mM each dNTP, 400 to 600 nM each primer, 10 U (set 1) or 1 U (set 3) AmpliTaq Gold DNA Polymerase (Applied Biosystems) or 10 U (set 4) or 1 U (set 2) HotMaster Taq DNA Polymerase (Eppendorf), 0.4 µl 100X BSA (Ambion) (sets 1 and 4), 1.89 µl 5M betaine (Sigma) (sets 1 and 4), 2 µl genomic DNA (20 to 40 ng). The cycling conditions were as follows: initial denaturation step 94 °C for 9 min (sets 1 and 3) or 2 min (sets 2 and 4), followed by 34 cycles at 94 °C for 30 s, annealing temperature (see Table 1a) for 30 s and extension at 72 °C (sets 2 and 3) or 65 °C (sets 1 and 4) for 30 s, followed by a single final extension step at 72 °C (sets 2 and 3) or 65 °C (sets 1 and 4) for 30 min. Fluorescently labeled products were

Table 1a. Description of 10 microsatellite loci in tick *Bothriocroton hydrosauri*. Cloned allele size is in basepairs (bp). The final annealing temperature (T_a) is in °C. Primer pairs assigned to the same multiplex set 1-4 were used to amplify template in a single reaction tube (see text for reaction mix details). *Dots represent non-motif bases. Cloned sequences are in GenBank under accession numbers.

Locus name	Primer sequence (5'-3')	Repeat motif in clone*	Cloned allele size	Primer label	Multiplex set, T_a	GenBank accession
Bohy1	F: AGTCGGGCTTCAAAGGTTCA R: CCTACCCAGTCCCATTAAAGA	$(AAAG)_{18}$	224	PET	1, 59	EU051324
Bohy2	F: CACTACCTCCTGTTGCACACA R: GGGACTTGTCGTTTTTGCTGT	$(AAAG)_9$...$(AAAG)_2$...$(AAAG)_1$...$(AAAG)_2$	206	VIC	1, 59	EU051325
Bohy3	F: CCGACACCTTCGTTACCGA R: ATGTGGAACAGCGCCTCATTA	$(AAAG)_{11}$	261	NED	1, 59	EU051326
Bohy4	F: CGTCACACTTGATACGTTGTC R: AGGCGTAATTAATGACCGCT	$(AAAG)_{11}$	231	FAM	2, 53	EU051327
Bohy5	F: CGTTAGCGTTGTCTTGCACAA R: CAGAAATGGCTTGCTTCAGA	$(AC)_{10}$	219	NED	3, 48	EU051328
Bohy6	F: TGTGGCCAATCACTCTTTGT R: TTAGACTGCACTCGATGGCGT	$(AAAG)_{14}$...$(CAAG)_{10}$	200	VIC	4, 59	EU051329
Bohy7	F: ATGTGGAGGTAGTGGGTTCGA R: GTTTTTGAGCTGTTTTATGCG	$(AAAG)_7$	127	FAM	4, 59	EU051330
Bohy8	F: TACGCAGCGGATAGGCAAC R: TGGGTGATATTGTCAAAGGCT	$(AAAG)_{16}$...$(AAAG)_3$	246	FAM	4, 59	EU051331
Bohy9	F: TCTGTATTGGAACGTGTGACG R: CCAAGGAAGAGAGGTCATCAT	$(AC)_{19}$	161	NED	4, 59	EU051332
Bohy10	F: GCGAGCCGATGTAGTGAAA R: CTGCACATAAATGTAGATAGC	$(AC)_{28}$	192	PET	4, 59	EU051333

Table 1b. Description of 10 microsatellite loci in tick *Amblyomma limbatum*. Cloned allele size is in basepairs (bp). The indicated annealing temperature (T_a, in °C) is the optimum for each primer pair if the primers are unlabeled. TD indicates a Touch Down PCR program. *Dots represent non-motif bases.

Locus name	Primer sequence (5'-3')	Repeat motif in clone*	Cloned allele size	Primer label (MRT)	T_a (unlabeled)
Amli1	F: AGATTGCCTGTAAGCTCA R: ATTCGCTACTGTCGCTCA	$(AG)_9$...$(AC)_{26}$…$(AC)_4$…$(AC)_{12}$…$(AC)_3$	410		TD 55→47
Amli2	F: CCTTGCCCTATTCCTCAT R: ACACTGTAGTTACCGCAC	$(AAAG)_{26}$	288	VIC	TD 55→47
Amli3	F: GTATAAGACGGCGACGAC R: AAAGGCAAATATAGAGAACA	$(AAAG)_{15}$…$(AAAG)_1$…$(AAAG)_3$	176	VIC	TD 62→54
Amli4	F: CTAGAGCCTATGCCATAG R: CATATAGAGACTGTGCGAC	$(AAAG)_{13}$	343		TD 55→47
Amli5	F: GAGTATCACACAGGGACG R:ACAACTGCTTGGCCGAAC	$(AAAG)_{18}$…$(AAAG)_2$	345	PET	TD 64→56
Amli6	F: CGCCATAACCTCTGCTGC R: TAGTTCTGTGTATCTCCG	$(AAAG)_9$…$(AAAG)_{16}$…$(AAAG)_5$	215	PET	TD 53→45
Amli7	F: CAGTACAGGTTGAATATC R: TTTCTCTTTTGCTACATG	$(AAAG)_{16}$…$(AAAG)_{10}$	397	NED	TD 54→49
Amli8	F: CCCTGCTTGGACCTGTGC R: AATTTCATGCCTGTCTGC	$(AAAG)_{14}$	276	NED	TD 55→47
Amli9	F: GCTCTATTTACTGCGTTGAC R: CCTTTACACTGCCACTCTG	$(AC)_{14}$...$(TC)_{19}$	215	FAM	TD 62→54
Amli10	F: TTTCCGTGTCATGGTGCG R: CAGTTTTATCCCTATTGG	$(AAAG)_{17}$	278	FAM	TD 57→49

separated on an ABI 3730 DNA Analyzer and sized and scored using the program GeneMapper® version 3.7 (Applied Biosystems). Allele sizes and patterns did not differ between the multiplex and singleplex amplifications for the same loci.

PCR conditions - Amblyomma limbatum

Amblyomma limbatum DNA was amplified with the ten primer pairs listed in Table 1b following the recently developed Multiplex Ready Technology (MRT) (Hayden *et al*. 2008). This system fluorescently labels the product during PCR amplification without the need to permanently label primers, which is highly cost-effective and allows for increased flexibility in the choice of the dyes. Moreover all marker essays are performed under standardized conditions that do not require optimization, thus potentially allowing many primer pairs with differing optimum annealing temperatures to amplify template in a single reaction.

PCR was performed in two stages, the first of which comprised an initial denaturation at 95 °C for 10 min, followed by 5 cycles at 92 °C for 60 s, 50 °C for 90 s, 72 °C for 60 s, followed by 20 cycles at 92 °C for 30 s, 63 °C at 90 s and 72 °C at 60 s. In this stage, the target loci were amplified with the locus-specific primers (Table 1b), each tagged at its 5' end with a non-complementary nucleotide tag sequence, such that upon its completion the tag sequence was fully incorporated into the amplified product, at both the 3' and the 5' ends. These sequences act as universal primer binding sites for the second PCR stage (40 PCR cycles of 92 °C for 15 s, 54 °C for 60 s and 72 °C for 60 s, with a final extension step at 72 °C for 10 min) in which short, dye labeled primers that are complementary to the specialized tag sequence were used to amplify and label (dye chosen to label each locus listed in Table 1b) the first phase PCR product.

PCR amplifications were performed individually for each locus in 12-µl reaction volumes, containing 1X Multiplex-Ready PCR buffer (containing 5X Immolase PCR buffer, 7.5 mM $MgCl_2$, 1 mM each dNTP, 1 ml 100X BSA and sterile water), 75 nM fluorescent-colour labeled forward tag primer (tagF), 75 nM reverse tag primer (tagR), 20 nM or 40 nM the locus specific primer, 0.15 U Immolase polymerase (Bioline) and 2 µl DNA (20 to 40 ng). Initial screening showed that two (*Amli1* and *Amli4*) of the ten primer pairs did not amplify consistently, despite excellent previous unlabeled amplification (performed under

different PCR conditions – see Table 1b). Fluorescently labeled products were separated, sized and scored as for *B. hydrosauri*.

Pedigree analysis

Crossing experiments were conducted in order to examine whether the isolated *B. hydrosauri* and *A. limbatum* markers are inherited in a Mendelian fashion

In September 2006, 10 lizards were collected from the Mt Mary study site (from the same region that was sampled for the population genetic estimates – see Table 2 for coordinates of each capture) and bagged individually for transport to the Flinders University Animal House. Each lizard was infested with at least one virgin female and, with the exception of three hosts, at least one conspecific male (Table 2). Unfed, unengorged females lacking a white spermatophore plug on their abdomens were classified as virgins (Andrews and Bull 1980). In a couple of cases, the female was slightly or substantially engorged, but as only a single male was found on that same host, he was presumed to be that female's mating partner.

In captivity the lizards were housed separately indoors, with 12 hours/day exposure from a heat lamp. The lizards were monitored everyday to record the reproductive status of the tick females and the movement of the males. After the mated female ticks fell off the host they were housed individually in small plastic containers at 25° C and 80-85% relative humidity with a 12:12 photoperiod. DNA was extracted from parents and 40 larvae from each of eight broods (six *B. hydrosauri,* two *A. limbatum*) following the DNeasy Tissue kit (QIAGEN) protocol. The *B. hydrosauri* and *A. limbatum* parents and 20 larvae/brood with the highest DNA quantity (approximately 20 to 40 ng) were genotyped as described above. The time line of the “main events” for the 8 families that were used for pedigree analysis is presented in Table 3.

Predictions of genotypic proportions under the expectation of Mendelian inheritance were tested with chi-squared contingency tests with the *P* value significant if less than the Bonferroni corrected value (Hochberg 1988).

Results

Specificity of the isolated markers

Table 2. *Bothriocroton hydrosauri* and *A. limbatum* males and virgin females used in the crossing experiments to examine the inheritance of the isolated microsatellite loci were collected from ten *Tiliqua rugosa* lizards captured at the Mt Mary sampling site. Subsequently these lizards and the ticks infesting them were transported to the Flinders University Animal House. The location and date of each capture were as listed above. M and F indicates male and female ticks, respectively. H represents *Bothriocroton hydrosauri* ticks and L represents *Amblyomma limbatum* ticks. *These ticks were not present on the host when it was captured and were put on it at a later date. As a total of 15 female ticks were infesting the ten hosts, potentially 15 different broods could have been reared. However, because of female mortality either prior to or after mating (due to such causes as desiccation or the host ingesting the tick) eight broods were reared (six *B. hydrosauri* and two *A. limbatum*).

Host number	Capture coordinates	Capture date	Tick infestation
1	S34º 06.010' / E139º 26.220'	26/09/06	1H F, 2H M
2	S34º 08.135' / E139º 26.187'	26/09/06	2H F, 1H M, 1L M
3	S34º 03.634' / E139º 26.916'	27/09/06	2L F, 1L M, 3H M
4	S34º 03.942' / E139º 26.818'	27/09/06	2H F, 1L M, 1H M*
5	S34º 04.600' / E139º 26.608'	27/09/06	2H F, 7L M, 1H M*
6	S34º 04.733' / E139º 26.566'	27/09/06	1H F, 1H M, 1L M
7	S34º 05.872' / E139º 26.215'	27/09/06	2H F, 1H M, 1L M
8	S33º 59.058' / E139º 27.470'	26/09/06	1L F, 6H M, 1L M*
9	S33º 55.856' / E139º 25.495'	26/09/06	1L F, 3L M
10	S34º 01.829' / E139º 26.455'	27/09/06	1H F, 1H M

Table 3. Time line of the main events for the eight families that were used in the pedigree study. Note that the dates specified for Eggs laid and Larvae hatched are the starting dates for these events, i.e. when these events were first observed for each family. Families BH1 and BH2 share the same father as do families BH3 and BH4.

Family	Species	Host	Mating	Female detached	Male killed	Eggs laid	Female killed	Larvae hatched	Larvae killed
BH1	*B. hydrosauri*	4	29/09/06	13/10/06	21/10/06	12/11/06	12/12/06	31/12/06	05/01/07
BH2	*B. hydrosauri*	4	07/10/06	21/10/06	21/10/06	23/11/06	12/12/06	07/01/07	09/01/07
BH3	*B. hydrosauri*	5	28/09/06	10/08/06	22/10/06	12/11/06	12/12/06	31/12/06	05/01/07
BH4	*B. hydrosauri*	5	01/10/06	22/10/06	22/10/06	23/11/06	12/12/06	31/12/06	05/01/07
BH5	*B. hydrosauri*	6	Prior to 27/09/06	28/09/06	28/09/06	02/11/06	30/11/06	23/12/06	25/12/06
BH6	*B. hydrosauri*	7	02/10/06	18/10/06	18/10/06	24/11/06	12/12/06	12/01/07	16/01/07
AL1	*A. limbatum*	3	Prior to 27/09/06	06/10/06	06/10/06	15/10/06	27/10/06	10/12/06	12/12/06
AL2	*A. limbatum*	9	02/10/06	01/11/06	01/11/06	10/11/06	27/12/06	27/12/06	05/01/07

Origin of the DNA extract used to isolate the microsatellite loci from was confirmed as tick DNA due to lack of amplification from that extract with the host specific primers. Moreover, host DNA was not amplified with any of the tick microsatellite markers. Hence all the markers are tick specific. *Amblyomma limbatum* DNA could not be reliably amplified with the *B. hydrosauri* microsatellite markers and vice-versa, at the conditions optimum for each primer.

Inheritance of Bothriocroton hydrosauri *loci*

Pedigree analysis of the six *B. hydrosauri* families showed nine of the ten loci to be inherited in a Mendelian manner, as the Bonferroni corrected *P* values at those loci were nonsignificant for all six broods (Table 4). Null alleles or large allele dropout were not observed at any of the loci.

Locus *Bohy5* was inherited in a Mendelian manner in three families. In families BH5 and BH6 both parents were 216/216 homozygotes and so were all the scored offspring, whereas in family BH4 (*P* value 0.3711), the mother was a 216/216 homozygote and the father a 216/222 heterozygote, and the offspring were either 216/216 homozygotes or 216/222 heterozygotes (Table 4). In the three families in which this locus was inherited in a non-Mendelian manner (*P* value significant even after Bonferroni correction – Table 4) both parents were 216/222 heterozygotes. However, these families lacked 222/222 homozygous offspring and family BH3 also lacked 216/216 homozygotes (thus all scored offspring were 216/222 heterozygotes).

One explanation for the phenotypes observed in the three families at which *Bohy5* was inherited in a non-Mendelian manner is that the two alleles (216 and 222) belong to two different (most likely monomorphic) loci that have been co-amplified due to a partial complementarity of the priming sites. However, sequencing of a 216/216 individual and a 222/222 individual showed the flanking regions of these two alleles to be identical. This locus was not considered in the subsequent analyses.

Inheritance of Amblyomma limbatum *loci*

Genotype analysis of the two *A. limbatum* families indicated presence of multiple F_1 offspring whose genotypes did not conform to expectations under Mendelian inheritance proportions (Table 5a and Table 5b). Offspring of family AL1 had

Table 4. Inheritance of 10 microsatellite loci in six *Bothriocroton hydrosauri* families (BH1 – BH6). Scored F_1 genotypes indicates the number of larvae (maximum 20) that were successfully genotyped at each locus for each family. For each locus and each family shown are the parental (Mother and Father) genotypes and all the possible Mendelian larval (F_1) genotypes. Observed indicates how many larvae per brood exhibited each genotype. Expected indicates how many F_1 offspring should be exhibiting each genotype if the loci were inherited in a Mendelian manner. Whether the observed genotypic proportions differed significantly from those expected (thus indicating non-conformance to Mendelian expectations) was tested with a chi-squared contingency test with the *P* value significant if less than the Bonferroni corrected value. **P* value significant after sequential Bonferroni correction**.**

Locus	Family	Scored F_1 genotypes	Mother	Father	F_1	Observed	Expected	*P* value
Bohy1	BH1	19	222/246	222/254	222/222	6	4.75	0.9014
					222/246	4	4.75	
					222/254	4	4.75	
					246/254	5	4.75	
	BH2	19	246/246	222/254	222/246	11	9.5	0.4912
					246/254	8	9.5	
	BH3	19	246/246	246/254	246/246	7	9.5	0.2513
					246/254	12	9.5	
	BH4	20	222/246	246/254	222/246	2	5	0.2615
					222/254	8	5	
					246/246	6	5	
					246/254	4	5	
	BH5	20	230/246	222/246	222/230	7	5	0.3080
					222/246	4	5	
					230/246	2	5	
					246/246	7	5	
	BH6	19	246/246	230/254	230/246	13	9.5	0.1083
					246/254	6	9.5	
Bohy2	BH1	20	224/224	216/224	216/224	7	10	0.1797
					224/224	13	10	
	BH2	19	224/224	216/224	216/224	10	9.5	0.8186
					224/224	9	9.5	
	BH3	19	224/224	224/228	224/224	10	9.5	0.8186
					224/228	9	9.5	
	BH4	20	224/224	224/228	224/224	8	10	0.3711
					224/228	12	10	
	BH5	20	224/224	216/224	216/224	10	10	1.0000
					224/224	10	10	
	BH6	19	224/224	216/224	216/224	11	9.5	0.4912
					224/224	8	9.5	
Bohy3	BH1	19	274/290	290/314	274/290	7	4.75	0.5909
					274/314	5	4.75	
					290/290	4	4.75	
					290/314	3	4.75	
	BH2	18	274/274	290/314	274/290	11	9	0.3460
					274/314	7	9	
	BH3	19	274/290	274/290	274/274	6	4.75	0.1388
					274/290	12	9.5	
					290/290	1	4.75	

	BH4	20	274/274	274/290	274/274	10	10	1.0000
					274/290	10	10	
	BH5	20	290/314	274/274	274/290	7	10	0.1797
					274/314	13	10	
	BH6	20	314/314	290/290	290/314	20	20	1.0000
Bohy4	BH1	20	264/264	260/260	260/264	20	20	1.0000
	BH2	17	264/264	260/260	260/264	17	17	1.0000
	BH3	19	260/264	260/260	260/260	7	9.5	0.2513
					260/264	12	9.5	
	BH4	19	260/260	260/260	260/260	19	19	1.0000
	BH5	18	260/260	260/264	260/260	11	9	0.3460
					260/264	7	9	
	BH6	18	268/268	256/260	256/268	13	9	0.0954
					260/268	6	9	
Bohy5	BH1	20	216/222	216/222	216/216	3	5	0.0047*
					216/222	17	10	
					222/222	0	5	
	BH2	18	216/222	216/222	216/216	3	4.5	0.0111*
					216/222	15	9	
					222/222	0	4.5	
	BH3	18	216/222	216/222	216/216	0	4.5	0.0001*
					216/222	18	9	
					222/222	0	4.5	
	BH4	20	216/216	216/222	216/216	8	10	0.3711
					216/222	12	10	
	BH5	19	216/216	216/216	216/216	19	19	1.0000
	BH6	19	216/216	216/216	216/216	19	19	1.0000
Bohy6	BH1	20	220/220	212/220	212/220	8	10	0.3711
					220/220	12	10	
	BH2	20	220/220	212/220	212/220	9	10	0.6547
					220/220	11	10	
	BH3	20	220/224	212/220	212/220	5	5	0.0937
					212/224	9	5	
					220/220	1	5	
					220/224	5	5	
	BH4	19	220/220	212/220	212/220	9	9.5	0.8186
					220/220	10	9.5	
	BH5	19	212/220	220/220	212/220	12	9.5	0.3441
					220/220	8	9.5	
	BH6	20	212/224	220/244	212/220	6	5	0.8495
					212/244	4	5	
					220/224	4	5	
					224/244	6	5	
Bohy7	BH1	20	140/166	140/162	140/140	7	5	0.5724
					140/166	3	5	
					140/162	4	5	
					162/166	6	5	
	BH2	20	140/140	140/162	140/140	8	10	0.3711
					140/162	12	10	
	BH3	20	132/140	140/158	132/140	8	5	0.4235
					140/140	4	5	
					132/158	3	5	

					140/158	5	5	
	BH4	19	140/166	140/158	140/140	7	4.75	0.2243
					140/166	2	4.75	
					140/158	3	4.75	
					158/166	7	4.75	
	BH5	20	166/166	140/140	140/166	20	20	1.0000
	BH6	20	140/140	132/140	132/140	10	10	1.0000
					140/140	10	10	
Bohy8	BH1	19	278/282	262/262	262/278	13	9.5	0.1083
					262/282	6	9.5	
	BH2	20	278/290	262/262	262/278	10	10	1.0000
					262/290	10	10	
	BH3	20	262/274	262/274	262/262	4	5	0.3012
					262/274	8	10	
					274/274	8	5	
	BH4	19	270/274	262/274	262/270	3	4.75	0.8012
					262/274	6	4.75	
					270/274	5	4.75	
					274/274	5	4.75	
	BH5	20	262/274	262/262	262/262	7	10	0.1797
					262/274	13	10	
	BH6	20	274/274	262/262	262/274	20	20	1.0000
Bohy9	BH1	20	158/176	158/176	158/158	5	5	0.0907
					158/176	14	10	
					176/176	1	5	
	BH2	20	176/176	158/176	158/176	12	10	0.3711
					176/176	8	10	
	BH3	20	158/176	158/176	158/158	8	5	0.2592
					158/176	7	10	
					176/176	5	5	
	BH4	19	158/158	158/176	158/176	9	9.5	0.8186
					176/176	10	9.5	
	BH5	20	158/176	158/176	158/158	6	5	0.8187
					158/176	10	10	
					176/176	4	5	
	BH6	20	158/158	158/176	158/176	8	10	0.3711
					176/176	12	10	
Bohy10	BH1	20	184/202	184/202	184/184	2	5	0.1653
					184/202	14	10	
					202/202	4	5	
	BH2	20	184/204	184/202	184/184	5	5	0.4235
					184/202	8	5	
					184/204	3	5	
					202/204	4	5	
	BH3	20	184/202	184/202	184/184	7	5	0.3867
					184/202	7	10	
					202/202	6	5	
	BH4	19	184/206	184/202	184/184	8	4.75	0.0753
					184/202	1	4.75	
					184/206	3	4.75	
					202/206	7	4.75	
	BH5	19	202/206	184/184	184/202	10	9.5	0.8186

				184/206	9	9.5	
	BH6	20	184/206	202/206	184/202	4	5	0.5724
					184/206	3	5	
					202/206	6	5	
					206/206	7	5	

Table 5a. Inheritance of eight microsatellite loci in an *Amblyomma limbatum* family (AL1). Scored F_1 genotypes indicate the number of larvae (maximum 20) that were successfully genotyped at each locus. For each locus and each family shown are the parental (Mother and Father) genotypes and all the possible Mendelian larval (F_1) genotypes. Observed indicates how many larvae per brood exhibited each genotype. Expected indicates how many F_1 offspring should be exhibiting each genotype if the loci were inherited in a Mendelian manner. Whether the observed genotypic proportions differed significantly from those expected (thus indicating non-conformance to Mendelian expectations) was tested with a chi-squared contingency test with the *P* value significant if less than the Bonferroni corrected value. The test was not performed for those loci at which larval genotypes were observed that are not possible under Mendelian inheritance laws (Non-Mendelian F_1).

Locus	Scored F_1 genotypes	Mother	Father	F_1	Observed	Expected	Non-Mendelian F_1	Observed	*P* value
Amli2	15	322/330	322/322	322/330	8	7.5	330/330	3	
				322/322	4	7.5			
Amli3	16	206/206	206/218	206/206	11	8			0.1336
				206/218	5	8			
Amli5	13	343/343	343/343	343/343	13	13			1.0000
Amli6	17	232/232	182/232	182/232	11	8.5			0.2253
				232/232	6	8.5			
Amli7	14	435/439	396/400	396/435	4	3.5			0.4818
				396/439	5	3.5			
				400/435	4	3.5			
				400/439	1	3.5			
Amli8	17	307/315	315/315	307/315	7	8.5	307/307	6	
				315/315	4	8.5			
Amli9	18	245/245	245/245	245/245	18	18			1.0000
Amli10	15	335/335	295/335	295/335	3	7.5	295/295	6	
				335/335	6	7.5			

Table 5b. Inheritance of eight microsatellite loci in an *Amblyomma limbatum* family (AL2). Scored F_1 genotypes indicate the number of larvae (maximum 20) that were successfully genotyped at each locus. For each locus shown are the parental (Mother and Father1, also Father2 and Father3 – see text) genotypes and all the possible Mendelian larval (F_1) genotypes. Observed indicates how many larvae per brood exhibited each genotype. Expected indicates how many F_1 offspring should be exhibiting each genotype if the loci were inherited in a Mendelian manner. Whether the observed genotypic proportions differed significantly from those expected (thus indicating non-conformance to Mendelian expectations) was tested with a chi-squared contingency test with the *P* value significant if less than the Bonferroni corrected value. The test was not performed for those loci at which larval genotypes were observed that are not possible under Mendelian inheritance laws (Non-Mendelian F_1).

	Scored F_1								Non Mendelian		
Locus	**genotypes**	**Mother**	**Father1**	**Father2**	**Father3**	**F_1**	**Observed**	**Expected**	**F_1**	**Observed**	**P value**
Amli2	18	286/376	286/286	376/376	286/300	286/286	12	9			0.1573
						286/376	6	9			
Amli3	20	174/174	190/202	218/218	190/214	174/190	2	10	190/190	8	
						174/202	7	10	202/202	3	
Amli5	19	399/399	343/347	343/399	343/383	343/399	1	9.5	343/343	6	
						347/399	4	9.5	347/347	8	
Amli6	19	202/232	220/220	182/182	198/250	202/220	5	9.5	202/202	5	
						220/232	1	9.5	232/232	8	
Amli7	18	377/435	373/435	393/435	435/435	373/377	0	4.5	373/373	2	
						373/435	4	4.5	377/377	5	
						377/435	1	4.5			
						435/435	6	4.5			
Amli8	19	288/299	311/311	288/288	271/271	288/311	4	9.5	288/288	5	
						299/311	6	9.5	299/299	4	
Amli9	20	217/234	234/234	217/217	217/234	217/234	6	10	217/217	6	
						234/234	8	10			
Amli10	20	288/288	284/288		288/299	284/288	0	10	284/284	9	
						288/288	11	10			

genotypes not possible under Mendelian expectations at three loci: *Amli2*, *Amli8* and *Amli10* (Table 5a). For instance, at locus *Amli2*, three of the 15 successfully scored offspring had a 330/330 genotype, which is not possible under Mendelian inheritance, as only the mother had a 330 allele (Table 5a). Also, at locus *Amli8*, six of the 17 successfully scored offspring had a 307/307 genotype, which is not possible under Mendelian inheritance, as again only the mother had a 307 allele (Table 5a). The case is similar for locus *Amli10*, although in this case it was the father who was heterozygous for allele 295, which was not present in the mother, but for which six of the 15 successfully scored offspring were homozygous (Table 5a). For the five remaining loci, at which all the scored offspring contained genotypes in accordance with Mendelian inheritance proportions, the *P* values ranged from 0.1336 to 1 (Table 5a) thus confirming these loci to be inherited in a Mendelian manner.

A large proportion of the successfully scored AL2 offspring exhibited genotypes impossible under Mendelian inheritance laws at seven of the eight loci (Table 5b). For example at locus *Amli3* eight of the 20 successfully scored offspring had a 190/190 genotype and three had a 202/202 genotype but none of these alleles were present in the mother (Table 5b). At locus *Amli7* two of the 18 successfully scored offspring had a 373/373 genotype and five had a 377/377 genotype but neither of these alleles was present in both parents (Table 5b).

Ticks labeled Father2 and Father3 were genotyped (Table 5b) as both of them were present on the same host (host 9 – see Table 2) as female AL2 and Father1 when that host was captured. Thus even though they were subsequently removed, there was a possibility that one of them was the mating partner of the female and not Father1 - the presumed father of AL2 offspring as only he was observed in the copulatory position with the female. This possibility was discounted as none of the AL2 offspring exhibited a genotype consistent with inheritance either from Father2 and Father3 and female AL2 at all eight loci tested (Table 5b).

Possible reasons for the apparent non-Mendelian genotypes among F_1 offspring may be null alleles (presuming that the homozygous parent carries a null allele - N) or large allele dropout (presuming that in the offspring apparently homozygous for the smaller of the two parental alleles the larger parental alleles did not amplify). Thus, chi-square tests were performed for those loci at which offspring were observed to have non-Mendelian genotypes, with an allowance for null allele presence (Table 6a) or large allele dropout (Table 6b). After such correction,

Table 6a. Recalculations of the chi square contingency tests for three loci in family AL1 and six loci in family AL2 with an allowance for null allele (N) presence in one of the parents and hence also in a proportion of the F_1 offspring. **P* value significant after sequential Bonferroni correction.

Family AL1						
Locus	**Mother**	**Father**	**F_1**	**Observed**	**Expected**	***P* value**
Amli2	322/330	322/N	322/330	8	3.75	0.0369
			322/322, 322/N	4	7.5	
			330/N	3	3.75	
Amli8	307/315	315/N	307/315	7	4.25	0.0958
			315/315, 315/N	4	8.5	
			307/N	6	4.25	
Amli10	335/N	295/335	295/335	3	3.75	0.4066
			335/335, 335/N	6	7.5	
			295/N	6	3.75	
Family AL2						
Locus	**Mother**	**Father1**	**F_1**	**Observed**	**Expected**	***P* value**
Amli3	174/N	190/202	174/190	2	5	0.1577
			174/202	7	5	
			190/N	8	5	
			202/N	3	5	
Amli5	399/N	343/347	343/399	1	4.75	0.1310
			347/399	4	4.75	
			343/N	6	4.75	
			347/N	8	4.75	
Amli6	202/232	220/N	202/220	5	4.75	0.1570
			220/232	1	4.75	
			202/N	5	4.75	
			232/N	8	4.75	
Amli8	288/299	311/N	288/311	4	4.75	0.9014
			299/311	6	4.75	
			288/N	5	4.75	
			299/N	4	4.75	
Amli9	217/234	234/N	217/234	6	5	0.6703
			234/234, 234/N	8	10	
			217/N	6	5	
Amli10	288/N	284/288	284/288	0	5	0.0158*
			288/288, 288/N	11	10	
			284/N	9	5	

Table 6b. Recalculations of the chi square contingency tests for one locus in family AL1 and two loci in family AL2 with an allowance for non-amplification of the larger allele. The bold allele was inherited by a proportion of the F_1 offspring but it was not amplified in some F_1 offspring possibly due to large allele dropout PCR error.

Family AL1						
Locus	**Mother**	**Father**	**F_1**	**Observed**	**Expected**	***P* value**
Amli10	335/335	295/335	295/**335**	9	7.5	0.4386
			335/335	6	7.5	
Family AL2						
Locus	**Mother**	**Father1**	**F_1**	**Observed**	**Expected**	***P* value**
Amli5	399/399	343/347	343/**399**	7	9.5	0.2513
			347/**399**	12	9.5	
Amli7	377/435	373/435	373/377	0	4.5	0.1116
			373/**435**	6	4.5	
			377/**435**	6	4.5	
			435/435	6	4.5	

inheritance of all of the loci for both families was consistent with Mendelian expectations (nonsignificant *P* after Bonferroni correction), except for locus *Amli10* in family AL2 (Tables 6a and 6b). Null allele presence could explain the observed lack of Mendelian inheritance proportions for three loci in family AL1 (null alleles present in the father at two loci and in the mother at one locus) and five loci in family AL2 (null alleles present in the father at three loci and in the mother at two loci) (Table 6a). Large allele dropout was presumed to be the cause of the observed non-Mendelian inheritance at a single locus in family AL1 (40 bp difference between the scored alleles) and at two loci in family AL2 (around 50 bp difference between the scored alleles) (Table 6b). The observed non-Mendelian inheritance patterns at locus *Amli10* for family AL1 and locus *Amli5* for family AL2 could be explained either by null allele presence or by large allele dropout (Tables 6a and 6b).

The high frequency of null alleles or large allele dropout observed for eight newly developed *A. limbatum* microsatellite loci could be either due to features of the template DNA itself (such as mutations at the priming sites) or possibly due to the MRT labeled primers amplifying the product but not labeling all of it, thus rendering some of the alleles not visible. As MRT is a relatively new primer labeling technology that has only been extensively used to genotype several well-studied organisms (e.g. wheat or barley), it is unknown how it may perform under other circumstances. In order to test the performance of the MRT labeled primers, seven *A. limbatum* individuals (AL1 and AL2 parents and up to three larvae per family (Table 8)) were amplified at all eight loci with unlabeled primers, at each primer pair's optimum annealing temperature (Table 2). To label the product, fluorescent dUTPs were added to the reaction mix. The products were separated on a Gelscan acrylamide gel system (Corbett) and the allele patterns were analysed.

There were not any new alleles detected for any of the eight loci for any of the seven individuals after amplification with the unlabeled primers, compared to the MRT labeled products (Table 7). Therefore it appears that the genotypes impossible under Mendelian laws of inheritance observed for a large proportion of the F_1 offspring were not due to MRT-specific reasons, as those same genotypes were observed after amplification with the non-labeled primers (Table 7). However, the lack of the larger allele observed in some AL2 F_1 offspring at loci *Amli5* and *Amli7* (non-Mendelian genotypes) seems to be due to lack of amplification or labeling of that allele with the MRT labeled primers as these alleles were clearly amplified with

Table 7. Comparison of genotypes assigned the AL1 and AL2 parents and three F_1 offspring (L) after amplification with MRT (MRT score) labeled and non-labeled (dUTP score) primers for the eight *Amblyomma limbatum* loci. In bold are the F_1 offspring that displayed non-Mendelian genotypes. Highlighted in bold and italicized are individual genotypes that differed after amplification with the MRT labeled and unlabeled primers. Note that the size of a fluorescent dUTP labeled product was smaller than the size of an equivalent MRT labeled product due to the lack of the non-complementary nucleotide tag sequence.

Locus	Tick	MRT score	dUTP score
Amli2	AL1 Mother	322/330	322/330
	AL1 Father	322/322	322/322
	AL2 Mother	286/376	286/376
	AL2 Father1	286/286	286/286
	AL1qL	**330/330**	**330/330**
	AL1rL	**330/330**	**330/330**
	AL1sL	322/330	322/330
Amli3	AL1 Mother	206/206	206/206
	AL1 Father	206/218	206/218
	AL2 Mother	174/174	174/174
	AL2 Father1	190/202	190/202
	AL2aL	174/202	174/202
	AL2c1L	**190/190**	**190/190**
	AL2h1L	**202/202**	**202/202**
Amli5	AL1 Mother	343/343	Did not amplify
	AL1 Father	343/343	343/343
	AL2 Mother	399/399	399/399
	AL2 Father1	343/347	343/347
	AL2bL	343/399	343/399
	AL2dL	***343/343***	***343/399***
	AL2gL	***347/347***	***347/399***
Amli6	AL1 Mother	232/232	232/232
	AL1 Father	182/232	182/232
	AL2 Mother	202/232	202/232
	AL2 Father1	220/220	220/220
	AL2dL	202/220	202/220
	AL2vL	**232/232**	**232/232**
	AL2eL	**202/202**	**202/202**
Amli7	AL1 Mother	435/439	Did not amplify
	AL1 Father	396/400	393/400
	AL2 Mother	377/435	377/435
	AL2 Father1	373/435	Did not amplify
	AL2aL	373/435	373/435
	AL2cL	***377/377***	***377/435***
	AL2gL	***373/373***	***373/435***
Amli8	AL1 Mother	307/315	Did not amplify
	AL1 Father	315/315	315/315
	AL2 Mother	288/289	288/289
	AL2 Father1	311/311	311/311
	AL1tL	**307/307**	**307/307**
	AL2nL	**299/299**	**299/299**
	AL2pL	**288/288**	**288/288**
Amli9	AL1 Mother	245/245	245/245
	AL1 Father	245/245	245/245

	AL2 Mother	217/234	217/234
	AL2 Father1	234/234	234/234
	AL2iL	217/234	217/234
	AL2oL	**217/217**	**217/217**
	AL2wL	**217/217**	**217/217**
Amli10	AL1 Mother	335/335	335/335
	AL1 Father	295/335	295/335
	AL2 Mother	288/288	288/288
	AL2 Father1	284/288	284/288
	AL1oL	**295/295**	**295/295**
	AL2gL	**284/284**	**284/284**
	AL2uL	**284/284**	**284/284**

the unlabeled primers, as represented by strong bands on the acrylamide gel (compare the MRT score and dUTP score for individuals AL2dL and AL2gL at locus *Amli5* and AL2cL and AL2gL at locus *Amli7*, Table 7). Thus labeling these eight *A. limbatum* specific markers with another system (such as ABI that was used to label *B. hydrosauri* specific markers) would not have made them usable for a population genetics study, save perhaps *Amli5* and *Amli7*.

Since none of the tested *A. limbatum* microsatellite loci were inherited in accordance with Mendelian laws in both families, this suite of markers was deemed unsuitable for a population genetics study. Due to time and monetary constraints more *A. limbatum* specific markers were not developed and no further analyses were performed on this species. To perform a population genetics study on this tick species a very high number of microsatellite markers would need to be developed, as the marker attrition rate was very high.

Bothriocroton hydrosauri *loci characteristics*

Genotypes of at least 56 adult *B. hydrosauri* ticks (males and females) from Mt Mary were used for the analyses described below. Number of alleles per locus, allele size range, frequency of the most common allele and the observed (H_O) and expected (H_E) heterozygosities were calculated in GENALEX 6 (Peakall and Smouse 2006) and CERVUS 3.0 (Kalinowski *et al*. 2007) (Table 8). The number of alleles per locus ranged from 2 to 7 (mean = 5.11) and the expected heterozygosity ranged from 0.399 to 0.755. Observed heterozygosity values (average 0.538) were lower than the expected heterozygosity (average 0.583) at six of the nine loci (Table 8). The slight heterozygote deficit at these loci may suggest null alleles, large allele dropout, inbreeding or the Wahlund effect. Null allele estimates were obtained for each locus with CERVUS 3.0 (Table 8), which showed the null allele frequencies to be positive for six of the loci, the highest value being 0.1955 (locus *Bohy7*).

Tests of loci conformance to Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium were performed in GENEPOP web version 3.4 (Raymond and Rousett 1995). Markov chain parameters were: dememorization at 10000, 10000 batches and 10000 iterations per batch. Two loci significantly deviated from HWE (*Bohy6* and *Bohy7*), one locus (*Bohy6*) even after sequential Bonferroni correction (Table 8). No linkage disequilibrium was observed for any of the loci after sequential

Table 8. The properties of nine microsatellite loci within a sample of *Bothriocroton hydrosauri*. The number of individuals genotyped (N), number of alleles (N_A), allele size range in basepairs (bp), the most common allele and its frequency (F), observed (H_O) and expected (H_E) heterozygosities, the probabilities associated with the exact Hardy-Weinberg test (P_{HW}), and the null allele frequency (F_N) are listed for each locus. *P_{HW} significant after sequential Bonferroni correction.

Locus	N	N_A	Allele size range (bp)	Most common allele (F)	H_o	H_e	P_{HW}	F_N
Bohy1	56	6	222→266	254 (0.446)	0.643	0.680	0.628	0.015
Bohy2	57	4	216→232	224 (0.561)	0.649	0.594	0.640	-0.054
Bohy3	56	6	274→314	274 (0.625)	0.500	0.554	0.319	0.045
Bohy4	53	5	256→272	260 (0.566)	0.509	0.612	0.051	0.077
Bohy6	57	6	200→244	220 (0.640)	0.456	0.556	0.002*‡	0.112
Bohy7	58	3	140→166	140 (0.741)	0.276	0.399	0.0110*	0.196
Bohy8	57	7	262→294	274 (0.412)	0.684	0.755	0.450	0.050
Bohy9	58	2	158→176	158 (0.716)	0.431	0.411	1.000	-0.029
Bohy10	58	7	184→208	202 (0.483)	0.690	0.689	0.090	-0.016

Bonferroni correction. Thus these loci should be valuable markers for population genetic analysis of *B. hydrosauri* though properties of locus *Bohy6* should be investigated in a wider sample.

Discussion

The nine newly developed *B. hydrosauri* microsatellite loci exhibited allelic richness of 5.11 within a sample of at least 56 adult ticks. Thus these loci are less variable than 17 microsatellite loci of tick *Ixodes ricinus*, which is the main vector for important infectious diseases in both humans and animals [average allelic richness of 8.71 within a sample of up to 24 individuals (Roed *et al*. 2006)], and less variable than nine microsatellite loci of a seabird tick *I. uriae* [average allelic richness of 9.22 alleles per locus within a sample of up to 64 ticks originating from two Atlantic puffin colonies (McCoy and Tirard 2000)]. For the nine *I. ricinus* microsatellite loci the H_E ranged from 0.08 to 0.94 (average 0.62) (McCoy and Tirard 2000), whereas for the 17 *I. ricinus* loci the H_E ranged from 0.40 to 0.87 (average 0.69) (Roed *et al*. 2006), thus a similar range to that of the nine *B. hydrosauri* loci described above. The H_O values were much lower than the H_E values for most of the 17 *I. ricinus* loci and seven of the loci deviated significantly from HWE expectations after Bonferroni correction, which was suggested to be due to relatively high frequency of null alleles for these loci (Roed *et al*. 2006). H_O was lower than H_E for six of the nine *B. hydrosauri* loci though null allele presence was moderate for only one locus and low for the other loci. Null alleles were thought to be present at one of the eight newly developed Southern cattle tick *Boophilus microplus* microsatellite loci within a sample of 94 individuals (Koffi *et al*. 2006a).

Pedigree analysis showed all but one of the ten newly developed *B. hydrosauri* microsatellite loci to be inherited in a Mendelian manner in all six families, but for all of the eight tested *A. limbatum* loci a proportion of the F_1 offspring displayed genotypes not possible under Mendelian laws of inheritance in at least one of the two tested families. For seven of the loci the non-Mendelian offspring genotypes could be explained either by the presence of null alleles or large allele dropout. It is not known whether null alleles are wide spread throughout the *A. limbatum* genome or whether they are specifically associated with microsatellites comprising (AAAG), (AC), (AG)(AC) or (AC)(TC) repeat motifs. Primers

amplifying loci comprising other motifs would have to be designed to investigate this further.

However, for two loci, one *B. hydrosauri*-specific (*Bohy5*) and one *A. limbatum*-specific (*Amli10*), technical causes could not explain the aberrant F_1 offspring genotypes observed in at least some of the tested families. Locus *Bohy5* was inherited in a non-Mendelian fashion in three *B. hydrosauri* families, where both parents were heterozygous for the same two alleles (216 and 222). Two of these families lacked 216/216 homozygous offspring and one family also lacked 222/222 homozygous offspring (all of the scored offspring had the 216/222 genotype). The reasons for such genotype ratios are unknown but it seems that there is a bias against homozygous F_1 offspring at this locus, especially those exhibiting the 222/222 genotypes. Locus *Amli10* was inherited in a non-Mendelian fashion in one *A. limbatum* family where the father was a 284/288 heterozygote and the mother a 288/288 homozygote. Eleven of the offspring exhibited a 288/288 genotype and nine a non-Mendelian 284/284 genotype. Moreover, no 284/288 heterozygous offspring were scored. If the mother carried a null allele, then this could explain the 284/284 F_1 genotypes, though the genotypic ratios would still differ significantly from those expected under Mendelian laws of inheritance due to the lack of 284/288 offspring. Non-amplification of the 288 bp allele in the offspring scored as 284/284 homozygotes due to preferential amplification of the shorter (284 bp) allele would bring the observed genotype ratios in line with Mendelian expectations (i.e. nine offspring exhibiting a 284/288 genotype and 11 a 288/288 genotype). However large allele dropout seems unlikely in this case as there is only a 4 bp size difference between the 284 bp and 288 bp alleles.

Non-Mendelian microsatellite inheritance patterns that cannot be explained by PCR artefacts have also been observed in other organisms and several different reasons have been given as explanation. Examples include the Eastern oyster *Crassostrea virginica* [possibly due to strong zygotic selection (Reece *et al*. 2004)], Pacific abalone *Haliotis discus hannai* [reasons unknown but proposed to occur due to such events as gene conversion, nonrandom segregation of chromosomes during meiosis, differential viability or functionality of gametes, or linkage to a second locus with a deleterious dominant allele (Li *et al*. 2003)], or plant pathogen *Phytophthora cinnamomi* [best explained by nondisjunction at meiosis in the parents resulting in aneuploid progeny (Dobrowolski *et al*. 2002)].

Non-Mendelian patterns of inheritance that could not be explained by null alleles were observed for four recently characterized *I. ricinus* microsatellite loci in at least one of the five tested families (Roed *et al*. 2006). Another study, which investigated the inheritance of five other *I. ricinus* microsatellite loci found two loci to be inherited in a Mendelian manner in all families tested, two loci that were not inherited in a Mendelian manner, though this discrepancy could be explained by the parents carrying null alleles, and one locus which non-Mendelian inheritance observed in some of the families could not be explained by null allele presence (de Meeûs *et al*. 2004). For those pairs, maternal alleles appeared to be experiencing some difficulties in being amplified in the offspring, which was proposed to be occurring due to maternal imprinting via methylation of the template DNA (de Meeûs *et al*. 2004). However, as experiments comparing the efficiency of amplification of the methylated and un-methylated templates did not show any significant differences between the two treatments, methylation does not explain the observed non-Mendelian offspring genotypes (de Meeûs *et al*. 2004). Short allele dominance (amplification bias in favour of the shortest alleles) was proposed as another explanation for the behaviour of this locus (de Meeûs *et al*. 2004).

An intriguing inheritance pattern of three microsatellite loci has recently been described in the human body louse *Pediculus humanus* (McMeniman and Barker 2006). If the male was heterozygous for any of these loci (which was the case in six of the eight tested families) then he only passed one of his two alleles to his offspring, i.e. all of the scored F_1 offspring of a heterozygous male had the same paternal allele at that particular locus (McMeniman and Barker 2006). This resulted in non-Mendelian inheritance ratios, which could not be explained by null allele presence or large allele dropout. To further investigate these observations two separate F_1 crosses were set up, involving two males that were F_1 brothers and had identical genotypes at the three loci (and were heterozygous at two) and two F_1 sisters (not related to the males). Interestingly, inheritance was Mendelian at the two informative loci (the loci the males were heterozygous for) in one family whereas in the other family none of the F_2 offspring inherited, from the male, alleles of grand-paternal origin (alleles this male received from his P_1 father) (McMeniman and Barker 2006). This result hinted at an extreme case of transmission ratio distortion of paternal alleles, possibly occurring due to paternal genome elimination (males transmit to their offspring only their maternal set of chromosomes as their paternal

set were eliminated at some stage during development), which could occur due to meiotic drive following nonindependent assortment of maternal and paternal chromosomes during spermatogenesis or postmeiotic selection prior to fertilization (McMeniman and Barker 2006). Furthermore, as one of the F_1 males did transmit his paternally inherited alleles to his offspring in a Mendelian manner, this suggested that the mother of the two F_1 males that fathered the F_2 offspring may have been heterozygous for a genetic element that eliminates alleles in male *P. humanus* (McMeniman and Barker 2006). Paternal genome elimination would affect all loci and I observed non-Mendelian inheritance patterns that cannot be explained by PCR artifacts at just one *B. hydrosauri* and one *A. limbatum* locus, thus this phenomenon cannot be the cause of the non-Mendelian inheritance patterns at these two loci. To better understand the reasons for the observed non-Mendelian inheritance patterns at these two loci further crosses will need to be set up.

Chapter 3 - Molecular genetic data provide support for a model of transmission dynamics in an Australian reptile tick *Bothriocroton hydrosauri*

Introduction

Since the free-living stages of many parasite species have low dispersal capability, and some parasites may even completely lack free-living stages, the population structure and dynamics of individual parasite species may be intimately connected to the ecology and movement patterns of their host species (Nadler 1995, Criscione *et al*. 2005). The ecological challenges for a parasite can be extremely different in the environment they experience when they are with a host and when they are in the external environment away from a host. Hosts can be regarded as spatially and temporally patchy habitats within a hostile matrix (Criscione *et al*. 2005). Price (1977, 1980) suggested that this might lead to locally isolated parasite populations, each derived from a small number of founders. He suggested there would be low dispersal of parasites among host individuals and reduced opportunities for outbreeding in local parasite populations. These predictions particularly apply to parasites that can go through multiple generations on the same host individual (Criscione *et al*. 2005). However, many animal macroparasites release offspring into the external environment, where progeny from different sources can become mixed, before infesting another definitive host. The degree of mixing within populations of an individual parasite species may fall somewhere along a continuum from isolation on individual hosts to complete mixing among hosts. The position of a parasite species between these two extremes of population structure may depend on the life history strategy of the parasite species considered, on the density, movement and social overlap of its hosts and on the severity of the external environment. The more frequently hosts contact each other, and the less severe the off-host conditions, the more likely that parasite individuals in a population will become mixed.

The population genetic structure of a parasite species may provide an indication of where it fits on that continuum. This in turn provides evidence about the transmission dynamics of the parasite. For instance a parasite that can disperse widely among its host population, and that has extensive mixing, would be expected to show low levels of relatedness among individuals infesting the same host, and

high levels of outbreeding and heterozygosity within host populations. Parasites that are mostly confined to a local subset of the host population may show opposite trends. My study describes aspects of the population genetic structure of the Australian reptile tick, *Bothriocroton hydrosauri*. My aim was to explore the transmission dynamics, and to test specific predictions based on previous ecological and behavioural studies.

Previous studies of tick populations have described different patterns of genetic variation both among and within tick species, and have inferred the probable role of host movement. Bull *et al*. (1984) reported lower levels of isozyme variation in six species of Australian reptile ticks, than either Healy (1979a, 1979b) or Hilburn and Sattler (1986) found for ticks with more mobile hosts. Lampo *et al*. (1998) found that most of the genetic variation in the tick *Amblyomma dissimile* resulted from differences in allele frequencies among ticks from different host individuals. They suggested that host dispersion influenced the genetic structure of populations of that tick. Similarly, McCoy *et al*. (2003) found that populations of the seabird tick *Ixodes uriae* had different genetic structure in colonies of two seabirds with differing mobilities.

Bothriocroton hydrosauri is a three-host tick that infests large reptiles in south-eastern Australia (Smyth 1973). In my study area the major host for all life stages of the tick is a large skink, the sleepy lizard, *Tiliqua rugosa* (Smyth 1973). Larvae attach to a host, engorge on blood or lymph, detach and moult to nymphs. These attach to a second host, engorge, detach and moult to adults. These in turn attach to a third host. Adult male ticks do not engorge, but mate on the host with attached females. A female will mate with a single male, whereas males can mate with multiple partners (Andrews and Bull 1980). Mated females engorge, detach and lay several thousand eggs, which hatch into the next generation of larvae. Engorged ticks detach while their hosts are in refuge shelters, moult to the next stage and then wait in the refuge for another host. As males remain attached to hosts after mating, feeding sparingly, many more males than females are found on the lizard hosts. Bull (1978b) argued that the limited movement of host lizards is likely to restrict the distance that ticks disperse.

Models of the population dynamics of this tick species (Bull 1991, Bull and Possingham 1995, Tyre 1999, Tyre *et al*. 2006) have invoked a process called the ‘ripple effect’ (Bull, unpublished data). It is based on the use of multiple shelter

refuges by each individual host lizard within its home range which averages 3-5 ha (Bull and Freake 1999, Kerr *et al*. 2003), the overlap of lizard home ranges (especially male overlap of female home ranges) (Kerr and Bull 2006a), the non-synchronous sharing of refuges by different lizards (Dubas and Bull 1992, Kerr and Bull 2006a), and the high susceptibility of detached ticks to predation (Bull *et al*. 1988, Dawes-Gromadzki and Bull 1997a, 1997b) and desiccation (Chilton and Bull 1993c). Desiccation is particularly severe on the small larval stage in the hot Australian spring and summer when their lizard hosts are most active. The ripple model assumes that many ticks in lizard refuges may have to wait for the next host for longer than they can survive. This will be particularly the case at the lower rainfall edge of the tick distribution, where most of the previous studies have been conducted (Bull and Possingham 1995, Bull and Burzacott 2001).

In the ripple model, an engorged female tick deposits her egg clutch in a lizard refuge. The hatched larvae aggregate while waiting for a host (Petney and Bull 1981), but many complete clutches of larvae die before a host lizard uses the refuge. However, in a surviving clutch, many of the aggregated larvae attach to the first host that uses the refuge. Thus larvae occur in high densities on a few hosts that represent rare focal points in the landscape (Bull 1978a). As those larvae engorge on the host they detach over a number of days, and into a number of refuges that the host lizard visits (Bull 1978b, Kerr and Bull 2006a). A subset of these detached larvae survive and moult, to attach as nymphs onto separate hosts that enter the several refuges. These in turn engorge over a period of time and are scattered into further refuges where they moult to adults.

Thus, in this model, the progeny of a single female will be spread further from their single point of origin with each life stage. In a landscape with high tick mortality, each of the few clutches that contribute to the population can be imagined as creating a ripple of ticks that spreads from the central clutch deposition site. The harsher the habitat, the fewer ripples across the landscape and the less likely it is that there will be mixing of different clutches. In the continuum of parasite population structure referred to above, the ripple model is positioned towards the isolation and inbreeding end. The ripple model predicts different levels of mixing at the different life stages.

The model predicts that:

a) there will be higher relatedness among ticks on individual hosts than in the overall population, and on individual hosts relatedness will be higher among larvae than among nymphs or adults

b) spatial genetic structuring within the tick population will extend further for larvae than for the later life stages, because there is less mixing among clutches at the larval stage

c) there will be detectable inbreeding within the tick population, because adult ticks are likely to encounter related adults on a host for mating, particularly where "ripples" from different clutches have low overlap.

Here I tested these predictions with data from microsatellite DNA genotypes from *B. hydrosauri* ticks.

Materials and Methods

Sampling and genotyping

The study was conducted during spring and early summer (Sept – Dec) of 2004 and 2005, along a 29 km unsealed road transect about 10 km east of Bundey Bore Station, South Australia (139° 21' E, 33° 55'S). The chenopod shrubland habitat has been described previously by Kerr *et al*. (2003). The average annual rainfall at Bundey Bore is 241 mm. The study transect was perpendicular to and close to the abrupt distributional boundary of *B. hydrosauri* (Bull and Possingham 1995, Bull and Burzacott 2001), and within the most arid part of the species' range. I captured a total of 155 active (feeding or basking) sleepy lizards by hand along roadsides, 109 (70.3%) of which were infested with *B. hydrosauri* ticks (average 9.57 *B. hydrosauri* ticks per host). All of the lizard capture locations, which were recorded by GPS, were in close proximity to large bushes (potential host refuge sites, hence potential *B. hydrosauri* transmission locations).

I collected all *B. hydrosauri* ticks attached to the captured *T. rugosa* hosts and placed them in individual vials that were immediately frozen in liquid nitrogen. Tick DNA was extracted following the DNeasy Tissue kit (QIAGEN) protocol and genotyped at nine polymorphic microsatellite loci as described in Guzinski *et al*. (2008), which also presents details of the variability of the markers based on genotypes of at least 56 *B. hydrosauri* adults from the study site. I used data from

848 ticks that were successfully genotyped for at least six loci. These included 464 larvae, 140 nymphs and 244 adults (222 males and 22 females), which were collected from 40 hosts captured in 2004, and an additional 56 new lizards, plus two recaptures in 2005 (a total of 98 samples from 96 hosts).

Relatedness

I tested the prediction that there was higher relatedness (*R*) among larvae than among nymphs or adults on a host using estimates of pairwise *R* derived from RELATEDNESS 5.0.8 (Queller and Goodnight 1989). Allele frequencies for the whole sample were estimated in GENALEX 6.1 (Peakall and Smouse 2006) from the genotypes of the adult ticks to ensure that the reference allele frequencies were based on a random sample of minimally related individuals (allele frequencies did not differ significantly at the majority (six) of the loci between the 2004 and 2005 adult samples and there was no significant difference in the multilocus allelic richness and gene diversity estimates between the two samples). Standard errors of *R* estimates were obtained by jackknifing over loci.

For each tick life stage in each year, I derived two relatedness coefficients. The first measure was the mean *R* among ticks of the same life stage on the same host (On Host *R*). For each host infected with more than one of a particular tick life stage I estimated the mean *R* for all pairs of that life stage on that host. Then I took the mean of these values over all multiply-infected hosts. The second measure was the mean *R* value for ticks of that life stage in the whole sample (Total *R*). For hosts with more than one individual of a particular life stage, I randomly chose one tick to include in the analysis. The number of hosts included in these two estimates differed because hosts infested with a single individual of a particular life stage could be included in the Total *R* but not On Host *R* calculations.

I tested for differences between mean values of Total *R* and On Host *R* in each life stage, and between On Host *R* values among different life stages and years using the jackknife resampling technique (over loci), followed by a standard unpaired *t*-test in RELATEDNESS 5.0.8.

Spatial genetic structure

I investigated the spatial extent of genetic population structure for each of the three *B. hydrosauri* life stages using spatial autocorrelation analysis as implemented in SPAGEDI 1.2 (Hardy and Vekemans 2002). All ticks collected from the same host were assigned the capture location of their host. In this case SPAGEDI treats all individuals collected from the same host as belonging to one "spatial group" and permutes "spatial group" locations to estimate the 95% confidence interval. In order to test whether the degree of relatedness among individual ticks was dependent on geographical distance, I modified one of the coordinates of each individual by altering the third decimal point of the easting such that each individual had a unique set of spatial coordinates. In this way, I permuted the locations of individual ticks and not host i.e. "spatial group" locations. I specified distance intervals of 650 meters. This interval was the smallest that had over 50% of individuals participating at least once in a pairwise comparison within an interval and a coefficient of variation <1 for the number of times each individual participated in a pairwise comparison within an interval, conditions recommended for robust analysis by Hardy and Vekemans (2002).

I calculated for each distance interval a mean of the pairwise relatedness coefficients for all pairs of individuals within that distance interval, using Li's relationship coefficient (*L*) as a measure of pairwise relatedness (Li *et al*. 1993).

I considered the genetic structure to be significantly positive (individuals more related to each other than would be expected by chance) for distance intervals where *L* (including standard errors) was positive and exceeded the 95% confidence interval, determined from 10 000 random permutations, about the null hypothesis of random genetic structure. For this analysis I used 374 larvae, 109 nymphs and 145 adults collected in 2005. Sample sizes from 2004 were too low for analyses.

Inbreeding

RELATEDNESS 5.0.8 was used to calculate the mean relatedness coefficient (On Host *R*) for all pairs of opposite sex adult ticks collected from the same host (10 hosts were infested with at least one male and one female tick). This mean was compared with a Total *R* value, derived as the mean of the pairwise relatedness values for all pairs of opposite sex adults in the whole sample, using a single tick (chosen randomly) of each sex per host.

I estimated the F_{IS} coefficient within the sample of the 244 adult ticks in SPAGEDI 1.2. I tested the significance by performing 10,000 random permutations of genes among individuals within the sample. Standard error was estimated by jackknifing over loci.

Results

Testing the power of the loci to identify individuals

Preliminary analysis of the 848 multilocus tick genotypes, performed in MICROSATELLITE TOOLKIT v.3.1 (Parks 2001), identified 24 sets of two to six *B. hydrosauri* individuals (14 pairs of larvae, five sets comprising a larva and a nymph, a set comprising two larvae and a nymph, a set comprising four larvae and a nymph, a set comprising four larvae and two nymphs, a pair of adult males and a set comprising an adult male and an adult female) that had identical genotypes at all nine unlinked loci (Guzinski *et al*. 2008). One possible explanation is clonal reproduction, which has been reported in several ixodid tick species (e.g. Stone 1963, Oliver 1989), although not in *B. hydrosauri*. I explored the alternative explanation that genetically unique, but closely related individuals could not be distinguished because of the low power of the nine microsatellite loci that I used. API-CALC 1.0 (Ayers and Overall 2004) was used to estimate an average multilocus infrapopulation Probability of Identity [P_{ID} – this index is the probability that two randomly selected individuals that belong to a particular sample will have the same multilocus genotype (Waits *et al*. 2001)] for 64 infrapopulations that comprised at least four ticks of any life stage. Following Bush *et al*. (1997), I defined an infrapopulation as all conspecific parasitic individuals infesting a particular host at the same time.

Assuming all individuals within an infrapopulation to be unrelated, the mean $P_{(ID)}$ value was 0.00175, indicating that two individuals with identical genotypes would be expected to be randomly encountered every 571 samples. When all individuals within an infrapopulation were assumed to be siblings, the mean $P_{(ID)}$ value was 0.079 indicating that two individuals with identical genotypes would be encountered roughly every 13 samples. I was not able reliably to determine the exact proportion of sibling individuals making up each infrapopulation, but in infrapopulations which comprised largely juvenile, and especially larval, ticks the

proportion was likely to be substantial. Hence the true mean within infrapopulation $P_{(ID)}$ value likely lies somewhere between these two extremes though closer to 0.079, i.e. relatively large.

This result is a strong indication that some genetically distinct individuals in my sample could have been scored with identical genotypes due to a low discriminatory power of the loci. Most cases of identical genotypes were from larvae or nymphs, and all cases were either from a single host or from hosts collected less than 280 meters apart. This is consistent with a sexually reproducing population following the predictions of the model because if clonal reproduction occurred in this tick species then I would expect to encounter identical genotypes amongst a higher proportion of adult individuals than observed and from a wider geographic range, i.e. adults with identical multilocus genotypes collected from hosts captured at distances further than a diameter of a single host range. Although the nine loci have incomplete discriminatory power, they still have sufficient power to estimate variation in *R* between the different life stages as indicated by narrow standard error estimates about this coefficient (Table 9).

Relatedness

The mean relatedness among pairs of ticks on individual hosts (On Host *R*) was consistently higher than the overall relatedness among pairs of ticks (Total *R*) (Table 9). The difference was strongly significant for each life history stage in each year. The adult On Host *R* value was significantly lower than for nymphs or larvae in each year, and On Host *R* for nymphs was significantly lower than for larvae in 2004 ($P < 0.001$ in all cases).

Spatial genetic structure

Larval ticks showed significant positive genetic structuring over the first three distance intervals. Individual larvae on hosts up to 1950 m apart were significantly more related to each other than random (Fig. 2a). For nymphs and adults positive genetic structure was detected within the first distance interval, but not in subsequent intervals below 10 km (Fig. 2b and c). For adults, positive genetic structure was also detected for ticks sampled approximately 10 km apart (distance interval 10400 –

Table 9. Estimates of mean relatedness (*R*) among pairs of ticks on the same host (On Host *R*) and among pairs of ticks on different hosts (Total *R*) for each tick life stage and for each year of the study and also for pairs of adult ticks of opposite sex (both years of the study combined). N, number of hosts from which ticks were collected. SE, the jackknifed over loci standard error about *R*. *P*, probability of whether the On Host *R* and Total *R* values are significantly different for each category

		On Host *R*		Total *R*		
Life Stage	**Year**	**N**	***R* (SE)**	**N**	***R* (SE)**	***P***
Adults	2004	27	0.315 (0.025)	38	-0.021 (0.031)	<0.001
	2005	32	0.282 (0.045)	45	-0.017 (0.013)	<0.001
Nymphs	2004	3	0.414 (0.048)	8	0.062 (0.068)	<0.001
	2005	28	0.439 (0.033)	38	0.002 (0.026)	<0.001
Larvae	2004	13	0.500 (0.031)	17	0.056 (0.052)	<0.001
	2005	38	0.454 (0.044)	46	-0.041 (0.017)	<0.001
Male/Female	2004+2005	10	0.280 (0.081)	83	0.026 (0.030)	<0.001

A

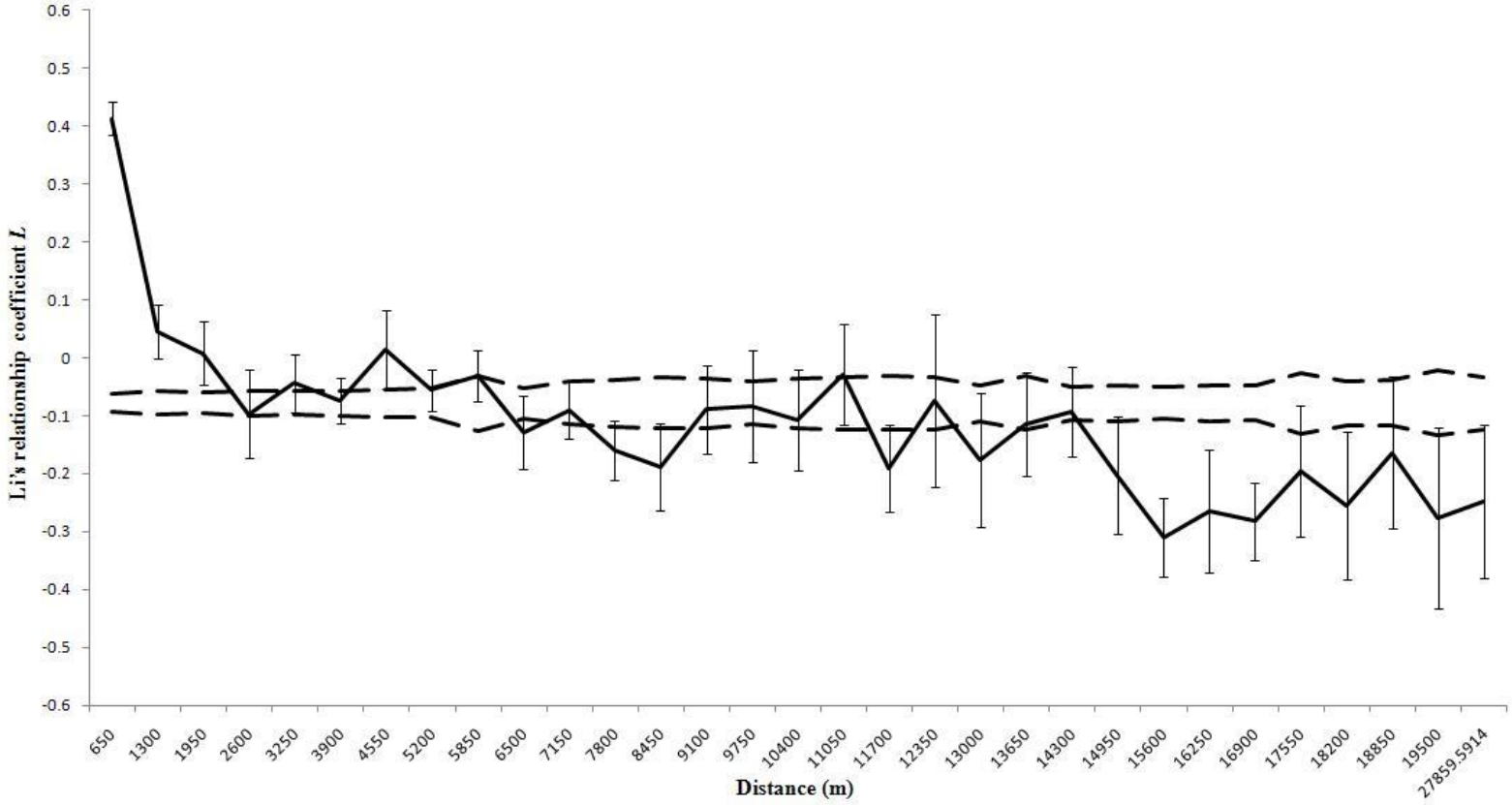
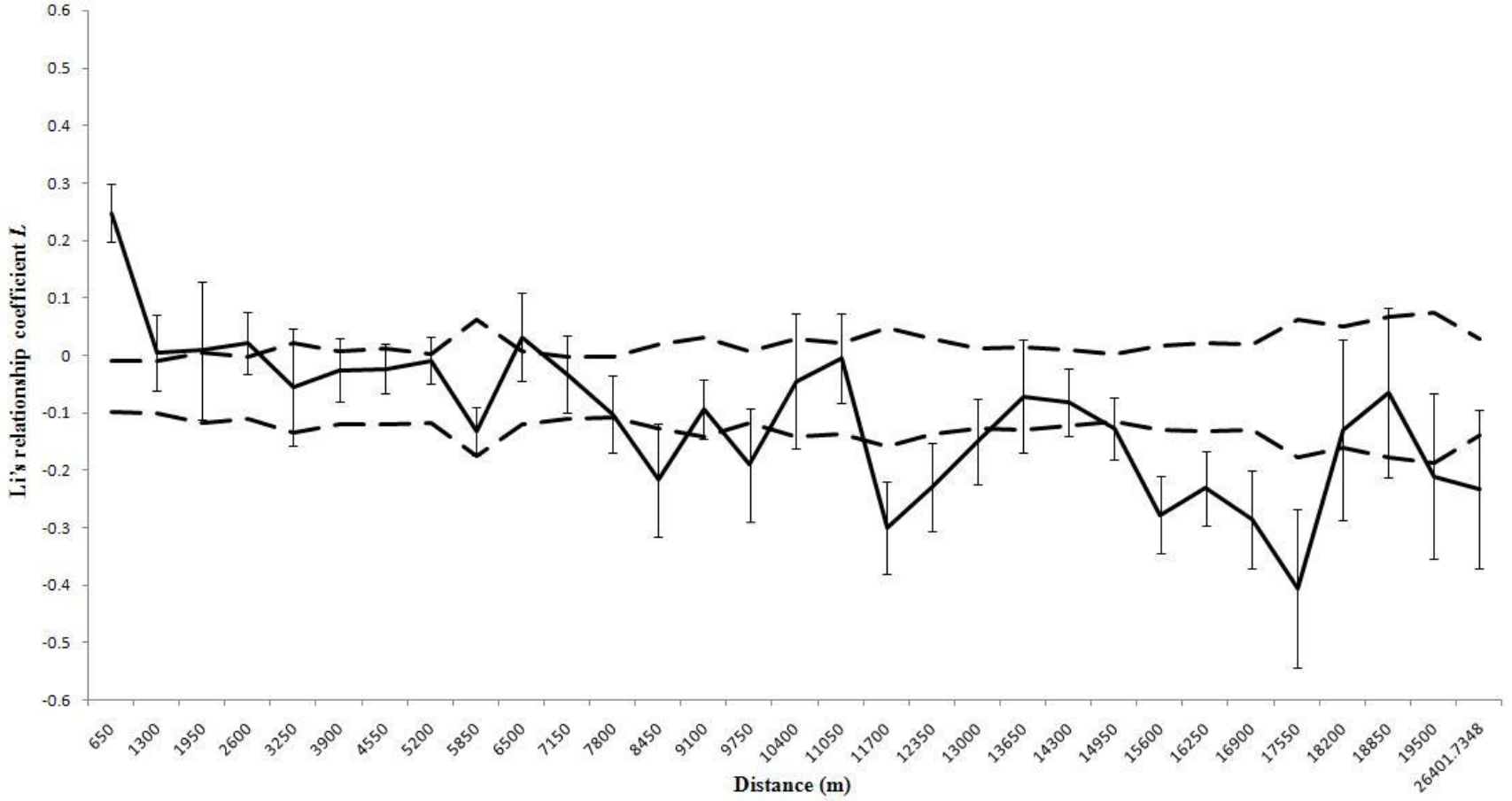
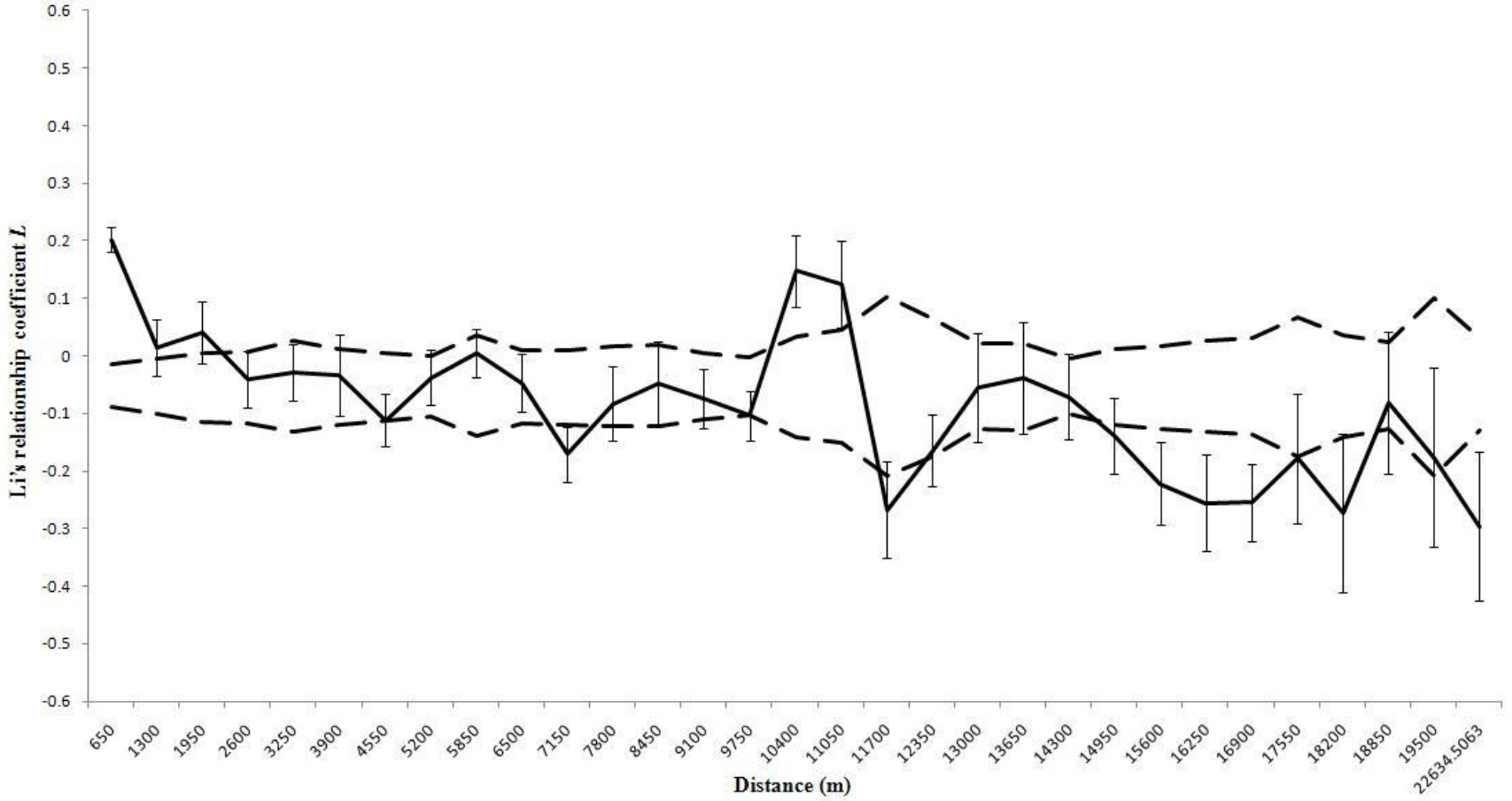


Figure 2. Spatial autocorrelation analysis performed on **A.** 374 *B. hydrosauri* larvae, **B.** 109 nymphs and **C.** 145 adults collected in 2005 from 46, 38 and 45 *T. rugosa* hosts respectively. Ticks collected from the same host were assigned slightly different spatial coordinates. Significantly positive genetic structure is present within distance classes in which in which Li's relationship coefficient (*L*), including standard errors, is positive and exceeds the 95% confidence interval envelope (dashed lines) about the null hypothesis of random genetic structure.

B


0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
Li's relationship coefficient L
650
1300
1950
2600
3250
3900
4550
5200
5850
6500
7150
7800
8450
9100
9750
10400
11050
11700
12350
13000
13650
14300
14950
15600
16250
16900
17550
18200
18850
19500
26401.7348
Distance (m)

C


0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
Li's relationship coefficient L
650
1300
1950
2600
3250
3900
4550
5200
5850
6500
7150
7800
8450
9100
9750
10400
11050
11700
12350
13000
13650
14300
14950
15600
16250
16900
17550
18200
18850
19500
22634.5063
Distance (m)

11050 meters) although this result should be interpreted cautiously given the smaller fraction of individuals available for analysis at this distance class (Fig. 2c).

Inbreeding

Adult ticks of opposite sex infesting the same host (thus potential mating partners) were significantly more related to each other than a random pair of opposite sex ticks (Table 9). The estimated F_{IS} coefficient for the adult sample of 0.156 (SE = 0.037) was significantly positive. The F_{IS} coefficients obtained for each of the nine loci were: *Bohy1* 0.190 [$P < 0.001$ (two-sided test)], *Bohy2* -0.050 ($P = 0.241$), *Bohy3* 0.098 ($P = 0.028$), *Bohy4* 0.165 ($P = 0.002$), *Bohy6* 0.082 ($P = 0.074$), *Bohy7* 0.253 ($P < 0.001$), *Bohy8* 0.257 ($P < 0.001$), *Bohy9* 0.302 ($P < 0.001$), *Bohy10* 0.113 ($P < 0.001$). Since the majority (seven) of the loci exhibited a significantly positive $F_{IS,}$ a population level effect (e.g. inbreeding) is likely to be the cause rather than locus specific effects (e.g. selection, null alleles). Furthermore, I have shown that all of these loci are inherited in a Mendelian manner in six *B. hydrosauri* families (Guzinski *et al*. 2008).

Discussion

Each of the predictions of the ripple model of tick population dynamics was supported by my genetic data. Ticks on individual hosts were significantly more related to each other than ticks in the overall sample. On hosts, the relatedness was significantly higher among the juvenile stages than among adults in 2004 and 2005 and significantly higher among larvae than nymphs in 2004. For larvae and nymphs, mean relatedness values close to 0.5 were consistent with the prediction that many of the ticks that co-infested a single host were siblings. Moreover, as my observation of identical multilocus genotypes amongst juvenile ticks collected from the same host or nearby hosts indicated them to be full-sibs, this provided further support for the ripple model.

This genetical structure was maintained across groups of hosts collected close together. Samples of ticks from hosts located near to each other showed higher relatedness than random in the spatial autocorrelation analysis. I did not have a large enough sample for detailed fine spatial scale analysis, but larval ticks retained

positive genetic structure over a larger distance interval than the other two life stages. This was predicted by the model in which "ripples" of each successful clutch of eggs spread across the landscape, increasing the overlap from adjacent ripples, and the amount of mixing among different clutches, with later life stages. Because larvae are less mixed than nymphs or adults, they should retain genetic structure over greater distances.

Despite increased mixing at the adult stage, the ripple model still predicts that related adults are likely to co-infest the same host. This was supported by my finding of significantly high relatedness among adults on the same host. Because these ticks mate on their hosts, a consequence would be an enhanced probability of inbreeding. The genetic data showed high relatedness of potential mating partners, and a positive F_{IS} coefficient for the adult sample. The significant deficiency of heterozygote genotypes indicated by this result is consistent with the predicted inbreeding, although it could have been caused also, at least partially, by the Wahlund effect (Wahlund 1928), i.e. sampling together of differentiated genetic groups. If each group exhibits different allele frequencies then the overall heterozygosity within the pooled sample will be reduced. Thus, while interpretation of the F_{IS} coefficient remains ambiguous, nonetheless the rest of the data are consistent with the prediction of the ripple model for this tick sample.

My genetic analysis supported the predictions of a model for population structure that was derived from ecological and behavioural observations of one tick-host system. Can this model be generalised to other ticks? Although up to now, relatively few other studies of genetic structure within tick populations have been carried out, interestingly they do show some different patterns. Delaye *et al*. (1997) suggested that *Ixodes ricinus* was panmictic in Switzerland, and ascribed that to the large number of host species available for the ticks. Chevillon *et al*. (2007) ascertained that there was no correlation between the genetic relatedness and mating status of female-male pairs of cattle tick *Rhipicephalus microplus* in New Caledonia at both the cow and herd scales, thus indicating frequent transfers of sibling ticks between individual hosts in a cattle herd. McCoy *et al*. (2003) found that *Ixodes uriae* (seabird tick) populations of black-legged kittiwakes exhibited higher levels of genetic substructure than *I. uriae* populations of Atlantic puffins. This result concurred with their prediction that ticks infesting puffins should have greater

opportunities for dispersal than ticks infesting kittiwakes due to differences in the movement patterns and social behaviour of the two host species.

In each of these examples the factor that influences the genetic structure of the tick population is the dynamics of transmission among hosts. Where there is a relatively high rate of movement among hosts, the ticks become mixed and panmixia is more likely. Transmission dynamics may even vary within tick populations. In *Ixodes ricinus*, de Meeûs *et al*. (2002) suggested there were sex-specific host associations, with hosts of male ticks generating wider dispersal and greater mixing than hosts of female ticks. In my study species, I found strong genetic structure in an area where the off-host environment is stressful and where mortality of larvae is high. In more benign habitats for this species and/or where host species diversity is higher, there may be more successful female ticks. This would produce a higher density of "ripples", and more extensive overlap of adjacent clutches, reducing the level of genetic structuring. My study site, close to the low rainfall limit of the tick distribution, may have provided unusually harsh conditions for the ticks. Perhaps boundary populations like this one, develop more genetic structure than in the centre of the distribution. This might provide conditions for accelerated micro-evolutionary change in ticks (Magalhães *et al*. 2007) that are not typically available in the rest of the range.

More broadly however, the results suggest that for any parasite species, the population structure will be intimately linked to the mode of transmission of parasites among hosts, and that parasite-host systems will provide a rich source of variability in population genetic structure for future study.

Chapter 4 - The Australian reptile tick, *Bothriocroton hydrosauri*, exhibits marked genetic population substructure at the edge of its distribution range

Introduction

Various classical theoretical models have been developed to explain processes that could generate the observed levels of population structure in a species. These include panmixia, island, stepping stone, metapopulation, and source-sink models. Traditional use of population genetic predictions of models to determine which model best describes population structure typically requires that individuals are aggregated into groups *a priori*, on the basis of demographic information such as morphology or capture location, and subsequently the groups are investigated for patterns of genetic connectivity (Waples and Gaggiotti 2006). However, it is uncertain that *a priori* designated groups can provide an objective basis for accurately examining population structure (Pearse and Crandall 2004). The main advance of the recently developed field of landscape genetics (Manel *et al*. 2003, Storfer *et al*. 2007) has been the development of analytical methods that do not require assumptions of population boundaries based only on the known demographic data, rather these methods are individual-based. Several currently available software packages (for example Pritchard *et al*. 2000, Dawson and Belkhir 2001, Corander *et al*. 2004, Guillot *et al*. 2005b) operate within a Bayesian or maximum likelihood framework to group sampled individuals into an appropriate number of distinct genetic clusters ("populations"). These programs employ multi-locus genotypes of individuals (some also incorporating the capture location coordinates) to assign those individuals into groups in such a way as to minimise deviations from Hardy-Weinberg equilibrium and linkage equilibrium. This approach circumvents the issue of *a priori* group designation (Pearse and Crandall 2004). Importantly, the methodology of landscape genetics makes an individual the operational unit of the study, avoiding potential bias in identifying populations in advance, and allowing studies of population structure to be conducted at a finer scale (Manel *et al*. 2003). This approach can also identify cryptic genetic discontinuities resulting either from breaks in gene flow across populations where there are no obvious dispersal barriers,

or from secondary contact of previously isolated populations (Manel *et al*. 2003, Guillot *et al*. 2005a).

The landscape genetics approach is particularly suitable for parasites. Belonging to a diverse range of phyla, parasite species vary substantially in their morphology, physiology and natural history characteristics such as the number of life stages, the mating system or the mode of reproduction (Poulin and Morand 2004). Various levels of population genetic substructure are therefore expected to be exhibited in these organisms (Barrett *et al.* 2008). Parasite population structure depends on the physiology and ecology of the parasite, such as its ability to survive off the host, the range of hosts it infests, its life-cycle and the number of life stages and the mating system, and also on the ecology, sociality and vagility of the host (Huyse *et al*. 2005, Barrett *et al.* 2008). The influence of host behaviour on parasite population structure is particularly important for those parasites which themselves are poor dispersers and rely on host mediated dispersal. A traditional view is that the infrapopulation, defined as all parasites of a given species within or on an individual host (Bush *et al*. 1997), should be considered as the most relevant unit of parasite evolution. Price (1977, 1980) argued that low connectivity between disparate infrapopulations led to high levels of subdivision in parasite populations with limited gene flow among populations and low genetic diversity within populations. However Price based this prediction on observations of parasites that continually reinfest the same host each generation, and hence the model may be less relevant for some other parasite species (Criscione *et al*. 2005). Many parasite species emit their offspring into the external environment, creating opportunities for mixing of individuals originating from different broods and increasing the connectivity between traditional infrapopulations. Studies that use molecular data to explore how these processes influence the population genetic structure of parasites are lagging far behind those on free-living organisms (Criscione *et al*. 2005). The recent development of landscape genetics methodology now provides appropriate tools to study parasite systems without any preconceived bias about the identity of individuals on the same host.

A particularly powerful use of the landscape genetics approach is to examine the genetic predictions of population models as a test of the underlying demographic model. Here I focus on a parasitic tick species for which a specific population model has been developed to explain a parapatric boundary with another tick species,

highlighting the use of landscape genetic approaches for the analysis of general range boundary issues and parasite population biology.

Bothriocroton hydrosauri is an ixodid tick that infests large reptiles in south-eastern Australia (Smyth 1973). Its range extends over temperate to semi-arid climates (Bull and Smyth 1973). A variant of the source-sink model, termed the ridge and trough model, has been proposed to explain the population substructure of *B. hydrosauri* at the northern edge of its range, in a semi-arid region where it shares a parapatric range boundary with another reptile tick, *Amblyomma limbatum* (Bull and Possingham 1995, Bull and Burzacott 2001). Empirical data on the average number of ticks per host showed areas of high tick density interspersed with areas of low tick density along linear transects (Bull and Possingham 1995). The environment for *B. hydrosauri* was inferred to be a heterogenous matrix of areas of high fitness (ridges) and of low fitness (troughs). Host lizard density, the availability of refuge cover, and predation intensity are possible factors that could influence the probability of ticks surviving while off the host, and hence the habitat suitability (Bull and Possingham 1995). In the model, ridge populations act as sources, and trough populations as sinks where populations are sustained by a regular supply of immigrants. I used programs associated with landscape genetics procedures to test predictions of genetic structure arising from the ridge and trough model, and to explore other patterns of genetic structuring of populations of this tick.

I predicted that high density ridge populations of *B. hydrosauri,* identified *a priori* on the basis of a detailed demographic survey, would represent distinct genetic clusters, with low density trough areas acting as barriers to gene flow. I used nine highly polymorphic microsatellite loci in a landscape genetics approach, which did not require prior aggregation of samples, to detect genetically distinct groupings of ticks.

Materials and Methods

Ecology of the study species and its main host

Bothriocroton hydrosauri is a three-host tick that feeds on reptiles. The major host of *B. hydrosauri* in South Australia is a large skink, the sleepy lizard, *Tiliqua rugosa* (Smyth 1973). In the study area, these lizards are mostly active during spring and

early summer (Bull 1987, Kerr *et al*. 2003). They occupy home ranges with an average area of 4 ha that they maintain from season to season (Bull and Freake 1999). Except for a central core area, home ranges of adjacent lizards overlap extensively (Kerr and Bull 2006a).

Female ticks mate on-host, detach, and lay several thousand eggs (Chilton and Bull 1991). Larvae attach to a host, engorge on blood or lymph, detach and moult to nymphs. These attach to a second host, engorge, detach and moult to adults. These in turn attach to a third host. Male ticks remain attached for many months, waiting for mating opportunities, and are usually the most common developmental stage found on hosts (Andrews and Bull 1981). *Bothriocroton hydrosauri* disperse passively as their hosts move among multiple refuges within their home range. The ticks detach while their hosts are in refuge shelters and remain there to moult and wait for the next host (Bull 1978b, Petney *et al*. 1983, Petney and Bull 1984). Ticks that detach outside of the refuges are unlikely to survive desiccation (Bull and Smyth 1973) and predation (Dawes-Gromadzki and Bull 1997a, 1997b). I previously reported limited mixing on individual hosts amongst individual ticks originating from different broods and probable mating between related *B. hydrosauri* adults (Guzinski *et al*. 2009).

Tick sampling for the demographic and population genetic analyses

I sampled *B. hydrosauri* from *T. rugosa* hosts captured along a non-linear transect (Fig. 3) located 10 km east of Bundey Bore Station, South Australia (139° 21’ E, 33° 55’S), and 10 km east of the area where Bull and Possingham (1995) described tick density distributions. The collecting transect included the northern limit of the distribution of *B. hydrosauri*, where it abuts parapatrically with *A. limbatum* (Fig. 3). The habitat of chenopod shrubland has been described by Kerr *et al*. (2003).

There were two phases to the study. The first was in 1992 to 1994 when random encounter surveys over the period September – December in each year resulted in over 2,500 captures of lizards along 28.4 km of the transect. For each lizard I counted the number of *B. hydrosauri* ticks of each life stage that were attached. I then estimated the mean number of ticks per lizard for all lizards captured in each of the 284 100 m sections of the transect. For these estimates I only used data from the first capture in each year of lizards that were encountered multiple times in

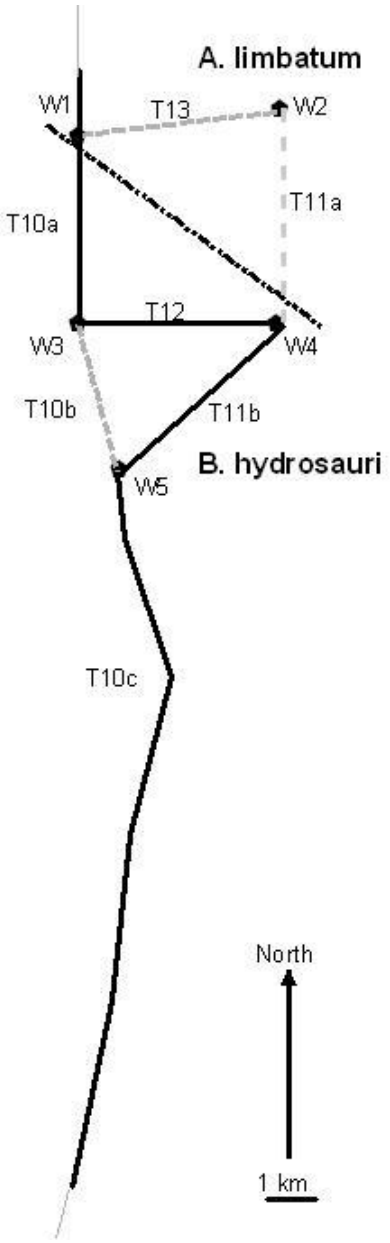


Figure 3. The sampling route that was followed during the 04-05 sampling seasons is represented by the thick black line. It comprises a series of unsealed road transects (T10a, T12, T11b and T10c), approximately 36 km in combined length. Some hosts were also captured on transects T13, T11a and T10b (dotted grey line) but no ticks were collected from them. These captures were used to estimate the position of the zone of parapatry between *B. hydrosauri* and *A. limbatum* represented by the dotted black line. In this area majority of the captured *T. rugosa* hosts were infested with both *B. hydrosauri* and *A. limbatum*, whereas south of it almost all hosts were infested just with *B. hydrosauri* and north of it almost all hosts were infested just with *A. limbatum*. The coordinates of the main points along the sampling route were: W1 - 33°55'40.9872"S, 139°25'34.0818"E; W2 - 33°55'19.7138"S, 139°28'20.7567"E; W3 - 33°58'16.305"S, 139°25'35.0034"E; W4 - 33°58'15.834"S, 139°28'18.6594"E; W5 - 34°00'16.794"S, 139°26'09.4600"E. Note that the sampling route that was followed during the 92-94 sampling seasons extended approximately 4.5 km further north and was shorter by approximately 12 km due south.

a year (1620 individuals). I used these data to develop distribution maps for areas of high and low tick density along the transect.

In the second phase of the study (2004-05) I collected 254 adult *B. hydrosauri* ticks along 36 km of the transect from 38 *T. rugosa* hosts captured in 2004 on 9 separate sampling days (22 Sep to 29 Oct) and from 45 *T. rugosa* hosts (including one recapture from 2004) captured in 2005 on 14 separate sampling days (22 Nov to 14 Dec). Active *T. rugosa* hosts were captured by hand, individually marked (via toe-clipping) for short-term recognition, and all *B. hydrosauri* adults were removed. I recorded the coordinates of each capture on a GPS (Garmin) unit. All *B. hydrosauri* adults collected from a particular host were assigned the GPS coordinates of their host. I also recorded the numbers of larval and nymphal *B. hydrosauri* ticks, to confirm previously recognised areas of high and low tick density along the transect, and the numbers of all life stages of *A. limbatum* to estimate the position of the zone of parapatry.

Microsatellite genotyping

I transported the adult ticks from the field in liquid nitrogen before DNA extraction (DNeasy Tissue kit, QIAGEN). Subsequently, each of the 254 ticks collected was genotyped at nine polymorphic microsatellite loci using the primers and the protocols described in Guzinski *et al*. (2008). In analyses I used the genotypes of 244 *B. hydrosauri* adult ticks (222 male ticks and 22 female ticks, collected from 83 *T. rugosa* hosts) that I successfully genotyped for at least six loci. Poor DNA quality was responsible for some PCR failures in the other ten ticks.

Population genetic structure analyses

The conformance of the loci to Mendelian expectations was assessed previously by Guzinski *et al*. (2008). Deviation from Hardy-Weinberg equilibrium (HWE) within the entire sample of 244 *B. hydrosauri* adults was used to infer a Wahlund effect, signifying population substructure. The HWE was examined for each of the nine loci using GENEPOP web version 3.4 (http://wbiomed.curtin.edu.au/genepop) (Raymond and Rousset 1995). The significance of any deviations from HWE was estimated through the Markov chain method using 10,000 dememorizations, 10,000 batches

and 10,000 iterations per batch [the "exact" HW test of Guo and Thompson (1992)]. Linkage disequilibrium between all pairs of loci was calculated for the whole sample (244 *B. hydrosauri* adult ticks) also in GENEPOP web version 3.4, using the same Markov chain parameters as for HWE calculations to calculate Fisher exact probabilities. All significance levels were adjusted using sequential Bonferroni corrections to allow for multiple tests on the same dataset (Rice 1989).

The program MICROCHECKER (van Oosterhout *et al*. 2004) determines whether any HWE departures are likely to have resulted from technical causes, such as null alleles, mis-scored alleles due to stuttering, or allelic drop-out due to short allele dominance. I used this program to check whether any highly significant HWE deviations that were observed in the data resulted from any of these scoring errors or technical artefacts. The frequencies of null alleles at each locus were estimated following the Brookfield 1 equation (Brookfield 1996), which is appropriate if there is uncertainty as to why samples did not amplify, i.e. whether this was because these samples were null homozygotes, had degraded DNA or there were technical issues with the PCR.

As there was no *a priori* information as to the likely number of *B. hydrosauri* populations across the sampling transect, I considered three commonly used assignment programs, all based on Bayesian approaches, to investigate the genetic structure within the sample. First I used STRUCTURE version 2.0 (Pritchard *et al*. 2000, Falush *et al*. 2003), a clustering program that assigns individual multi-locus genotypes into *K* genetic clusters so that deviations from HWE and linkage equilibrium are minimised within each cluster. In order to estimate the most likely number of *K*, ten independent runs of $K = 1\text{-}10$ were performed at 500,000 Markov chain Monte Carlo (MCMC) iterations following a 100,000 burn-in period. No prior information was entered and the model was run assuming correlated allele frequencies and admixture. These parameters are recommended by Falush *et al*. (2003) if subtle population structure is suspected. Other parameters were left at default levels. The optimal value of *K* was chosen following the approach of Evanno *et al*. (2005). Subsequently individuals were assigned to each of the *K* clusters based upon the highest proportion of membership (*q*), without the need to overcome any specific (threshold) value of *q*. The position of each cluster with respect to the other clusters was visualised by mapping the samples in ArcMap 8.3 (ESRI).

Second, I used GENELAND version 2.0.9 (Guillot *et al*. 2005b). This program also clusters individuals into *K* populations, such that each population conforms to HWE and linkage equilibrium, but the program also incorporates the spatial coordinates of each sample to infer the most likely population structure. Thus GENELAND assigns greater probability to genetic clusters that are continuous within a spatial landscape. In order to avoid any potential bias resulting from multiple genotypes being associated with a single set of spatial coordinates, I modified one of the coordinates of each individual by altering the third decimal point of the easting such that each individual had a unique set of spatial coordinates. The program was accessed through R 2.5.1 (Ihaka and Gentleman 1996). Initially GENELAND was implemented five times (to verify the consistency between runs) with variable K = 1-10 with these MCMC parameters: 1,000,000 iterations, with thinning of 100, and a burn-in period of 1,000. Other parameters were left at default levels. The Dirichlet model was used as a model for allelic frequencies. After establishing the most likely number of clusters, GENELAND was run 50 times with that fixed value of *K*, and with the same parameters as for the variable *K* runs. The 50 runs produced highly variable results – less than a third of the runs resulted in a consistent assignment of individuals to each of the *K* clusters. To overcome this, GENELAND was run a further ten times at the fixed value of *K* using 10,000,000 MCMC iterations, with thinning set to 500 and the burn-in period to 100.

The third assignment program was BAPS version 5.1 (Corander *et al*. 2003, Corander and Marttinen 2006), which also groups individuals into *K* distinct genetic clusters assuming HWE and linkage equilibrium within each cluster. Unlike STRUCTURE and GENELAND, BAPS uses stochastic optimization to infer the posterior mode of genetic structure. BAPS was run in a spatial mode, which uses individual geo-referenced multi-locus genotypes, multiple and variable (10, 16, 22, 100) times for each K = 1-20. However, this did not provide consistent results. In particular, the number of times BAPS was run for each *K* seemed to affect the final outcome. The likely reason for such inconsistency is that BAPS cannot confidently determine genetic population structure given the relatively sparse molecular information provided. This may be because the search through the posterior space is stochastic, and the results will vary if there are many weakly separated points (J. Corander, pers. comm.). In order to increase the strength of the input data, individual *B. hydrosauri* adults were *a priori* grouped into a population if they were collected

from the same host (J. Corander, pers. comm.). Thus the genotype input file (in GENEPOP format) contained 83 populations. These, together with the coordinate input file were run 10, 16 and 22 times in BAPS under the "spatial clustering of groups of individuals" mode for K = 1-20, which was sufficient to produce a consistent estimate of K. Subsequently, admixture analysis was performed to estimate the ancestry coefficient for each individual, with the number of iterations set to 10,000, the minimum population size set to 1 and the other parameters set at default values. Individuals were assigned to the cluster within which they exhibited the highest ancestry coefficient and mapped in ArcMap 8.3 (ESRI) so that the spatial arrangement of the clusters could be visualised.

Cluster analyses

I focussed further analysis on the four clusters defined by STRUCTURE, the assignment program that provided the most plausible interpretation of population structure in *B. hydrosauri* (see *Results*). HWE and linkage disequilibrium were assessed within each of the clusters using GENEPOP web version 3.4, with the same parameters as in the whole sample analysis as described above. For each locus within each cluster I calculated the observed (H_O) and expected (H_E) heterozygosities (Nei 1987) in CERVUS version 3.0 (Kalinowski *et al*. 2007) and estimated the null allele frequency following the Brookfield 1 equation (Brookfield 1996) in MICROCHECKER (van Oosterhout *et al*. 2004). To estimate the level of genetic differentiation between the clusters, pair-wise F_{ST} (Weir and Cockerham's (1984) estimator of pair-wise F_{ST} θ - the least biased and most widely used F statistic) values were computed for all the loci combined for all pairs of clusters in ARLEQUIN version 3.1 (Excoffier *et al*. 2005), with the significance level α set to 0.05 tested with 10,100 permutations. I used Analysis of Molecular Variance (AMOVA - Excoffier *et al*. 1992) to estimate variance components and to test the significance (via 10,100 permutations) of partitioning of microsatellite variation at three hierarchical levels: among clusters, among individuals within each cluster and within individuals. This analysis was performed in ARLEQUIN version 3.1. All significance levels were adjusted using sequential Bonferroni corrections (Rice 1989). To test for within-cluster inbreeding I estimated the F_{IS} coefficients within each of the four clusters in SPAGEDI 1.2 (Hardy and Vekemans 2002), and tested

their significance by performing 10,000 random permutations of genes among individuals within the sample. Standard errors were estimated by jackknifing over loci. Within each cluster I used cluster-specific allele frequencies from the genotypes of all ticks assigned to that cluster to compute mean relatedness (*R*) among ticks collected from the same host in RELATEDNESS 5.0.8 (Queller and Goodnight 1989). Thus for each host infected with more than one adult tick assigned to a particular cluster I estimated the mean *R* for all pairs of such ticks on that host. Then I took the mean of these values over all multiply infected hosts. Standard errors of *R* estimates were obtained by jackknifing over loci.

I used a non-equilibrium Bayesian method implemented in the software BAYESASS version 1.3 (Wilson and Rannala 2003) to estimate recent migration rates (within the last few generations) between the four clusters defined by STRUCTURE. This program requires linkage equilibrium, but not necessarily HWE, to be maintained within each cluster. It computes the immigrant ancestry of each individual and the generation in which immigration occurred. This allowed me to detect presumed recent immigrants and their recent descendants, because these individuals display genotypic disequilibrium relative to other members of their current cluster. BAYESASS assumes relatively low migration rates. The posterior probability of distribution for the migration rates between the clusters was calculated by running BAYESASS at 10,000,000 MCMC iterations, sampled every 10,000 iterations, of which the first 2,000,000 were discarded as burn-in because this value maximised the chain convergence. Delta values were adjusted ($\Delta p = 0.08$, $\Delta m = 0.33$ and $\Delta F = 0.1$) to optimize terminal proposed changes between chains (between 40% and 60% of the total number of iterations as recommended by the authors) to ensure sufficient parameter space was searched. I ran the program five times using these parameters, with a different starting point each time, to test the convergence of the chain and thus the consistency of the results.

To assess the relative strengths of immigration versus drift in the four clusters defined by STRUCTURE I used a coalescent-based MCMC method as implemented in 2MOD v.0.2 (Ciofi *et al*. 1999). Two models that were evaluated were the immigration-drift equilibrium model (gene frequencies within populations are determined by a balance between drift and gene flow), and pure drift model (ancestral population split into several independent units diverging purely by genetic drift). The key assumption of the program is that the effects of mutation are

negligible. To estimate the relative likelihood of each model, the MCMC stimulation was run for 1,000,000 iterations with the initial 10% data discarded as burn-in to avoid dependence on starting conditions. The program was run six times. The probability that two alleles are identical by descent (*F*) was calculated as a relative measure of the effect of drift within each of the four clusters. *F* was determined via density estimation using estimates of *F* from each step of the MCMC.

mtDNA sequencing

As a further test of population structure I examined the distribution of mtDNA haplotypes among the four clusters defined by STRUCTURE. An approximately 630 base pair fragment of the mitochondrial *CO1* gene was amplified and sequenced in 100 *B. hydrosauri* individuals selected from across the sampled geographic range of each of the clusters. To investigate a possible role of interspecific hybridisation, I also sequenced the same fragment in 8 *A. limbatum* adults. These ticks were collected during 2004 and 2005 from the northern end of the sampling transect, at most 3 km north of the parapatric boundary (Fig. 3). I included *CO1* sequences from three species of *Ixodes* as outgroups for phylogenetic analysis (GenBank accession numbers: FJ571509, FJ571510, FJ571511). Amplifications were performed in 50 µL reaction volumes containing 1x GeneAmp PCR Gold Buffer (Applied Biosystems), 2 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer (forward LCO1490 (Folmer *et al*. 1994), reverse HCO2198 (Folmer *et al*. 1994) for *B. hydrosauri* and forward 1718 (Simon *et al*. 1994), reverse HCO2198 (Folmer *et al*. 1994) for *A. limbatum*), 1 U Ampli*Taq* Gold DNA Polymerase (Applied Biosystems) and 5 µL genomic DNA (20-40 ng) extracted from bodies of whole ticks. PCR was performed in an Eppendorf thermal cycler under the following conditions: one denaturing cycle at 94 ºC for 9 min, followed by 34 cycles at 94 ºC for 45 s, 50 ºC for 45 s and 72 ºC for 1 min, followed by a single final extension step at 72 ºC for 6 min. The sequencing procedure was carried out at Macrogen (sequencing conducted under BigDye Terminator (Applied Biosystems) cycling conditions; sequencing products run on an Applied Biosystems Automatic Sequencer 3730xl). Samples were sequenced initially with the forward primer only. If the sequence contained ambiguous sites it was sequenced again with the reverse primer.

Sequences were edited in SEQED v1.02 and aligned by eye in SE-AL v2.0a11 (Rambaut 1996). An exemplar of each haplotype was deposited in GenBank (accession numbers: FJ584422 to FJ584435). Genetic variation at two hierarchical levels of genetic structure (among clusters and within clusters) was estimated with AMOVA, a method, which uses information on the nucleotide diversity of haplotypes, as well as their frequencies (Excoffier *et al*. 1992), in ARLEQUIN version 3.1. Significance of the covariance components associated with the different levels of genetic structure was tested via 20,220 permutations. A haplotype network was constructed using statistical parsimony in TCS v1.21 (Clement *et al*. 2000).

Results

Tick demographic data

Data from 1620 captures of *T. rugosa* lizards during the three field seasons of 1992-1994 showed a heterogeneous distribution of *B. hydrosauri* infections along the transect (Fig 4a). Higher densities of *B. hydrosauri* were found in two areas, segments 16 to 84 and segments 135 to 154 (Fig. 4a). The number of different host individuals captured in each 100 m segment (Fig. 4b) is an indirect estimate of host density. Many hosts caught within segments 85 to 134 were uninfested, implying an area where ticks have lower fitness or that dispersal into this area is reduced. Hosts within segments 154 to 284 were lightly infested with *B. hydrosauri* (Fig. 4a) but more heavily infested with *A. limbatum* (data not shown). Fewer lizards were sampled in 2004-2005 but a spatially heterogeneous distribution of infection with *B. hydrosauri* ticks (Fig. 5e) was consistent with the earlier pattern. Direct comparisons between the two sampling periods of transect areas favourable to ticks were constrained by the lower sampling effort in the latter period.

Population structure analyses

When all 244 *B. hydrosauri* adults were treated as a single sample, seven of the nine loci deviated significantly from HWE (Table 10). Three pairs of loci (*Bohy1*/*Bohy10*, *Bohy8*/*Bohy10* and *Bohy9*/*Bohy10*) exhibited significant linkage disequilibrium after sequential Bonferroni correction. However physical linkage between these loci

A

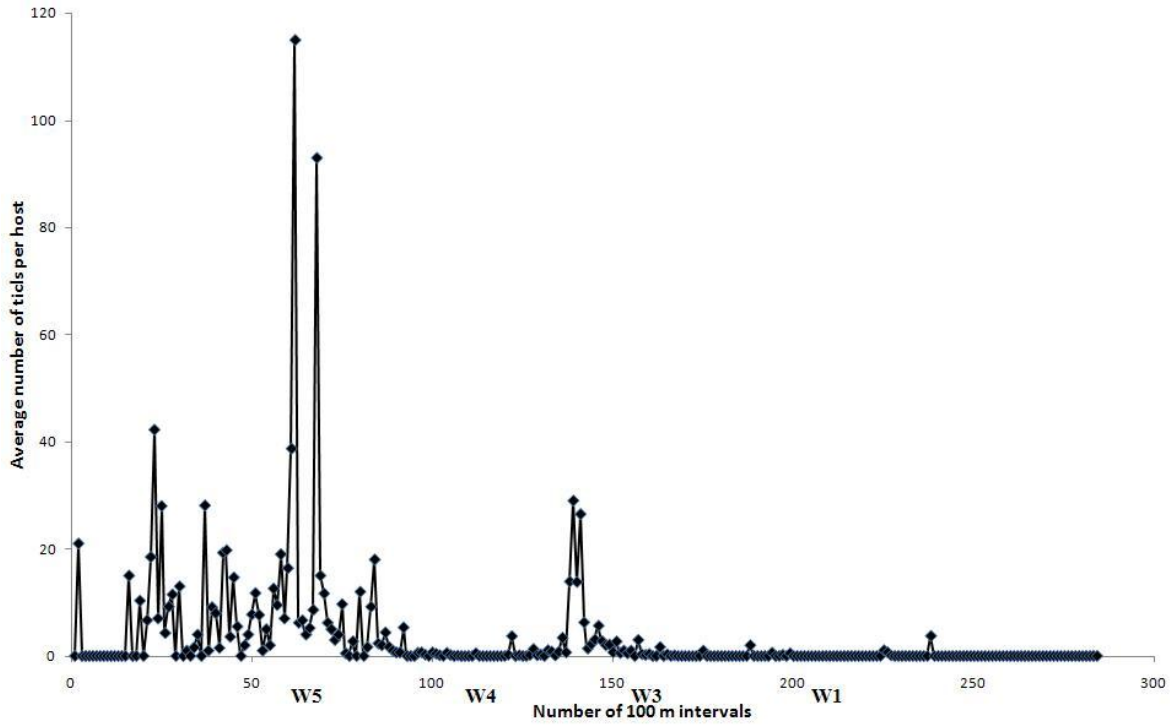
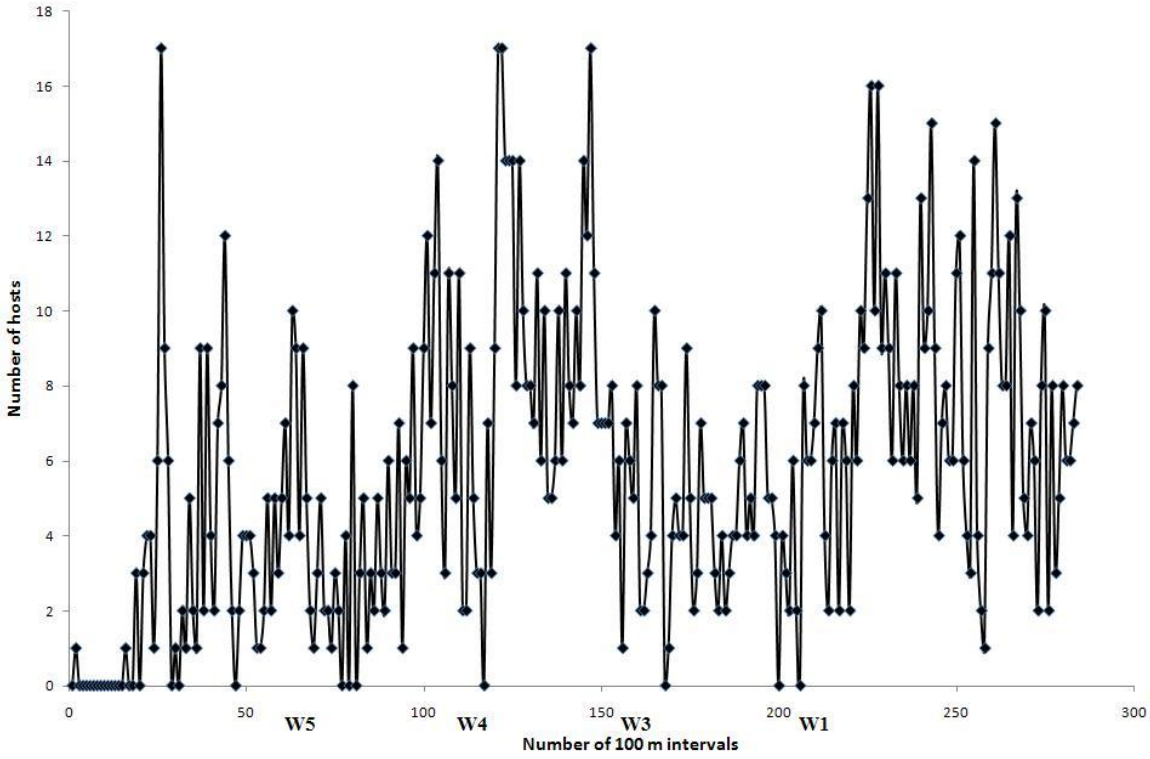


Figure 4. A. Average number of *B. hydrosauri* ticks (of any life stage) per *T. rugosa* host collected during the 1992, 1993, 1994 sampling seasons within each of the 284 100-meter segments covering the sampling transect. The distance between the first and the last segments is not a straight-line distance due to some segments covering transects T11b and T12. Two distinct *B. hydrosauri* density peaks can be identified, one covering segments 16 to 84 and the other covering segments 135 to 154. **B.** The number of *T. rugosa* hosts caught during the 1992, 1993, 1994 sampling seasons within each of the 284 100-meter segments covering the main sampling route at the sampling transect. For these estimates data were used only from the first capture when lizards were encountered multiple times in a year. Letters W1, W3, W4 and W5 indicate the main points along the sampling route, i.e. the points where the transects cross as shown on Fig. 3.

B


Number of hosts
0
2
4
6
8
10
12
14
16
18
0
50
100
150
200
250
300
W5
W4
W3
W1
Number of 100 m intervals

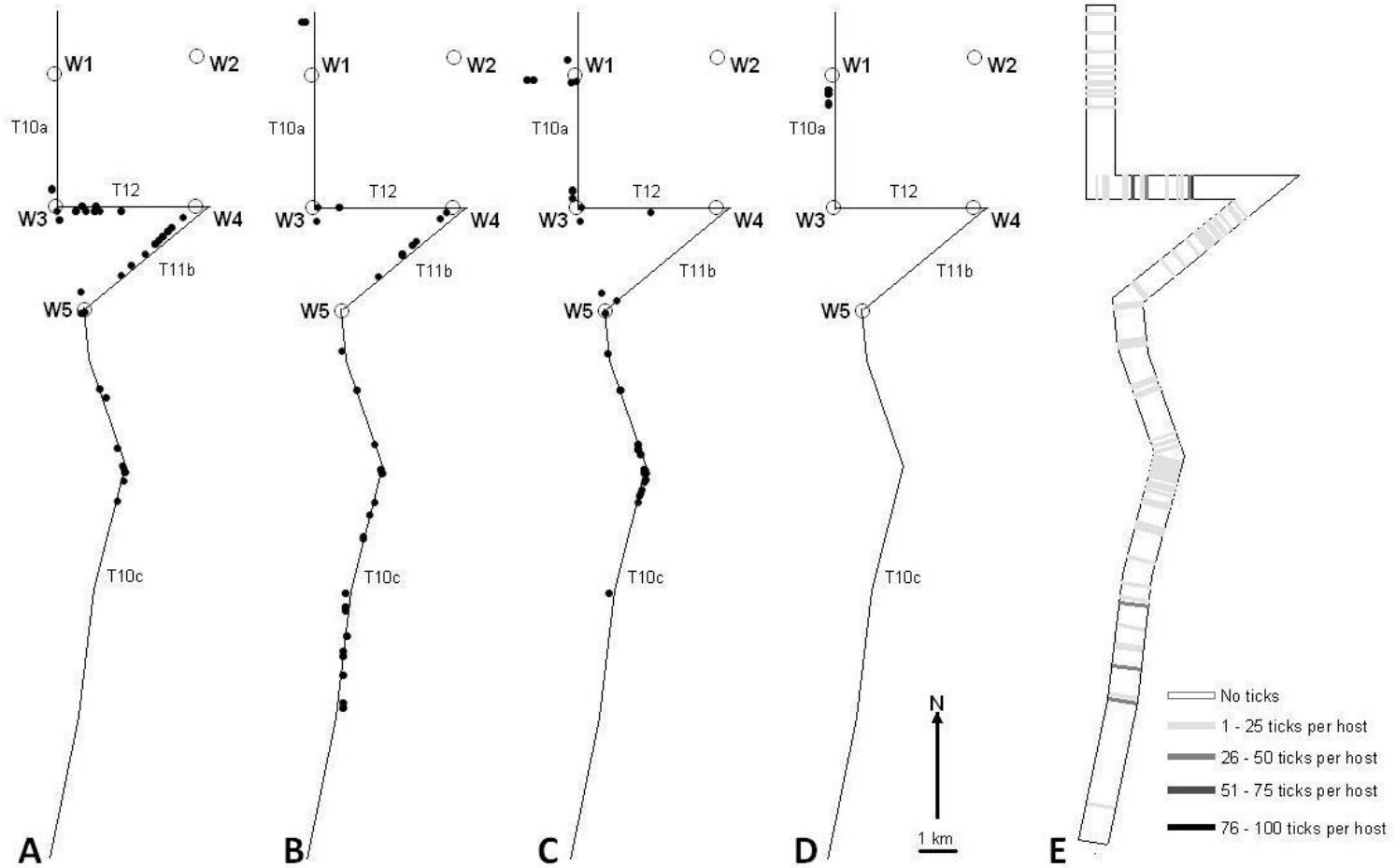


Figure 5. Geographical range of the four STRUCTURE identified *B. hydrosauri* clusters: **A.** cluster 1 comprised 74 adult ticks from 36 hosts, **B.** cluster 2 comprised 71 adult ticks from 34 hosts, **C.** cluster 3 comprised 83 adult ticks from 39 hosts, **D.** cluster 4 comprised 16 adult ticks from 4 hosts. Each dot represents a host capture location. **E.** *B. hydrosauri* abundance plot indicating the average number of ticks (any life stage) per host in each 100 m interval of the sampling transect in the 2004 and 2005 sampling season.

Table 10. Deviations from HWE at the nine loci if all 244 *B. hydrosauri* adults are treated as a single population. *these loci significantly deviate from HWE expectations after sequential Bonferroni correction. Null Allele Frequency (NAF) was estimated following Brookfield 1 equation (Brookfield 1986).

	HWE	
Locus	**P-value**	**NAF**
Bohy1	<0.0001*	0.078
Bohy2	0.2145	-0.0198
Bohy3	0.0043*	0.0354
Bohy4	0.0014*	0.0587
Bohy6	0.0367	0.0198
Bohy7	<0.0001*	0.0638
Bohy8	<0.0001*	0.1088
Bohy9	<0.0001*	0.1018
Bohy10	<0.0001*	0.0475

seemed unlikely since no linkage was detected between loci *Bohy1*/*Bohy8*, *Bohy1*/*Bohy9* or *Bohy8*/*Bohy9*. Thus I interpreted the linkage disequilibrium as resulting from population substructuring and continued to include all loci in further analyses. Further tests within subsequently defined genetic clusters did not reveal any consistent linkage disequilibrium amongst any locus combinations (see *Cluster analyses*).

MICROCHECKER inferred null alleles at the seven loci that were not in HWE (Table 10). Each locus had an excess of homozygotes. The highest null allele frequencies were estimated for loci *Bohy8* (10.88%) and *Bohy9* (10.18%) (Table 10). Null allele frequencies of this level are not considered high enough to warrant concern for population genetic analyses (Dakin and Avise 2004). Moreover, these null estimates likely are overestimates given the probability of population substructure.

The three assignment programs used to investigate genetic structure within the sample of *B. hydrosauri* produced similar outcomes. STRUCTURE generated four distinct genetic clusters from the 244 *B. hydrosauri* adults. The probability with which each individual was assigned to a cluster (q) varied very little across the ten independent STRUCTURE runs at the perceived true value of $K = 4$. For the three larger clusters (1, 2 and 3) about 70% of individuals were assigned to their particular cluster with greater than 75% assignment probability. For cluster 4 all but one individual were assigned to that cluster with greater than 75% assignment probability (Table 11). The lowest q value used to assign an individual to a cluster was 38.2% (Table 11). The geographical ranges of each cluster across the sampling transect are shown in Fig. 5a-d where each dot represents a capture location of a *T. rugosa* lizard from which ticks were collected. In cases where a particular host was infested with multiple ticks that were assigned to more than one cluster, that host was included on each of the appropriate cluster maps. There were 59 hosts from which multiple ticks were collected, of which 25 (42.4%) were infested with ticks assigned to more than one cluster. The three larger clusters overlapped extensively in their ranges (Fig. 5a-c), and can be classified as syntopic. However, I could still define areas of the sampling transect where one cluster was the most prevalent. The ranges of these three clusters were not constrained to single areas of high tick density (compare Fig. 5a-c and Fig. 5e) and thus the genetic data mismatched the demographic data. The smallest cluster in the sample (cluster 4; Table 11) was collected from four host

Table 11. The number of *B. hydrosauri* adults (*N*) assigned to each of the four STRUCTURE identified genetic clusters. Average *q* is the average of the assignment probability values for all individuals assigned to a particular cluster, % of samples assigned with > 75% *q* indicates how many individuals were assigned to a cluster with more than 75% assignment probability and lowest *q* is the lowest probability of assignment within each of the four clusters [but for these individuals this probability of assignment value (to that particular cluster) was the highest].

Cluster	*N*	Average *q*	% of samples assigned with > 75% *q*	Lowest *q*
1	74	0.807	70.2	0.382
2	71	0.811	71.8	0.395
3	83	0.786	67.5	0.419
4	16	0.948	93.8	0.723

lizards found close to each other at the northern end of the transect (Fig. 5d). All 16 *B. hydrosauri* adults collected from those lizards were assigned to cluster 4. Cluster 4 adults were sampled only in 2005, whereas ticks from the other three clusters were collected in both sampling years but never from the same host lizards as ticks from cluster 4.

GENELAND also subdivided the sample of 244 *B. hydrosauri* adults into four distinct clusters (Fig. 6). The ten 10,000,000 iterations runs all produced identical outcomes. Despite alterations of the coordinate input file such that each individual had a unique set of spatial coordinates, GENELAND always assigned all ticks collected from the same host to the same cluster, all with exactly the same probability of assignment. Assignment probabilities were higher than 0.9 for most ticks. No hosts were infested with ticks belonging to more than one cluster and the fine-scale areas occupied by each cluster were clearly separated. However the overall geographic distributions of the four clusters were similar to those defined by STRUCTURE (Fig 5; Fig 6). Cluster 4 retained the same individual ticks in both assignment programs, while the composition of the other three clusters differed slightly between programs.

BAPS 5.1 produced consistent results only when all ticks that shared the same host were *a priori* grouped into a single "population". Without that restriction the number of clusters and the assignment of individuals to clusters differed substantially between runs. With that restriction applied, multiple runs of BAPS produced a consistent result, with $K = 7$ genetic clusters. Assignment of individuals to each cluster was consistent across the multiple BAPS runs, with almost all individuals assigned to their cluster with 100% probability. Five individuals were assigned to a different cluster from other ticks on the same host. There was high concordance between the composition of the seven BAPS clusters and the composition of the four STRUCTURE and GENELAND clusters, in that BAPS appears to have split up each of the three larger clusters (1, 2, 3) into two. In what I assumed to be the most likely scenario, based on the results of the other two assignment programs, each of the clusters comprising a majority of individuals (Main BAPS cluster) was paired with a cluster that comprised only a few individuals (Small BAPS cluster) (Fig. 7). Cluster 4 in BAPS contained the same individuals as in the other two assignment programs.

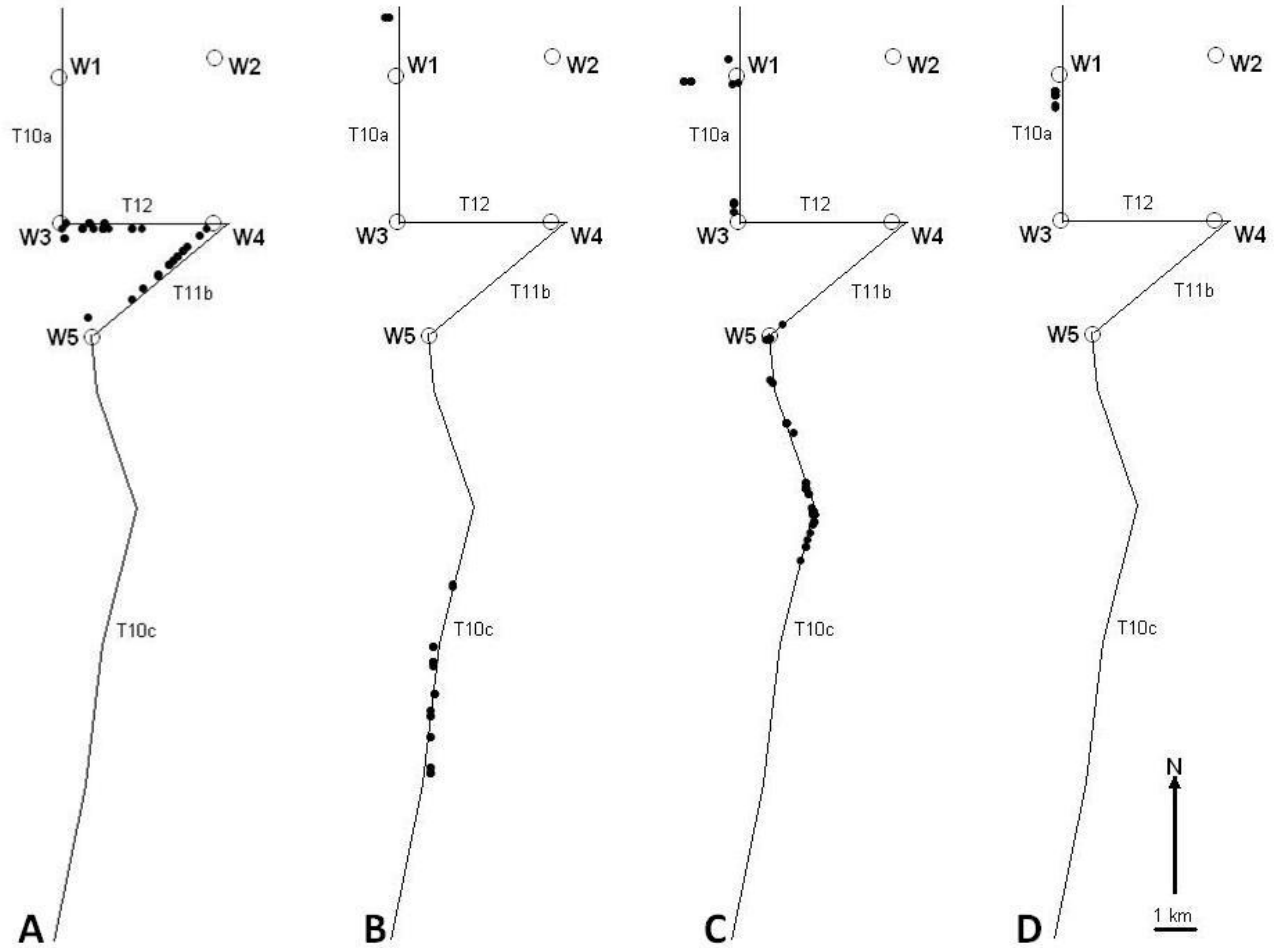


Figure 6. Geographical range of the four GENELAND identified *B. hydrosauri* clusters: **A.** cluster 1 comprised 72 adult ticks from 26 hosts), **B.** cluster 2 comprised 42 adult ticks from 14 hosts), **C.** cluster 3 comprised 114 adult ticks from 39 hosts), **D.** cluster 4 comprised 16 adult ticks from 4 hosts). Each dot represents a host capture location.

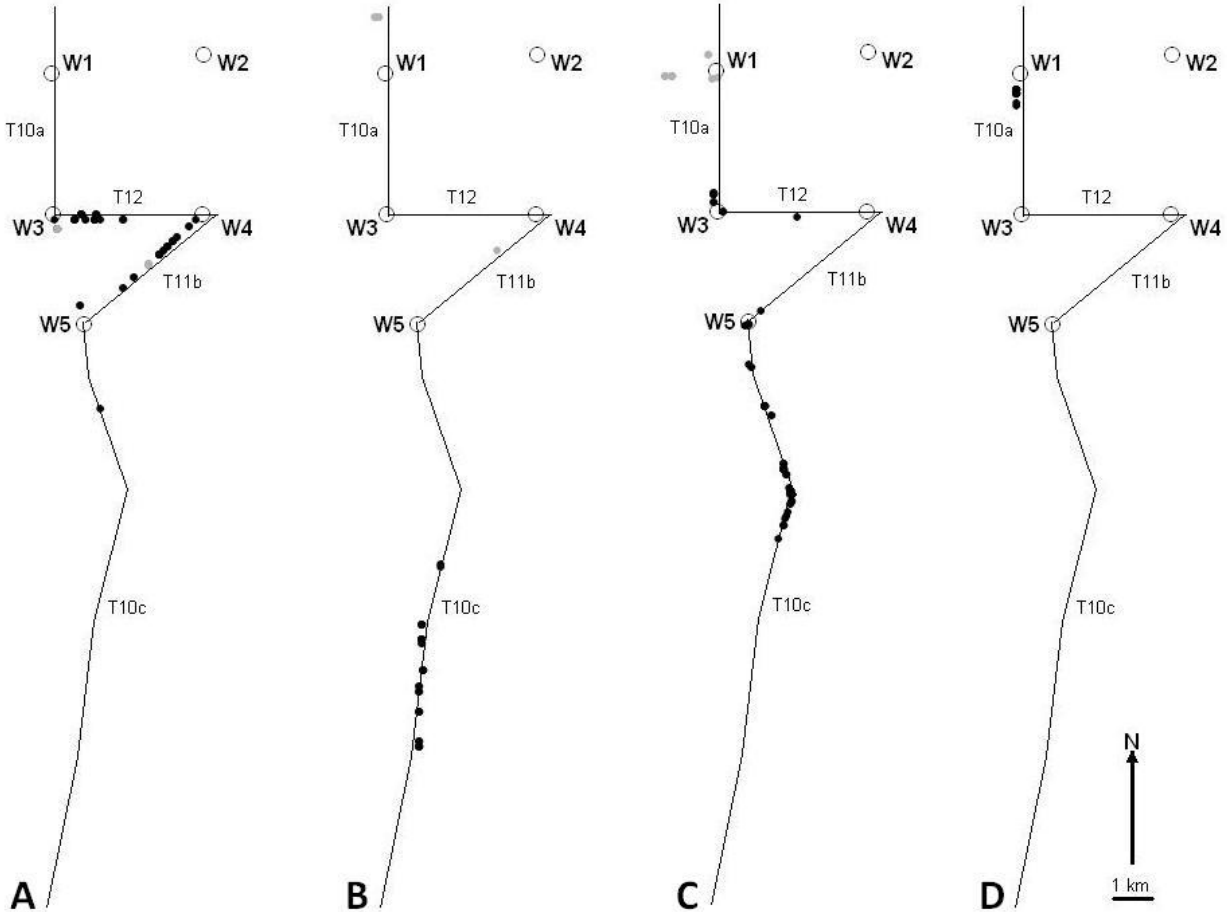


Figure 7. Geographical range of the four BAPS identified *B. hydrosauri* clusters: **A.** cluster 1 comprised 62 adult ticks from 23 hosts (black dots – BAPS Main) and 7 adult ticks from 3 hosts (grey dots – BAPS Small), **B.** cluster 2 comprised 38 adult ticks from 12 hosts (black dots – BAPS Main) and 5 adult ticks from 3 hosts (grey dots – BAPS Small), **C.** cluster 3 comprised 102 adult ticks from 35 hosts (black dots – BAPS Main) and 15 adult ticks from 7 hosts (grey dots – BAPS Small), **D.** cluster 4 comprised 16 adult ticks from 4 hosts. Each dot represents a host capture location.

All three assignment programs indicated that the sample of *B. hydrosauri* adults contained individuals belonging to multiple genetic clusters. Although the programs differed in the number of clusters they identified and in the fine detail of the assignment of individuals to particular clusters, the geographical distribution of the clusters broadly coincided. The consistency of results across the different procedures that I used is strong evidence for population genetic substructure within the sample of 244 *B. hydrosauri* adults. The clusters defined by STRUCTURE are probably the most biologically meaningful. Since GENELAND always and BAPS almost always assigned ticks from the same host to the same cluster by using geographic proximity as one of the clustering properties, they reduced the opportunity to detect possible substructuring among ticks that co-infest a host. Pre-assigning individuals to genetic clusters based on their geographical proximity introduced an *a priori* bias that I had wanted to avoid, leading me to favour the analysis in STRUCTURE. As clustering of individuals was independent of the year of collection I pooled individuals collected in different years for all subsequent analyses.

Cluster analyses

Exact tests for HWE within each of the four clusters defined by STRUCTURE showed just three out of 36 possible cases where loci deviated significantly from HWE proportions after sequential Bonferroni correction, and no cases where a locus consistently deviated in more than one cluster (Table 12). There was only a single case of significant linkage disequilibrium, between the loci *Bohy4* and *Bohy10* within cluster 3. The observed (H_O) and expected (H_E) heterozygosity values were variable within the four clusters (Table 12). Null allele frequency was generally low within each cluster, with no values above 10.8% (Table 12). Cluster 3 had nine unique alleles, cluster 2 had eight, cluster 1 had one, while cluster 4 had no unique alleles (Table 13).

All pair-wise F_{ST} values were significantly greater than zero (Table 14; overall $\alpha = 0.05$, level $P < 0.0001$). The highest F_{ST} values all involved cluster 4 (Table 14). The AMOVA showed that about 84% of the variation was at the individual level, 5% was among individuals within clusters and 11% among clusters,

Table 12. Deviations from HWE at each of the nine loci within the four STRUCTURE defined clusters. *indicates significant deviation from HWE after sequential Bonferroni correction. The observed (H_O) and expected (H_E) heterozygosity values for each locus within the four clusters are also presented. NAF - Null Allele Frequency. # - locus *Bohy7* was monomorphic within cluster 4.

	Cluster 1				Cluster 2			
Locus	**H_O**	**H_E**	**HWE (P-value)**	**NAF**	**H_O**	**H_E**	**HWE**	**NAF**
Bohy1	0.459	0.521	0.1675	0.0383	0.662	0.694	0.6646	0.0163
Bohy2	0.671	0.616	0.6538	-0.0372	0.643	0.592	0.5822	-0.0344
Bohy3	0.5	0.554	0.2833	0.0326	0.551	0.595	0.0169	0.0249
Bohy4	0.414	0.484	0.4194	0.0445	0.508	0.602	0.0823	0.056
Bohy6	0.25	0.244	1	-0.0059	0.471	0.544	0.008	0.0443
Bohy7	0.438	0.494	0.0896	0.035	0.296	0.422	0.0279	0.0866
Bohy8	0.529	0.701	0.002*	0.0985	0.681	0.767	0.4832	0.0457
Bohy9	0.176	0.32	0.0002*	0.1078	0.479	0.44	0.2925	-0.0289
Bohy10	0.658	0.673	0.0821	0.0067	0.676	0.694	0.0882	0.0076

	Cluster 3				Cluster 4			
Locus	**H_O**	**H_E**	**HWE (P-value)**	**NAF**	**H_O**	**H_E**	**HWE**	**NAF**
Bohy1	0.61	0.708	0.001*	0.0552	0.625	0.462	0.3157	-0.1228
Bohy2	0.6	0.605	0.307	0.0006	0.688	0.466	0.0949	-0.1629
Bohy3	0.519	0.592	0.2741	0.0441	0.6	0.577	0.3165	-0.0271
Bohy4	0.474	0.552	0.0104	0.0479	0.545	0.485	1	-0.0565
Bohy6	0.169	0.159	1	-0.0092	0.5	0.401	0.6341	-0.0802
Bohy7	0.108	0.105	1	-0.004	0	0	#	0
Bohy8	0.563	0.628	0.2172	0.0378	0.125	0.123	1	-0.0052
Bohy9	0.407	0.495	0.0875	0.0568	0.438	0.486	1	0.0226
Bohy10	0.695	0.704	0.222	0.0028	0.438	0.353	0.5432	-0.0713

Table 13. Allele frequencies for the nine loci within each STRUCTURE defined cluster. Alleles that are unique for any of the clusters are highlighted in bold. Sample sizes (in italics) are on the same row as the locus name.

Locus/allele	1	2	3	4
Bohy1	*74*	*68*	*82*	*16*
222	0.662	0.301	0.317	0.688
226	0.027	0.037	0.061	0.031
230	0.061	0.007	0.067	
234			0.018	0.281
238		**0.007**		
242			**0.012**	
246	0.061	0.221	0.427	
254	0.189	0.412	0.055	
262			**0.043**	
266		**0.015**		
Bohy2	*70*	*70*	*80*	*16*
216	0.207	0.257	0.213	
224	0.521	0.564	0.569	0.656
228	0.271	0.164	0.169	
232		0.014	0.006	0.344
240			**0.044**	
Bohy3	*74*	*69*	*81*	*15*
274	0.601	0.580	0.562	0.600
286	0.027	0.014	0.074	
290	0.284	0.239	0.296	
294		**0.014**		
306		0.029	0.006	
314	0.081	0.123	0.049	0.233
318	0.007		0.012	0.167
Bohy4	*70*	*65*	*78*	*11*
256	0.050	0.085		
260	0.679	0.569	0.462	0.636
264	0.236	0.254	0.487	0.364
268		**0.085**		
272		0.008	0.019	
276			**0.026**	
280	0.036		0.006	
Bohy6	*72*	*70*	*83*	*16*
200		**0.014**		
212	0.125	0.157	0.006	0.219
216			**0.024**	
220	0.861	0.650	0.916	0.750
224		0.086		0.031
232	0.014	0.071	0.054	
244		**0.021**		
Bohy7	*73*	*71*	*83*	*16*

132			**0.012**	
140	0.671	0.725	0.946	1.000
158	0.233	0.007	0.006	
162	0.041	0.035		
166	0.055	0.232	0.036	
Bohy8	*70*	*69*	*80*	*16*
258			**0.006**	
262	0.479	0.152	0.563	
266	0.014		0.006	
270	0.136	0.043	0.019	0.938
274	0.143	0.391	0.150	0.031
278	0.186	0.174	0.181	
282		0.159	0.063	
286	0.043		0.006	
290		**0.065**		
294		0.014	0.006	0.031
Bohy9	*74*	*71*	*81*	*16*
158	0.189	0.683	0.414	0.031
176	0.804	0.310	0.580	0.656
178	**0.007**			
184			**0.006**	
186		0.007		0.313
Bohy10	*73*	*71*	*82*	*16*
184	0.315	0.211	0.134	
186		0.014		0.219
199		**0.014**		
201			**0.006**	
202	0.226	0.479	0.402	0.781
204	0.425	0.042	0.140	
206	0.014	0.063	0.317	
208	0.021	0.176		

Table 14. Pairwise F_{ST} comparison between the four STRUCTURE defined clusters. All comparisons are significant (*P* values shown in the upper half of the matrix).

	1	2	3	4
1	-	<0.001	<0.001	<0.001
2	0.11619	-	<0.001	<0.001
3	0.08927	0.08534	-	<0.001
4	0.20719	0.16842	0.20815	-

with all three sources statistically significant (Table 15). Specifically, this indicated significant genetic structuring among clusters. The estimated F_{IS} coefficients within clusters 1, 2 and 3 were significantly positive whereas the estimated F_{IS} coefficient within cluster 4 was significantly negative (Table 16). For the three larger clusters the mean relatedness (*R*) among ticks collected from the same host was positive, higher than half-sib level for cluster 1 and around 0.2 for clusters 2 and 3 (Table 16). The relatedness estimate was negative for cluster 4 (Table 16).

BAYESASS 1.3 indicated very low levels of recent migration among the four STRUCTURE defined clusters (Table 17). As the five runs all produced very similar results, results are reported based on a single run. Almost all individuals within each cluster were identified as non-migrant, although cluster 4 had the highest proportion of migrants (Table 17). The program indicated that the highest migration rates, 0.6% to 0.7%, were from clusters 1, 2 and 3 into cluster 4 (Table 17). Migration rates into clusters 1, 2 and 3 were generally lower from cluster 4 than from the other three clusters (Table 17). However, the large overlap of the 95% confidence intervals did not allow me to conclude whether the differences in migration rates among the clusters were significant.

Such low levels of contemporary gene flow between the four clusters are surprising given the overlap of their geographic ranges (Fig. 5a-c), and the fact that a number of sampled hosts carried ticks assigned to two or three different clusters. The genetic data were sufficiently detailed to estimate migration rates as the confidence intervals recovered from the data (Table 17) were considerably smaller than confidence intervals obtained from the null hypothesis of no data (0.675 to 0.992, see BAYESASS documentation for details).

2MOD analysis revealed that the immigration-drift equilibrium model was a more likely explanation of the four STRUCTURE defined genetic clusters of *B. hydrosauri* than the pure drift model. 100% of the simulated data points were consistent with the immigration-drift equilibrium model after the burn-in period in all six runs. The probability of genes being identical by descent (*F*) was 2.5 to 7 times higher in cluster 4 than in other clusters in all six runs (average *F* for cluster 4 0.49), indicating that drift was greatest for this cluster and implying its greater genetic isolation.

mtDNA data

Table 15. AMOVA of microsatellite data performed on the four STRUCTURE-defined clusters.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	*P*
Among clusters	3	107.023	0.29122	11.52	<0.0001
Among individuals within clusters	240	565.364	0.11842	4.68	0.00158
Within individuals	244	517	2.11885	83.8	<0.0001
Total	487	1189.387	2.52849		

Table 16. Estimates of the inbreeding coefficient F_{IS} within each of the STRUCTURE defined clusters. *P* is the probability that F_{IS} is significantly different from zero. Also shown for each cluster is the estimate of mean relatedness (*R*) among pairs of ticks on the same host. *N* host is the number of hosts that were infested with at least two ticks assigned to the same cluster (only these ticks were included in the mean *R* calculations, see text for details). SE is the standard error about F_{IS} and *R* jackknifed over loci.

Cluster	F_{IS} (SE)	*P*	*R* (SE)	*N* host
1	0.112 (0.047)	<0.001	0.306 (0.029)	19
2	0.0072 (0.072)	0.007	0.223 (0.052)	19
3	0.089 (0.025)	0.002	0.180 (0.030)	22
4	-0.188 (0.078)	0.032	-0.115 (0.044)	4

Table 17. Mean +/- SD and (95% CI) posterior distributions for migration rates among STRUCTURE defined genetic clusters of *B. hydrosauri*. Values along the diagonal (bold) are the proportion of individuals derived from the source cluster (or non-migrant) each generation.

	Migration rate from			
To	**1**	**2**	**3**	**4**
1	**0.992 +/- 0.008 (0.967-1)**	0.002 +/- 0.004 (0-0.013)	0.004 +/- 0.006 (0-0.022)	0.002 +/- 0.003 (0-0.010)
2	0.005 +/- 0.007 (0-0.027)	**0.988 +/- 0.012 (0.956-1)**	0.005 +/- 0.008 (0-0.028)	0.002 +/- 0.003 (0-0.011)
3	0.003 +/- 0.004 (0-0.015)	0.002 +/- 0.003 (0-0.013)	**0.993 +/- 0.007 (0.976-1)**	0.001 +/- 0.002 (0-0.008)
4	0.007 +/- 0.009 (0-0.032)	0.006 +/- 0.010 (0-0.036)	0.007 +/- 0.011 (0-0.037)	**0.980 +/- 0.019 (0.932-1)**

Analyses of 593 bp of the mitochondrial *CO1* gene revealed seven polymorphic sites and six haplotypes within a sample of 30 *B. hydrosauri* adults from cluster 1, 30 from cluster 2, 28 from cluster 3 and 12 from cluster 4 (100 individuals in total) (Table 18). The majority of individuals from the three larger clusters, but none from cluster 4, had haplotype 1 (Table 18). All individuals from cluster 4 had haplotype 2, which was also present, although at low frequency, in each of the other three clusters. Haplotypes 3, 4 and 5 were represented by single individuals, whereas haplotype 6 was found in two individuals from cluster 3. Haplotype 4 was the most different from other haplotypes, differing by at least 3 bp (from haplotype 1) and up to 6 bp (from haplotype 3) (Fig. 8). AMOVA indicated significant among-cluster variation (39.4%) only if the analysis included all four clusters. The among cluster variation was non-significant if cluster 4 was excluded.

None of the six *CO1* haplotypes observed in *B. hydrosauri* were observed in any *A. limbatum* ticks also collected across the sampling transect, indicating *A. limbatum* is unlikely to have contributed to any genetic structure in the *B. hydrosauri* sample via matriline. In fact the haplotypes were quite different between the two species, differing from 19.6 to 20.4% uncorrected sequence divergence. By comparison, the three outgroup *Ixodes* were only slightly more divergent, showing 22 to 26% sequence divergence from both *B. hydrosauri* and *A. limbatum*.

Discussion

This study was initiated to test the predictions of a model formulated by Bull and Possingham (1995) to explain *B. hydrosauri* population structure at the edge of its range. I confirmed that *B. hydrosauri* ticks collected across our sampling transect exhibited a similarly patchy distribution to that described in Bull and Possingham (1995). Surveys made in 1992-94 indicated two distinct areas of high tick density on hosts, separated by areas where hosts had relatively lower levels of tick infestation. Host density, as indicated by the number of captured hosts, was similar across all areas. Ten years later, similar heterogeneity of tick densities was apparent. Thus tick density data appear to support the ridge and trough model that Bull and Possingham (1995) proposed.

I rejected panmixia as the population model for ticks in the study area. From my sample of 244 *B. hydrosauri* adults I found strong evidence for the presence of

Table 18. Relative frequencies of the six mitochondrial *CO1* haplotypes within the four STRUCTURE identified genetic clusters of *B. hydrosauri* adults. Numbers in brackets after the cluster names are the number of individuals sequenced.

Haplotype	**1** (30)	**2** (30)	**3** (30)	**4** (12)
1	0.967	0.833	0.786	
2	0.033	0.1	0.107	1
3		0.0333		
4		0.0333		
5			0.0357	
6			0.0714	

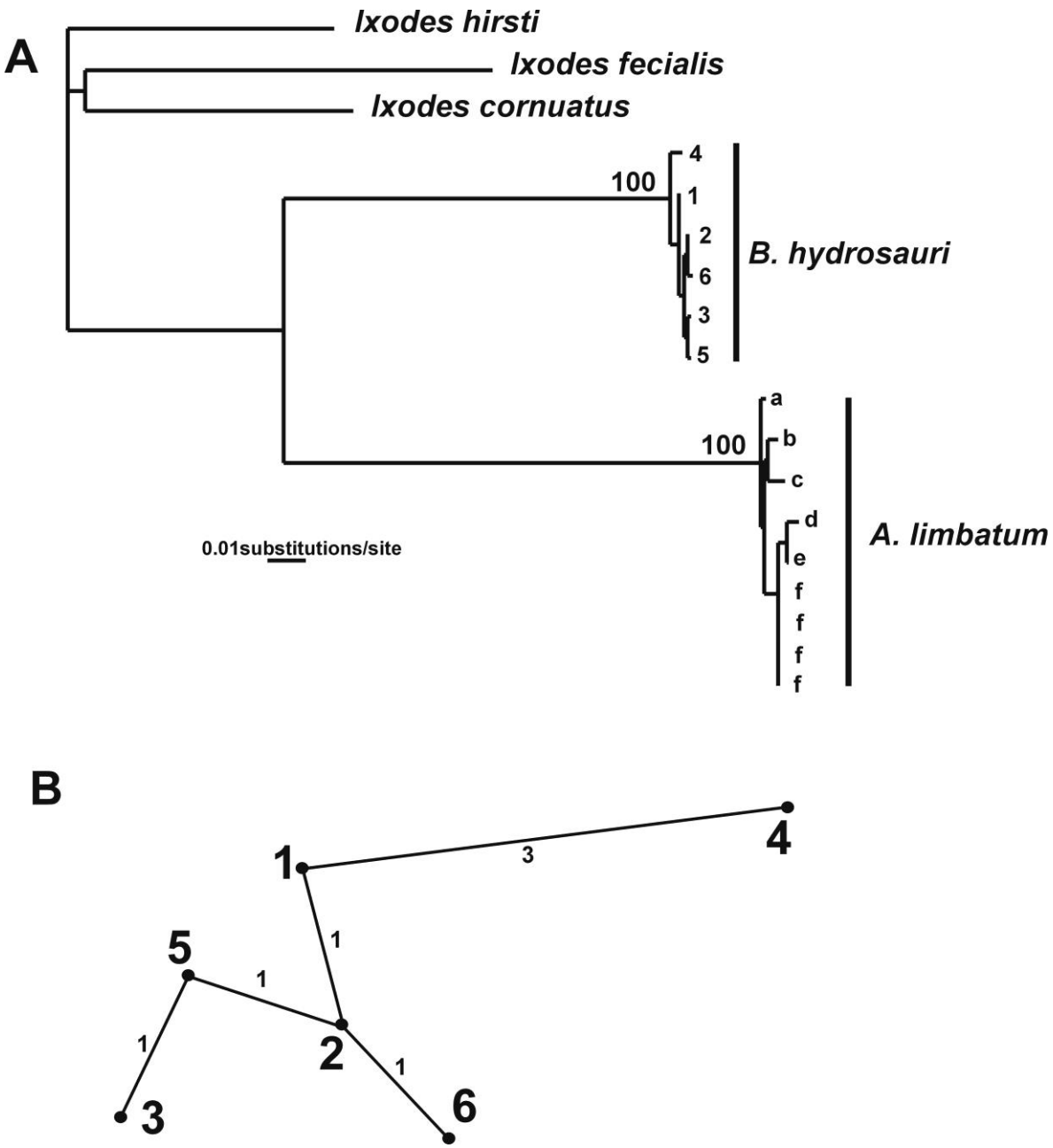


Figure 8. A. Neighbour-joining tree of mitochondrial *CO1* haplotypes in *B. hydrosauri* (1-6) and *A. limbatum* (a-f) from across the sampling transect, based on Kimura 2-parameter genetic distances between sequences. Only a single exemplar of each of the six haplotypes observed among the 100 *B. hydrosauri* is included. Numbers at nodes are non-parametric bootstrap proportions. **B.** An unrooted network among the six *B. hydrosauri* haplotypes. The numbers along branch are the number of substitutions between haplotypes. The numbers at nodes are the haplotype designations as per Table 18.

four genetic clusters. I also found very low levels of migration and gene exchange between the clusters. Genetic heterogeneity and low levels of gene exchange between ridge populations are predictions of the ridge and trough model, as each ridge population is assumed to be relatively isolated from adjacent ridges by trough areas of the environment. However, examination of the distribution of the genetic clusters found in my analysis showed they were not uniquely associated with specific areas of high tick density (ridges). Instead, and rather surprisingly, the genetic clusters overlapped in their distributions and in some cases ticks from different clusters were found on the same host individual. Given the geographic overlap of the clusters, the substantial level of genetic differentiation among clusters was an unexpected finding. I now discuss the evidence for the observed genetic population structure, its correspondence to and mismatch with the predictions from the demographic-based population model, explanations of causes of the genetic population structure and finally the wider evolutionary implications of the observed genetic population structure.

Evidence for genetic population structure

When all 244 adults were pooled into a single sample, seven of the nine microsatellite DNA loci deviated significantly from HWE. There are several possible explanations for the deficit of heterozygotes at these loci in a panmictic population. These include clonal reproduction, the presence of null alleles, or inbreeding. Alternatively, the Wahlund effect, where there is non-random mating among the sampled individuals, could produce a heterozygote deficiency.

Strict or facultative clonal reproduction is an unlikely explanation. Although I observed identical multi-locus genotypes in one pair of adult males and in one pair of an adult male and female, this could be explained by sexually reproducing ticks but with low discriminatory power of the loci (Guzinski *et al*. 2009).

Null alleles may cause some apparent reduction in heterozygote frequency, because actual heterozygotes will not be recognised. However, this would be unlikely to affect many loci in my study, given that I showed all alleles at these loci were inherited in a Mendelian manner in six *B. hydrosauri* families (Guzinski *et al*. 2008). Moreover, null allele frequencies were at most just over 10%, not high enough to warrant great concern (Dakin and Avise 2004). Finally and most

convincingly, when the samples were analysed in their separate STRUCTURE defined clusters, HWE was restored in the majority of loci in all clusters.

Inbreeding may increase homozygosity in a population (Wright 1921). I have shown previously that these ticks have limited opportunities to contact other adults originating from different broods, and that related adults that are potential mating partners may co-infest the same host (Guzinski *et al*. 2009). This suggests that inbreeding may take place within *B. hydrosauri* populations and should be considered as a contributor to the observed global deficit of heterozygous individuals.

However, given my data, the most likely explanation for the within-sample heterozygote deficit observed at a majority of the loci is that the entire sample of ticks includes individuals originating from more than one genetic cluster, that is, there is a Wahlund effect. *Bothriocroton hydrosauri* population substructure was indicated by three genetic clustering programs. STRUCTURE and GENELAND both grouped the 244 adult ticks into four genetic clusters, whereas BAPS further subdivided the three larger clusters to produce seven clusters. The geographic distributions of the clusters produced by each of the three programs were similar as was the assignment of individual ticks to the clusters. Thus three different assignment programs, each operating on a different premise, identified a similar pattern of genetic substructure. This strongly confirms the population substructure and supports the suggestion that the Wahlund effect was the main cause of the observed heterozygote deficit when the whole sample was pooled. Subsequent analysis of mtDNA haplotypes also supported the presence of population genetic substructure.

My study is the first to perform landscape genetics analyses on parasites with the three programs STRUCTURE, GENELAND and BAPS. Previous studies that have investigated population structure in non-parasite species (e.g. Frantz *et al*. 2006, Rowe and Beebee 2007) found less agreement in the outcomes of the different programs than I have observed in *B. hydrosauri*. Despite the high level of congruence in the output of the three programs, an important difference was that BAPS and GENELAND almost always assigned all individuals collected from the same host into a single cluster, whereas STRUCTURE assigned ticks from the same host into up to three different clusters. I surmised that this occurred because the geographic coordinates of each sample were included as clustering properties, and

these were identical (BAPS) or almost identical (GENELAND) for all individuals collected from the same host. I assumed the STRUCTURE clusters, defined without this bias, were closer to biological reality.

The algorithm implemented by STRUCTURE is not well suited for situations where there is isolation-by-distance and therefore in such situations caution is required in interpreting the results (Pritchard *et al*. 2007, Schwartz and McKelvey 2008). As my sampling was carried out along a transect, one could imply that there is a strong possibility of our samples exhibiting an isolation by distance pattern and hence not allowing a clear interpretation of the STRUCTURE results. However isolation by distance alone should produce clusters that replace each other along the transect rather than the overlapping clusters I observed. This increased my confidence in my interpretation of the STRUCTURE results. Moreover, the among-cluster F_{ST} levels were sufficiently high for the program to identify population substructure, as F_{ST} values of 3-5% or greater are regarded as sufficient for STRUCTURE to estimate population substructure (Rosenberg *et al*. 2002, Evanno *et al*. 2005).

Pattern of genetic population structure is not explained by the ridge and trough population model

Bull and Possingham (1995) had suggested that the overall population of *B. hydrosauri,* close to its northern distribution, consisted of a series of partially isolated populations in patches of good habitat (ridges) separated by poor habitat (troughs). With ridge populations separated by further than the dispersal distance for a tick, adjacent ridges required establishment of ephemeral populations in the troughs, and colonisation from them to maintain connections. In population genetics terms this is equivalent to the stepping stone model, and predicts that genetic structure should be characterised by genetically distinct and geographically isolated ridge populations. However, the genetic clusters I obtained were not geographically clustered. There was substantial geographic overlap in the ranges of the three larger clusters. Indeed, 42.4% of hosts from which multiple ticks were collected were infested with ticks assigned to different clusters.

Even though the clusters were syntopic they were highly divergent genetically, implying low levels of between cluster gene flow, in turn implying

limited mating between individuals belonging to disparate clusters. F_{ST} analysis indicated moderate to high levels of among-cluster differentiation, especially between cluster 4 and the other three clusters. This was confirmed by AMOVA of the microsatellite data, which showed significant between cluster variation. Estimates also indicated very low levels of contemporary gene flow between any pair of clusters. Cluster 4 was the most differentiated, with ticks in this cluster containing only a single mtDNA haplotype. This haplotype was at very low frequencies in all other clusters. However, 2MOD analysis indicated the immigration drift equilibrium model as a more likely model than pure drift for the differentiation of the four genetic clusters, thus suggesting that at least in the past there was some gene flow.

Alternative explanations for genetic clustering

My sample of ticks was not panmictic, but the genetic structuring did not coincide with the predictions of the ridge and trough model. I considered evidence for four possible alternative explanations for the origin and maintenance of these genetic clusters, and deduced that at least two of those were unlikely. First the clusters could result from inbreeding. The three larger clusters all exhibited a significantly positive F_{IS} coefficient and high relatedness among adult cluster members on the same host. However, the fact that more than a single mtDNA haplotype was scored within each of the three larger clusters and that these clusters were spread over a large range relative to the host home ranges implies that inbreeding alone cannot be an adequate explanation. Additionally we found ticks from different clusters on the same host lizards at the same time, providing opportunities for outbreeding.

A second possible explanation, especially for the genetic distinctiveness of cluster 4, which is closest to the zone of parapatry, is introgression from the adjacent *A. limbatum*. Although the two tick species are from different genera, they can co-occur on the same host. Hybridisation has previously been reported between other tick species (e.g. Spickett and Malan 1978). However Bull *et al*. (1981) and Andrews (1982) reported apparently insurmountable reproductive isolation between *B. hydrosauri* and *A. limbatum*. Moreover, I have established previously that the *B. hydrosauri* microsatellite primers used in my study do not successfully amplify *A. limbatum* DNA (Guzinski *et al*. 2008). Thus if cluster 4 contained introgressed individuals then it should appear deficient in heterozygotes due to a high proportion

of non-amplifying alleles. Instead cluster 4 exhibited significantly negative F_{IS}, i.e. a heterozygote excess. Additionally, the single mitochondrial haplotype found in cluster 4 was not observed in *A. limbatum* and this haplotype was at least 19.6% divergent from any of the *A. limbatum* haplotypes we found. This level of divergence is typical of divergence between genera in other arthropod groups (Lefebure *et al*. 2006). All of this evidence suggests introgression from *A. limbatum* is not an explanation for the genetic clusters we observed. No other tick species are found on the lizard hosts in this area.

Third, the clusters could be maintained by assortative mating. Although I did not record where ticks were attached, ticks from different clusters might preferentially attach to different parts of the host body and subsequently mate exclusively with other ticks at those sites. However, Andrews and Bull (1980) found that male ticks of both *B. hydrosauri* and *A. limbatum* are attracted by conspecific females attached to any part of the host. Alternatively clusters may vary in the pheromone cues that initiate courtship and mating in *B. hydrosauri* (Andrews and Bull 1981, Andrews and Bull 1982a, Andrews *et al*. 1986). Gene flow among ticks from different clusters may be reduced if they emit non-matching olfactory cues. Premating isolation due to incompatibility of mating pheromones has been reported for geographically separated populations of *B. hydrosauri* from Western Australia and from South Australia (Andrews and Bull 1983). However, no reproductive isolation was found among widely separated *B. hydrosauri* populations within South Australia (Andrews and Bull 1982b). Mating experiments will need to be performed to find out whether differences in olfactory cues are the main barrier to gene flow between ticks assigned to different genetic clusters.

A fourth possible explanation involves endosymbiotic bacteria that cause reproductive incompatibility between the clusters. Ixodid ticks harbour a range of endosymbiotic bacteria, from the alpha and gamma subclasses of proteobacteria (Noda *et al*. 1997, Duron *et al*. 2008). Endosymbiotic bacteria manipulate the reproduction of a variety of arthropod hosts including insects, isopods and mites. Of relevance to my findings is cytoplasmic incompatibility where embryonic death occurs after mating between males infected by certain strains of *Cardinium* or *Wolbachia* and females that are either uninfected or infected with an incompatible *Cardinium* or *Wolbachia* strain (Bandi *et al*. 2001, Duron *et al*. 2008). The absence, to date, of reports of reproductive manipulation by endosymbiotic bacteria in ticks,

likely reflects incomplete surveys. Given the arguments against the other three explanations, this at least deserves to be considered.

Evolutionary implications of genetic population structure

A major factor governing the dynamics of *B. hydrosauri* populations is the off-host environment, where ticks waiting for hosts can suffer desiccation and predation (Bull and Smyth 1973, Dawes-Gromadzki and Bull 1997a, 1997b). Past demographic and climatic events such as droughts, have probably had a major influence on the current *B. hydrosauri* population structure. Past droughts have probably led to greater patchiness in the tick distribution than is found today, particularly in the study area, which is at the most arid limit of the distribution of this species (Smyth 1973, Bull and Smyth 1973). Such conditions may have also led to reduced density and distribution of the hosts of *B. hydrosauri*. This environmental heterogeneity could have resulted in temporary but total isolation of *B. hydrosauri* in refugia of higher quality. After a sufficiently long time in isolation, the tick populations occupying the refugia could have diverged and reached a level of reproductive incompatibility, perhaps due to variation in the mating pheromones, thus speciating. Milder conditions allowed greater overlap of the tick and host ranges and hence the "new *B. hydrosauri* species" that previously were in allopatry could become syntopic. Traditionally, parasites have been considered to have a greater tendency to speciate in sympatry than do free-living organisms (White 1978, Price 1980, Bush 1994, de Meeûs *et al*. 1998), although more recently this prediction has been questioned (McCoy 2003). Thus my research could prove a valuable addition to this debate. In my model this is more likely to happen near the distributional limit of a species.

Lastly it is worth noting that the ridge and trough model may still apply to *B. hydrosauri* ticks but the population substructure I elucidated on the basis of the genetic data could be at a finer scale (i.e. "within" ridges). To achieve better understanding of *B. hydrosauri* demographic (and genetic) structure and of the extent of the ridge and trough pattern, sampling should be performed on several other transects, parallel to the one I followed, both close to the zone of parapatry and within the centre of *B. hydrosauri* range.

Chapter 5 – Summary and Future Direction

Bothriocroton hydrosauri and *Amblyomma limbatum* are two species of Australian reptile ticks that occupy contiguous ranges with very narrow overlap, such that in places one species can completely replace the other over a distance of less than 5 km (Smyth 1973, Bull *et al*. 1981). Despite a long term study of this ecological parapatry, the precise reasons for its formation and maintenance have not as yet been discovered (Bull and Burzacott 2001). The overall aim of this project was to investigate aspects of the population biology of these two tick species, such as population structure and dispersal, and to use this information to gain further insights into the cause and maintenance of this parapatric boundary. To this end I used molecular genetic markers, and specifically highly polymorphic microsatellite loci. Prior to commencement of this project, there were not any *B. hydrosauri* or *A. limbatum*-specific microsatellite primers available and thus they had to be developed during the course of this study. Crossing experiments were conducted within each species in order to examine whether the isolated markers are inherited in accordance with Mendelian laws of inheritance. *Chapter 2* describes isolation of the markers and setting up of the crossing experiments.

Inheritance of microsatellite loci

Despite difficulties associated with keeping the hosts and the ticks in captivity and the need for daily monitoring of the mating status of the tick females and later of the eggs and the hatched larvae, carrying out the crossing experiments was well worth doing as it uncovered a critical issue. If the newly developed *A. limbatum* microsatellite loci had been used to investigate the population structure of this tick species then most likely any conclusions drawn from that analysis would have been erroneous. None of the newly developed *A. limbatum* microsatellite loci were inherited in accordance with Mendelian laws in either of the tested families. Thus none of these loci were suitable for a population genetics study as using them would have resulted in incorrect allele frequency estimates. Due to time and monetary constraints more *A. limbatum* specific markers were not developed. To perform a population genetics study on this tick species a very high number of microsatellite markers would need to be developed due to an apparently very high marker attrition

rate. It is unknown whether null alleles, which were the cause of the observed non-Mendelian inheritance patterns for most of the newly developed *A. limbatum* loci, are specifically associated with microsatellites comprising the particular repeat motifs present in the current suite of loci, or are widespread throughout the *A. limbatum* genome. Developing primers amplifying loci comprising motifs other than (AAAG), (AC), (AG)(AC) or (AC)(TC) could be necessary. The use of "next generation" sequencing approaches on cDNA from *A. limbatum* would provide a very cost effective means of obtaining many microsatellite loci in this species.

Not being able to perform a population genetics study on *A. limbatum* forced me to change the overall aims of the project and investigating the population structure of *B. hydrosauri* became the sole focus. The crossing experiments identified one *B. hydrosauri* locus that was inherited in a non-Mendelian manner which was subsequently dropped from further analysis, highlighting the usefulness and even necessity of examining whether the microsatellite markers used for a population genetics study are inherited in accordance with Mendelian laws. Performing crossing experiments may not be practical for many organisms as some may not be possible to breed in captivity, have a long gestation period or a low number of offspring but perhaps it should be considered a standard requirement for molecular genetics studies of species where such problems can be overcome. This is especially relevant for parasites whose ecology, reproductive modes and strategies, dispersal and population structure and sizes can often only be assessed through the use of polymorphic molecular markers (de Meeûs *et al*. 2007). At least for ectoparasites, general features such as high fecundity and short generation time should allow for crossing experiments to be carried out without too much difficulty (though this may be complicated in cases where the host species is not possible to keep in captivity).

Transmission dynamics of *B. hydrosauri*

Although the lack of *A. limbatum* microsatellite markers appropriate for a population genetics analysis compromised the original aim of the study of investigating the parapatric boundary between this species and *B. hydrosauri*, nonetheless the data gained in this project have substantially increased our understanding of the ecology and population biology of *B. hydrosauri* at the low rainfall limit of its distribution. *Chapter 3* provides details on the transmission dynamics of *B. hydrosauri*. I showed

that for all life stages there was significantly higher relatedness among ticks infesting the same host than among pairs of ticks on different hosts. Moreover, within ticks on the same host, relatedness was significantly higher among larvae than among nymphs than among adult ticks. Spatial autocorrelation analysis showed that positive spatial genetic structure extended over a larger distance interval for larvae than for the later life stages, thus being in agreement with the relatedness analyses. These results indicate that mixing of *B. hydrosauri* ticks originating from disparate broods (clutches) is stronger for the later life stages, and they confirm the predictions of the ripple model, formulated on the basis of ecological and behavioural studies of *B. hydrosauri* and its main host in the area (sleepy lizard *Tiliqua rugosa*). The ripple model also predicted some inbreeding within the tick population. It was surmised that adults are likely to encounter related adults on a host for mating due to limited mixing of clutches even at the adult level because of high clutch mortality in the harsh environment. My data also supported this prediction of the ripple model, but the model remains to be tested in more benign conditions for this tick species, i.e. within the centre of its distribution range. In milder conditions more females would be expected to lay eggs and more clutches would be expected to survive thus resulting in a higher density of ripples and a greater overlap between ticks originating from disparate clutches. Presumably a host would be infested with adults originating from a number of clutches, thus allowing for greater gene flow between different broods and reduced levels of inbreeding compared to at the edge of the species range.

The sample of 244 adult *B. hydrosauri* ticks exhibited a global significantly positive F_{IS} coefficient, indicating a deficit of heterozygous individuals. In *Chapter 3* this result was interpreted to be caused by inbreeding, though it could have also been caused, at least partially, by the Wahlund effect (Wahlund 1928), i.e. sampling together of multiple differentiated gene pools (genetic clusters). If each cluster exhibits different allele frequencies (due to genetic drift having a more profound influence than gene flow) then the overall heterozygosity will be reduced. For example, admixture of genetically divergent stocks of cattle tick *Rhipicephalus microplus* was concluded to be the main cause of the significantly positive average F_{IS} estimate obtained for ticks infesting 20 cows (assuming no tick movement among cows) or four cow herds, each comprising five cows (assuming that ticks move among cows within each herd [which seems to be a more likely scenario for this tick species (Koffi et al. 2006b, Chevillon et al. 2007)]. Further analyses of the adult *B.*

hydrosauri sample revealed that it is made up of individuals belonging to multiple genetic clusters (described in detail in *Chapter 4*), thus strongly suggesting that the observed global significant heterozygote deficit could be explained by the Wahlund effect rather than by inbreeding. Does this result therefore undermine one of the predictions of the ripple model? Analyses within each of the genetic clusters showed that the three larger clusters all exhibited a significantly positive F_{IS} coefficient, which is not likely to have been due to the Wahlund effect but exclusively due to inbreeding. Thus the predictions of the ripple model should be tested at a cluster rather than global level. To be able to do that, juvenile individuals will first need to be partitioned into clusters. Such analysis could be performed in programs like STRUCTURE, GENELAND or BAPS, but the resulting partition could be incorrect. In population genetic analysis it is usually the adult individuals that are analysed as they are regarded as a random sample of minimally related individuals, and juveniles, especially in this case, cannot be considered as such.

An assignment test is a statistical method that could be used to overcome this problem (Waser and Strobeck 1998). This approach will allow one to work out which of the four STRUCTURE defined genetic clusters (see *Chapter 4*) is the population of origin of each juvenile. Two assignment tests that could be used in this situation are the exclusion test of Cornuet *et al*. (1999), which is based on the partial Bayesian method of Rannala and Mountain (1997) and the fully Bayesian test of Pritchard *et al*. (2000) implemented in software STRUCTURE. Both these methods assume HWE and independence and linkage equilibrium between loci within each putative population of origin (Manel *et al*. 2002), assumptions which are met by all four *B. hydrosauri* genetic clusters. These assumptions need to be upheld to accurately estimate the probability of origin of an individual in a population (Waser and Strobeck 1998). The exclusion methods use allele frequencies from each of the potential source populations to estimate a likelihood of the investigated individual (i.e. a genotype) originating from one of these populations. The exclusion methods operate by comparing the likelihood of the investigated genotype to a distribution of likelihoods of simulated genotypes (generated by Monte Carlo simulations of 10,000 independent individuals for each candidate population) for each of the potential source populations. A population can be excluded as the origin of the investigated individual if that individual's genotype likelihood is outside the tail of distribution (e.g. 0.001) for that population. If all but one of the putative source populations are

excluded in such a manner, that population will be assigned as the origin (Manel *et al*. 2002). An important feature of the exclusion method is that it does not assume that the true population of origin of the investigated individual has been sampled (i.e. is one of the putative source populations) as this method does not compare populations but rather treats each one separately. GENECLASS2 (Piry *et al*. 2004) is a program in which this sort of assignment test could be performed. The other type of approach, the fully Bayesian assignment test, estimates the posterior probability of the investigated genotype originating from each of the putative populations and subsequently compares that genotype's posterior probability of originating from each of the populations to a chosen threshold *T* (Manel *et al*. 2002). For example if *T*=0.999 (as is used for forensic applications) and if an individual genotype was estimated to originate from population A with a probability of 0.9999, from population B with probability of 0.0001 and from population C with probability of 0.0000, then this individual would be assigned to belong to population A and excluded from populations B and C. However if, for the same *T*, an individual genotype was estimated to originate from population A with a probability of 0.990, from population B with a probability of 0.000 and from population C with a probability of 0.10, then this individual could not be assigned to any of these populations because no probability of origin has a greater value than the chosen threshold (Manel *et al*. 2002). In this assignment test one could use prior information about the possible population structure, for example assuming no gene flow among the putative source populations. An assignment test will be more powerful if the putative source populations are genetically differentiated. The four genetic clusters that constituted the sample of the 244 *B. hydrosauri* adults exhibited pairwise F_{ST} values of at least 8.5%, thus indicating moderate to high levels of among cluster differentiation.

A comparison study found the GENECLASS exclusion test to perform worse than the assignment test of STRUCTURE in terms of accurate assignment of individuals (Manel *et al*. 2002) but the advantage of the exclusion method is that it does not assume that the true source population is one of the putative source populations. Thus if the exclusion method is used then false assignments will be avoided. The fully Bayesian test though could still assign an individual genotype to one of the putative source populations even if that individual's true source population is not among them as long as the posterior probability of that individual originating

from one these populations is higher than *T*. Manel *et al*. (2002) recommended using both these tests if the true source population is not present in the data set, as could be the case for some of the juveniles in my study. Besides the potential problem of not having sampled the true population of origin of some of the juveniles (i.e. none of the four STRUCTURE defined clusters is their source population), another issue is that clustering juvenile individuals could result in low numbers of ticks of the different life stages being present in some clusters. Thus analyses such as on-host relatedness comparison between the life stages or spatial autocorrelation could give misleading results due to too low sample size. Preferably more intense sampling should be performed before the ripple model can be tested at the cluster level.

Another useful addition to the study of *B. hydrosauri* transmission dynamics would be to estimate the average relatedness coefficient amongst *B. hydrosauri* ticks of the same life stage sampled from the same host refuge site. This would allow for a better understanding of the make up of the larval and nymphal aggregations and provide information on whether multiple females lay eggs within the same refuge site and if the nymphal aggregations are made up from individuals that had originated from multiple broods.

The applicability of the ripple model to other parasite species should also be tested. Likely these species would need to exhibit certain features, such as clumping of the first life stage (larvae), leading to most of the brood attaching to the same host, and some mechanism (perhaps host movement) that would result in attachment of siblings to different hosts at subsequent life stages. The ripple model predicts different levels of mixing at the different life stages thus most likely it could not be applied to parasite species whose offspring are spread across the landscape already at the first life stage, for example if the female lays eggs at multiple sites.

Population genetic structure of *B. hydrosauri*

In *Chapter 4* I investigated the population genetic structure of *B. hydrosauri*. Briefly, I found that these ticks exhibit a surprisingly complex population substructure at the northern limit of their distribution range. The sample of 244 *B. hydrosauri* adults, collected along a roughly 30 km linear sampling transect, comprised multiple genetic clusters, i.e. populations. STRUCTURE and GENELAND both identified four disparate clusters, whereas another assignment program called BAPS identified

seven clusters where the three larger clusters identified in the other two programs were each split into two. The level of congruence between the three programs was perhaps surprisingly high, with quite similar compositions of the clusters across the three programs. In both GENELAND and BAPS the user can add the geographic data (coordinates of the capture location of each sample) to sharpen cluster designation. Whether this is an improvement or a hindrance deserves further consideration and investigation in my opinion, especially in cases such as mine where multiple multi-locus genotypes were associated with a single set of spatial coordinates. This was because all individuals collected from the same host were assigned that host's spatial coordinates. I suspect that other parasite studies would deal with this issue the same way, rather than assigning each individual parasite a unique spatial coordinate. Despite attempting to overcome this issue with such "tricks" as slightly altering the last coordinate of each sample so that each tick would have a unique set of coordinates or implementing the "uncertainty on coordinates" option (choosing a value of 220, which is the square root of the area of a typical sleepy lizard home range), GENELAND always assigned all ticks collected from the same host to the same cluster. BAPS (as outlined in *Chapter 4*) could provide consistent results only if all individuals collected from the same host were *a priori* grouped into a population. This program assigned all but five adults collected from the same host to the same cluster. Thus possibly the priors introduced by these programs on the provided geographic information are too strong and the programs that do use the spatial coordinates as one of the priors are more applicable for investigation of population structure of organisms where each can be unambiguously assigned its own set of spatial coordinates such as large mammals for example. Given these points I assumed the STRUCTURE-defined clusters had the greatest biological reality.

If the STRUCTURE defined clusters are accepted as the biological reality then this makes for a very interesting situation as then three of the larger clusters are syntopic since multiple hosts were infested with ticks assigned to two or three clusters. Moreover, various analyses indicated these clusters to be highly divergent genetically. This result only partly concurs with the ridge and trough model (Bull and Possingham 1995), as although disparate genetic clusters were identified, as predicted by the model, they had overlapping distributions, whereas the model predicts each cluster to be confined to a specific (ridge) area of the environment. The

question that requires further research is why have these clusters formed and how they are maintained.

One possibility is that these clusters represent inbred entities. Supportive evidence for this suggestion is that the three larger clusters all exhibited a significantly positive F_{IS} coefficient and high relatedness among adult cluster members on the same host. Although most likely this is not the true explanation for the formation of the clusters (see *Chapter 4*), investigating the inbreeding status of the clusters would clarify this issue and would also be useful for testing the predictions of the ripple effect. Within cluster inbreeding could be inspected by testing for correlation between the genetic relatedness and the mating status of male-female pairs of ticks, following the method described in Chevillon *et al*. (2007). This approach will indicate whether mating takes place between related individuals, whether it is a random event (pangamy) or whether *B. hydrosauri* ticks actively avoid mating with related individuals. Certain identification of mating pairs should not be a problem as *B. hydrosauri* females mate only once and the partners are involved in a well described stereotyped six-phase courtship routine (Andrews and Bull 1980). Chevillon *et al*. (2007), who investigated the transmission patterns of cattle tick *Rhipicephalus microplus*, were able to almost categorically rule out mating between closely related individuals as a cause of the observed deficit of heterozygotes as they found evidence of pangamy (male and female ticks mating independently of their genetic relatedness) within infrapopulations and also at the cow-herd scale.

A further possibility is that the observed pattern is due to bacterial endosymbionts causing reproductive incompatibility between ticks from disparate clusters (*Chapter 4*). Currently it is not known whether *B. hydrosauri* ticks harbour these bacteria thus at minimum PCR based surveys for endosymbionts in *B. hydrosauri* would seem warranted and further experiments if these bacteria are shown to be present in *B. hydrosauri*.

Another possible factor to explain the clusters is that they are maintained by assortative mating. Ticks from disparate clusters may be unable to mate because the olfactory cues which *B. hydrosauri* emit to initiate courtship and mating activity are incompatible, which constitutes a barrier to gene flow. To test this possibility mating experiments involving adults from different clusters will need to be performed in the laboratory but also in the wild if possible to replicate the natural conditions. Also the

chemical composition of the mating pheromones and tick morphology (especially the genitalia) will need to be analysed and compared between ticks from different clusters. Ticks which will be used in these experiments will first need to have their cluster identity established. A male from for example cluster 1 not mating with a female from cluster 2 will strongly indicate that differences in the pheromone cues play a role in the maintenance of the clusters. If indeed further research will show strong evidence for reproductive incompatibility between ticks from disparate clusters then perhaps it will be necessary to review their taxonomic status. Karyotyping will also be useful to perform as it could reveal differences in the number and organization of chromosomes between individuals from different clusters, providing further support for the species status of the clusters.

Evolutionary processes that led to the establishment of the clusters should also be further investigated. Currently the clusters occur in sympatry, even syntopy, but they may have differentiated in allopatry. Sympatric speciation of parasites has been a recent topic of debate (McCoy 2003), with the exact definition of sympatry being heavily scrutinized. Two proposed mechanisms of sympatric speciation in parasites are through host choice [conspecific parasites infesting several different host species that occur in sympatry, leading to eventual host race formation, e.g. McCoy *et al*. (2001)] and through within-host habitat selection, termed synxenic speciation (McCoy 2003). However, neither of these causes appears applicable in this case as all clusters infest the same host species without any apparent niche partitioning. Thus the clusters most likely did not form in sympatry but rather in allopatry, as is explained in *Chapter 4*. One could speculate that the clusters formed when the environment was a lot more heterogeneous than today such that the patches of high quality habitat were scarce and very isolated, with ticks (and their hosts) confined to their patch. The connectivity between the patches would be extremely limited due to the habitat surrounding each patch being very poor quality. If there were different selective pressures operating within each patch, and given sufficiently long time, this could lead to differentiation of the mating signals and hence to reproductive incompatibility between ticks occupying different patches. With the conditions becoming milder, the host and the tick ranges could overlap, though the identity of the clusters would be maintained through (for example) differences in the mating pheromones. Past environmental heterogeneity would have also likely affected other species presently occupying this area thus investigating the population

structure of species with a similar life style and generation time to *B. hydrosauri* would be useful to carry out. If a similar population structure was identified for these species this would give credence to the environmental heterogeneity theory.

The population structure of *B. hydrosauri* was found to be different to that predicted by the ridge and trough model but further studies will be necessary to establish whether the ridge and trough model describes *B. hydrosauri* population structure at the edge of its range. It is possible that the population structure found in this study is at a finer scale than the model predicts, i.e. "within" ridges. Currently the extent and position of the ridges in relation to the zone of parapatry is unknown, thus sampling and subsequent genetic analyses will need to be performed on other transects, parallel and perpendicular to the one I followed, and also between transects if possible. Such a study would clarify the extent of the range of the four currently known genetic clusters and confirm the presence of any as yet unidentified clusters. Inspecting the mtDNA haplotypes of *B. hydrosauri* ticks collected from different transects, large distances from one another, and from across the species range in a large scale phylogeographic study will also be helpful for better understanding the historical processes responsible for the contemporary geographic distributions of individuals, thus providing information on the evolutionary and geographic processes that led to the formation of the clusters.

Investigating the population structure of *B. hydrosauri* within the centre of this species distribution range will be useful for testing the applicability of the ripple model in milder conditions than the edge of the species range and to work out the population substructure, but it will also be useful for comparing such parameters as allelic richness and gene diversity between the core and the edge populations and contribute to the debate on this topic. A commonly held assumption is that populations persisting at the edges of a particular species' range are less abundant and more prone to temporal variation in abundance than are the core populations and that migration rates into and between the edge populations are presumed to be lower than into and between the core populations. Peripheral populations have been proposed to be highly differentiated due to low connectivity. Drift would be more prevalent than gene flow in the peripheral populations making them more prone to genetic bottlenecks, resulting in depleted (neutral) genetic diversity. Hence the peripheral populations are predicted to exhibit lower heterozygosity and allelic richness at neutral loci (Sagarin *et al*. 2006). A theoretical study has indeed

suggested that central populations are more genetically diverse (Vucetich and Waite 2003).

However, many wild species are thought to display more complex demographic structures than the above-described model (Sagarin *et al*. 2006). Firstly, as few studies sample the entire species' range, firm conclusions on the relative abundance of peripheral and core populations can rarely be made (Sagarin *et al*. 2006). Moreover, even if the entire range was successfully sampled, it may not always be clear where is the core or centre of a given species' range (Sagarin *et al*. 2006). Even if the range and core populations have been adequately defined, studies on varied organisms have found no evidence of lower abundance of edge populations compared to the centre ones (see Sagarin *et al*. 2006 for examples). Similarly, empirical evidence for the greater diversity of core populations is equally ambiguous, with some studies suggesting that this may be the case (Lammi *et al*. 1999), and others providing no support or evidence to the contrary (Wendel and Parks 1985). Indeed, one theoretical study indicated that if migration rates increase as one moves away from the centre of a species' range towards its edges, then genetic variation will be higher in the edge populations, even if they exhibit a (slightly) lower effective population size than the core populations (Vucetich and Waite 2003). Lastly, in many cases it may not be clear how much influence historical geological events have on the current genetic makeup of species (Antunes *et al*. 2006). For example populations of Italian agile frog (*Rana latastei*) were not structured such that the core populations are more genetically variable than the edge populations, but rather genetic variation seemed to follow an east-to-west gradient of declining diversity best explained by post-glacial range expansion of that species (Garner *et al*. 2004). A comprehensive review of studies performed over the last 35 years that attempted to compare the genetic diversity and/or among population differentiation of peripheral versus central populations found that 64.2% of such studies detected peripheral populations to be less diverse and 70.2% of studies which tested for it concluded increased differentiation amongst peripheral populations. Moreover there was a positive association between these trends (Eckert *et al*. 2008). However, only a few studies have found the difference in genetic diversity between the peripheral and central populations to be substantial (Eckert *et al*. 2008).

A future project might provide new insights exploring these questions with *B. hydrosauri*. To test whether *B. hydrosauri* individuals from the edge of this species'

distribution range are less genetically variable at the neutral microsatellite loci than *B. hydrosauri* individuals from within the centre of this species' range, allelic richness and gene diversity could be compared amongst adult ticks collected from these areas. Allelic richness could be computed for each locus for each group and as an average for each group over all loci in FSTAT version 2.9.3 (Goudet 1995). To obtain estimates independent of sample size variation, the rarefaction method (El Mousadik and Petit 1996) would need to be applied with a standard sample size that corresponds to the group with the smallest number of adult ticks with a complete genotype at all nine loci. Gene diversity (Nei's unbiased expected heterozygosity) could be calculated for each locus within each group as well as average for each group over loci also in FSTAT version 2.9.3 (Goudet 1995). Levels of average (over loci) allelic richness and gene diversity could be compared between the groups using a Wilcoxon signed-rank test in STATMOST32 software. Differences in the allelic richness and gene diversity per locus among groups could also be tested with a two-way analysis of variance (ANOVA) without replication in R 2.5.1 (Ihaka and Gentleman 1996). The group should be entered as a factor in the model and each locus as a replicate, which, since the loci are the same in each group, should also be entered as a factor in the model.

In conclusion, the various additional analyses described in this *Chapter* should be performed to build on the results of my study. The situation already is very exciting but with the additional analyses, and hence a more clear picture, even more interesting conclusions on the population biology of *B. hydrosauri* will be able to be drawn. Furthermore, the development of *A. limbatum* specific microsatellite loci suitable for a population genetics study will allow the population structure and biology of this tick species to be examined, and perhaps the mystery of the *B. hydrosauri A. limbatum* parapatric boundary finally to be solved.

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Appendix I

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PERMANENT GENETIC RESOURCES

Development of microsatellite markers and analysis of their inheritance in the Australian reptile tick, *Bothriocroton hydrosauri*

JARO GUZINSKI,*† KATHLEEN M. SAINT,* MICHAEL G. GARDNER,†‡ STEPHEN C. DONNELLAN*‡ and C. MICHAEL BULL†

*Evolutionary Biology Unit, South Australian Museum, Adelaide, SA 5000, Australia, †School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia, ‡Australian Centre for Evolutionary Biology and Biodiversity, University of Adelaide, Adelaide, SA 5005, Australia

Abstract

Despite long-term study, the mechanism explaining the parapatric distribution of two Australian reptile tick species is not understood. We describe the development of primers amplifying 10 microsatellite *Bothriocroton hydrosauri* loci, for the study of population structure and dispersal patterns of this tick. The numbers of alleles per locus ranged from two to seven in ticks from the study site, and the observed heterozygosity between 0.28 and 0.69. Pedigree analysis indicates that one locus is inherited in a non-Mendelian manner in three families, which was not explained by null allele presence.

Keywords: *Bothriocroton hydrosauri*, inheritance, microsatellites, multiplex PCR, parapatric boundary, reptile tick

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Bothriocroton hydrosauri and *Amblyomma limbatum* are allopatrically distributed species of Australian reptile ticks. *Bothriocroton hydrosauri* occupies the wetter southeastern part of the continent and *A. limbatum* the arid zone to the north (Smyth 1973). The parapatric distributional boundary between these tick species has been studied in detail at Mount Mary, South Australia for the last 25 years. The most common host for the two tick species at the study site is the large scincid lizard *Tiliqua rugosa* (Bull *et al.* 1981). The boundary between the two tick species is very abrupt, with a very narrow overlap zone (Bull *et al.* 1981). Several mechanisms have been postulated to explain this tight boundary but none of them satisfactorily explain such a narrow overlap zone (Bull & Burzacott 2001). Some aspects of tick population biology that are not known presently, such as population structure and dispersal patterns, and how these vary between the different life stages, may provide further insights into the cause and maintenance of the parapatric boundary. We describe the isolation, characteristics and inheritance of 10 polymorphic microsatellite markers in *B. hydrosauri* for analysis of population structure and dispersal.

Correspondence: Jaro Guzinski, Fax: 61 883034364; E-mail: guzi0002@flinders.edu.au

Microsatellites were isolated following an enrichment protocol (Gardner *et al.* 2007). DNA was extracted following the DNeasy Tissue kit (QIAGEN) protocol from whole bodies of male ticks collected from *T. rugosa* hosts at Mount Mary. We confirmed the absence of host DNA in the extract used to isolate the microsatellite loci by polymerase chain reaction (PCR) amplification with host-specific mitochondrial DNA primers (Forstner *et al.* 1995). DNA was digested with *Rsa*I or *Bst*UI (New England Biolabs) and 300–1000 bp fragments were size selected. Those fragments to which 3′ biotinylated repeat oligos [$(AAAG)_6$; $(AAAC)_6$; $(AC)_{11}$] hybridized were isolated with Streptavidin MagneSphere Paramagnetic particles (Promega) and ligated into TOPO TA (Promega TA Cloning Kit, Invitrogen) vectors. One Shot Mach1 TIR (Promega TA Cloning Kit, Invitrogen) *Escherichia coli* cells were transformed with the plasmids and incubated overnight with ampicillin selection. Inserts containing clones were screened using hybridization with the appropriate biotinylated probe [$(AAAG)_6$; $(AAAC)_6$; $(AC)_{11}$] and an alkaline phosphatase/streptavidin colourimetric reaction (Boehringer Mannheim). Inserts were amplified with T7 promoter and M13 reverse plasmid vector primers and sequenced on both strands using the ABI PRISM Big Dye cycle sequencing kit in ABI 3700 sequencers

Table 1 Characterization of 10 microsatellite loci in the tick *Bothriocroton hydrosauri*. The final annealing temperature (T_a) is in °C. †Dots indicate the presence of nonmotif bases. The number of individuals genotyped (N), number of alleles (N_A), allele size range in basepairs (bp), observed (H_O) and expected (H_E) heterozygosities, and the probabilities associated with exact Hardy–Weinberg tests (P_{HW}) are listed for each locus. Primer labels: FAM, NED, PET, VIC. Cloned sequences are in GenBank under accession numbers. *P_{HW} significant after sequential Bonferroni correction

Locus name	Primer sequence (5′–3′)	Multiplex set, T_a	Repeat motif in clone†	Cloned allele size	Allele size	N	N_A	H_O	H_E	P_{HW}	GenBank accession
Bohy1	F: P·AGTCGGGCTTCAAAGGTTCA R: CCTACCCAGTCCCATTAAAGA	1, 59	$(AAAG)_{18}$	224	222–266	56	6	0.643	0.680	0.6275	EU051324
Bohy2	F: V·CACTACCTCCTGTTGCACACA R: GGGACTTGTCGTTTTTGCTGT	1, 59	$(AAAG)_9$ ... $(AAAG)_2$ ... $(AAAG)_1$ ... $(AAAG)_2$	206	216–232	57	4	0.649	0.594	0.6401	EU051325
Bohy3	F: N·CCGACACCTTCGTTACCGA R: ATGTGGAACAGCGCCTCATTA	1, 59	$(AAAG)_{11}$	261	274–314	56	6	0.500	0.554	0.3187	EU051326
Bphy4	F: F·CGTCACACTTGATACGTTGTC R: AGGCGTAATTAATGACCGCT	2, 53	$(AAAG)_{11}$	231	256–272	53	5	0.509	0.612	0.0591	EU051327
Bohy5	F: N·CGTTAGCGTTGTCTTGCACAA R: CAGAAATGGCTTGCTTCAGA	3, 48	$(AC)_{10}$	219							EU051328
Bohy6	F: V·TGTGGCCAATCACTCTTTGT R: TTAGACTGCACTCGATGGCGT	4, 59	$(AAAG)_{14}$ ... $(CAAG)_{10}$	200	200–244	57	6	0.456	0.556	0.0021*	EU051329
Bohy7	F: F·ATGTGGAGGTAGTGGGTTCGA R: GTTTTTGAGCTGTTTTATGCG	4, 59	$(AAAG)_7$	127	140–166	58	3	0.276	0.399	0.0197	EU051330
Bohy8	F: F·TACGCAGCGGATAGGCAAC R: TGGGTGATATTGTCAAAGGCT	4, 59	$(AAAG)_{16}$ ... $(AAAG)_3$	246	262–294	57	7	0.684	0.755	0.4500	EU051331
Bohy9	F: N·TCTGTATTGGAACGTGTGACG R: CCAAGGAAGAGAGGTCATCAT	4, 59	$(AC)_{19}$	161	158–176	58	2	0.431	0.411	1.000	EU051332
Bohy10	F: P·GCGAGCCGATGTAGTGAAA R: CTGCACATAAATGTAGATAGC	4, 59	$(AC)_{28}$	192	184–208	58	7	0.690	0.689	0.0901	EU051333

(Applied Biosystems). For 20 positive, unique clones, we used OLIGO 4.0 to design PCR primer pairs, complementary to the flanking regions of microsatellites that had at least five repeat motifs. Ten loci were discarded because they were monomorphic or had a high incidence of nonspecific bands in 30 adult ticks collected from the study site. The scorable polymorphic loci are listed in Table 1. Amplification of *A. limbatum* DNA was unsuccessful with any of the primer pairs.

Each forward primer was 5′ labelled with a fluorescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) (Table 1). PCR amplifications were performed for two multiplexes (sets 1 and 4) and two single locus reactions (sets 2 and 3) (Table 1) in 10 μL reaction volumes containing 2× reaction buffer (Applied Biosystems) and 2.5 mM $MgCl_2$ (sets 1 and 3) or 2× Eppendorf reaction buffer (including 2.5 mM $MgCl_2$) (sets 2 and 4), 0.2 mM each dNTP, 400–600 nM each primer, 10 U (set 1) or 1 U (set 3) Ampli*Taq* Gold DNA Polymerase (Applied Biosystems) or 10 U (set 4) or 1 U (set 2) HotMaster *Taq* DNA Polymerase (Eppendorf), 0.4 μL 100× BSA (Ambion) (sets 1 and 4), 1.89 μL 5 M betaine (Sigma) (sets 1 and 4), 2 μL genomic DNA (20–40 ng). The cycling conditions were: initial denaturation step at 94 °C for 9 min (sets 1 and 3) or 2 min (sets 2 and 4), followed by 34 cycles at 94 °C for 30 s, annealing temperature (see Table 1) for 30 s and extension at 72 °C (sets 2 and 3) or 65 °C (sets 1 and 4) for 30 s, followed by a single final extension step at 72 °C (sets 2 and 3) or 65 °C (sets 1 and 4) for 30 min. Fluorescently labelled products were separated on an ABI 3730 DNA Analyser and sized and scored using the program GENEMAPPER 3.7 (Applied Biosystems). Allele sizes and patterns did not differ between the multiplex and single amplifications for the same loci.

Six crosses were set-up to examine the inheritance of the 10 loci. Virgin *B. hydrosauri* females, kept separately, were mated with a conspecific male on-host, and after dropping off the host, were kept in individual containers at 25 °C and 80–85% relative humidity with a 12:12 photoperiod. Pedigree analysis of the six families (17–20 larvae per family) shows nine of the 10 loci to be inherited in a Mendelian manner (Table 2). Non-Mendelian inheritance of *Bohy5* in three families could not be explained by null allele presence or large allele dropout. Only two alleles have been scored at this locus, therefore non-Mendelian inheritance might be explained if the two alleles belonged to different loci, co-amplified because of a partial complementarity of the priming sites. However, sequencing of individuals homozygous for the alternate alleles confirmed the flanking regions to be identical, hence discounting that possibility. We did not consider this locus in subsequent analysis.

Table 2 Probability of conformance to Mendelian expectations of 10 microsatellite loci in six *Bothriocroton hydrosauri* families (AH1–AH6). Predictions of allelic proportions under the expectation of Mendelian inheritance were tested with chi-squared contingency tests with the *P* value significant if less than the Bonferroni corrected value. All loci conformed to Mendelian expectations in the tested families with the exception of locus *Bohy5* in families AH1, AH2 and AH3. **P* value significant after sequential Bonferroni correction

Locus	AH1	AH2	AH3	AH4	AH5	AH6
Bohy1	0.9014	0.4912	0.2513	0.2615	0.3080	0.1083
Bohy2	0.1797	0.8186	0.8186	0.3711	1.0000	0.4912
Bohy3	0.5909	0.3460	0.1388	1.0000	0.1797	1.0000
Bohy4	1.0000	1.0000	0.2513	1.0000	0.3460	0.0954
Bohy5	0.0047*	0.0111*	0.0001*	0.3711	1.0000	1.0000
Bohy6	0.3711	0.6547	0.0937	0.8186	0.3441	0.8495
Bohy7	0.5724	0.3711	0.4235	0.2243	1.0000	1.0000
Bohy8	0.1083	1.0000	0.3012	0.8012	0.1797	1.0000
Bohy9	0.0907	0.3711	0.2592	0.8186	0.8187	0.3711
Bohy10	0.1653	0.4235	0.3867	0.0753	0.8186	0.5724

Genotypes of at least 56 adult ticks, from Mount Mary, were used for the analyses described below and performed in GENALEX 6 (Peakall & Smouse 2006) and CERVUS 3.0 (Kalinowski *et al*. 2007). The number of alleles per locus ranged from two to seven (mean = 5.11) and the expected heterozygosity ranged from 0.399 to 0.755. Observed heterozygosity values (average 0.538) were lower than the expected heterozygosity (0.583) at six of the nine loci (Table 1). The slight heterozygote deficit at these loci may suggest inbreeding or the Wahlund effect. Tests of conformance to Hardy–Weinberg Equilibrium and linkage disequilibrium were performed in GENEPOP 3.4 (Raymond & Rousset 1995). *Bohy6* showed deviations from Hardy–Weinberg Equilibrium after sequential Bonferroni correction (Hochberg 1988) (Table 1). No linkage disequilibrium was found for any of the loci after sequential Bonferroni correction. Thus, eight of these nine polymorphic microsatellite loci should be valuable markers for population genetic analysis of *B. hydrosauri*.

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Appendix II

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Molecular genetic data provide support for a model of transmission dynamics in an Australian reptile tick, *Bothriocroton hydrosauri*

JARO GUZINSKI,*† C. MICHAEL BULL,† STEPHEN C. DONNELLAN*‡ and MICHAEL G. GARDNER†‡

*Evolutionary Biology Unit, South Australian Museum, Adelaide, SA 5000, Australia, †School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001, ‡Australian Centre for Evolutionary Biology and Biodiversity, University of Adelaide, Adelaide, SA 5005, Australia

Abstract

***Bothriocroton hydrosauri* is a three-host ixodid tick that infests large reptiles in southeastern Australia, where its most common host is a large scincid lizard *Tiliqua rugosa*. Based on previous ecological and behavioural studies of this system, we propose a 'ripple' model of tick population dynamics, where only a few female ticks succeed in producing surviving offspring. These females then are the centres of ripples of their progeny spreading into the broader landscape. The model predicts higher relatedness among larvae than among nymphs or adults on a host, and significant spatial autocorrelation in larvae extending further than for the later life stages. The model also predicts that adult ticks are likely to encounter related partners and that this will generate inbreeding within the population. We tested those predictions using nine polymorphic microsatellite loci on a sample of 848 ticks (464 larvae, 140 nymphs and 244 adults) collected from 98 lizard hosts from near Bundey Bore Station in South Australia. Our data support the predictions and indicate that the dynamics of transmission among hosts play an important role in parasite population structure.**

Keywords: inbreeding, lizard, microsatellite DNA, relatedness, tick

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Introduction

Since the free-living stages of many parasite species have low dispersal capability, and some parasites may even completely lack free-living stages, the population structure and dynamics of individual parasite species may be intimately connected to the ecology and movement patterns of their host species (Nadler 1995; Criscione *et al*. 2005). The ecological challenges for a parasite can be extremely different in the environment they experience when they are with a host and when they are in the external environment away from a host. Hosts can be regarded as spatially and temporally patchy habitats within a hostile matrix (Criscione *et al*. 2005). Price (1977, 1980) suggested that this might lead to locally isolated parasite populations, each derived from a small number of founders. He suggested that there would be low dispersal of parasites among host individuals and reduced opportunities for outbreeding in local parasite populations. These predictions particularly apply to parasites that can go through multiple generations on the same host individual (Criscione *et al*. 2005). However, many animal macroparasites release offspring into the external environment, where progeny from different sources can become mixed, before infesting another definitive host. The degree of mixing within populations of an individual parasite species may fall somewhere along a continuum from isolation on individual hosts to complete mixing among hosts. The position of a parasite species between these two extremes of population structure may depend on the life-history strategy of the parasite species considered, on the density, movement and social overlap of its hosts and on the severity of the external environment. The more frequently hosts contact each other, and the less severe the off-host conditions, the more likely that parasite individuals in a population will become mixed.

The population genetic structure of a parasite species may provide an indication of where it fits on that continuum.

Correspondence: Jaro Guzinski, Fax: 61 883034364;
E-mail: guzi0002@flinders.edu.au

This in turn provides evidence about the transmission dynamics of the parasite. For instance, a parasite that can disperse widely among its host population, and that has extensive mixing, would be expected to show low levels of relatedness among individuals infesting the same host, and high levels of outbreeding and heterozygosity within host populations. Parasites that are mostly confined to a local subset of the host population may show opposite trends. Our study describes aspects of the population genetic structure of the Australian reptile tick, *Bothriocroton hydrosauri*. Our aim was to explore the transmission dynamics, and to test specific predictions based on previous ecological and behavioural studies.

Previous studies of tick populations have described different patterns of genetic variation both among and within tick species, and have inferred the probable role of host movement. Bull *et al.* (1984) reported lower levels of isozyme variation in six species of Australian reptile ticks, than either Healy (1979a, b) or Hilburn & Sattler (1986) found for ticks with more mobile hosts. Lampo *et al.* (1998) found that most of the genetic variation in the tick *Amblyomma dissimile* resulted from differences in allele frequencies among ticks from different host individuals. They suggested that host dispersion influenced the genetic structure of populations of that tick. Similarly, McCoy *et al.* (2003) found that populations of the seabird tick, *Ixodes uriae*, had different genetic structure in colonies of two seabirds with differing mobilities.

Bothriocroton hydrosauri is a three-host tick that infests large reptiles in southeastern Australia (Smyth 1973). In our study area, the major host for all life stages of the tick is a large skink, the sleepy lizard, *Tiliqua rugosa* (Smyth 1973). Larvae attach to a host, engorge on blood or lymph, detach and moult to nymphs. These attach to a second host, engorge, detach and moult to adults. These in turn attach to a third host. Adult male ticks do not engorge, but mate on the host with attached females. A female will mate with a single male, whereas males can mate with multiple partners (Andrews & Bull 1980). Mated females engorge, detach and lay several thousand eggs, which hatch into the next generation of larvae. Engorged ticks detach while their hosts are in refuge shelters, moult to the next stage and then wait in the refuge for another host. As males remain attached to hosts after mating, feeding sparingly, many more males than females are found on the lizard hosts. Bull (1978b) argued that the limited movement of host lizards is likely to restrict the distance that ticks disperse.

Models of the population dynamics of this tick species (Bull 1991; Bull & Possingham 1995; Tyre 1999; Tyre *et al.* 2006) have invoked a process we here call the 'ripple effect'. It is based on the use of multiple shelter refuges by each individual host lizard within its home range which averages 3–5 ha (Bull & Freake 1999; Kerr *et al.* 2003), the overlap of lizard home ranges (especially male overlap of female home ranges) (Kerr & Bull 2006), the nonsynchronous sharing of refuges by different lizards (Dubas & Bull 1992; Kerr & Bull 2006), and the high susceptibility of detached ticks to predation (Bull *et al.* 1988; Dawes-Gromadzki & Bull 1997a, b) and desiccation (Chilton & Bull 1993). Desiccation is particularly severe on the small larval stage in the hot Australian spring and summer when their lizard hosts are most active. The ripple model assumes that many ticks in lizard refuges may have to wait for the next host for longer than they can survive. This will be particularly the case at the lower rainfall edge of the tick distribution, where most of the previous studies have been conducted (Bull & Possingham 1995; Bull & Burzacott 2001).

In the ripple model, an engorged female tick deposits her egg clutch in a lizard refuge. The hatched larvae aggregate while waiting for a host (Petney & Bull 1981), but many complete clutches of larvae die before a host lizard uses the refuge. However, in a surviving clutch, many of the aggregated larvae attach to the first host that uses the refuge. Thus larvae occur in high densities on a few hosts that represent rare focal points in the landscape (Bull 1978a). As those larvae engorge on the host, they detach over a number of days, and into a number of refuges that the host lizard visits (Bull 1978b; Kerr & Bull 2006). A subset of these detached larvae survive and moult, to attach as nymphs onto separate hosts that enter the several refuges. These in turn engorge over a period of time and are scattered into further refuges where they moult to adults.

Thus, in this model, the progeny of a single female will be spread further from their single point of origin with each life stage. In a landscape with high tick mortality, each of the few clutches that contribute to the population can be imagined as creating a ripple of ticks that spreads from the central clutch deposition site. The harsher the habitat, the fewer ripples across the landscape and the less likely it is that there will be mixing of different clutches. In the continuum of parasite population structure referred to above, the ripple model is positioned towards the isolation and inbreeding end. The ripple model predicts different levels of mixing at the different life stages.

Our model predicts that:

1 There will be higher relatedness among ticks on individual hosts than in the overall population, and on individual hosts relatedness will be higher among larvae than among nymphs or adults.
2 Spatial genetic structuring within the tick population will extend further for larvae than for the later life stages, because there is less mixing among clutches at the larval stage.
3 There will be detectable inbreeding within the tick population, because adult ticks are likely to encounter related adults on a host for mating, particularly where 'ripples' from different clutches have low overlap.

Here we tested these predictions with data from microsatellite DNA genotypes from *B. hydrosauri* ticks.

Materials and methods

Sampling and genotyping

The study was conducted during spring and early summer (September–December) of 2004 and 2005, along a 29-km unsealed road transect about 10 km east of Bundey Bore Station, South Australia (139°21′E, 33°55′S). The chenopod shrubland habitat has been described previously by Kerr *et al*. (2003). The average annual rainfall at Bundey Bore is 241 mm. The study transect was perpendicular to and close to the abrupt distributional boundary of *Bothriocroton hydrosauri* (Bull & Possingham 1995; Bull & Burzacott 2001), and within the most arid part of the species range. We captured a total of 155 active (feeding or basking) sleepy lizards by hand along roadsides, 109 (70.3%) of which were infested with *B. hydrosauri* ticks (average 9.57 *B. hydrosauri* ticks per host). All of the lizard capture locations, which were recorded by global positioning system, were in close proximity to large bushes (potential host refuge sites, hence potential *B. hydrosauri* transmission locations).

We collected all *B. hydrosauri* ticks attached to the captured *Tiliqua rugosa* hosts and placed them in individual vials that were immediately frozen in liquid nitrogen. Tick DNA was extracted following the DNeasy Tissue kit (QIAGEN) protocol and genotyped at nine polymorphic microsatellite loci as described in Guzinski *et al*. (2008), which also presents details of the variability of the markers based on genotypes of at least 56 *B. hydrosauri* adults from the study site. We used data from 848 ticks that were successfully genotyped for at least six loci. These included 464 larvae, 140 nymphs and 244 adults (222 males and 22 females), which were collected from 40 hosts captured in 2004, and an additional 56 new lizards, plus two recaptures in 2005 (a total of 98 samples from 96 hosts).

Relatedness

We tested the prediction that there was higher relatedness (*R*) among larvae than among nymphs or adults on a host using estimates of pairwise *R* derived from Relatedness 5.0.8 (Queller & Goodnight 1989). Allele frequencies for the whole sample were estimated in GENALEX 6.1 (Peakall & Smouse 2006) from the genotypes of the adult ticks to ensure that the reference allele frequencies were based on a random sample of minimally related individuals (allele frequencies did not differ significantly at the majority (six) of the loci between the 2004 and 2005 adult samples and there was no significant difference in the multilocus allelic richness and gene diversity estimates between the two samples). Standard errors of *R* estimates were obtained by jackknifing over loci.

For each tick life stage, in each year we derived two relatedness coefficients. The first measure was the mean *R* among ticks of the same life stage on the same host (On Host *R*). For each host infected with more than one of a particular tick life stage, we estimated the mean *R* for all pairs of that life stage on that host. Then we took the mean of these values over all multiply infected hosts. The second measure was the mean *R* value for ticks of that life stage in the whole sample (Total *R*). For hosts with more than one individual of a particular life stage, we randomly chose one tick to include in the analysis. The number of hosts included in these two estimates differed because hosts infested with a single individual of a particular life stage could be included in the Total *R* but not On Host *R* calculations.

We tested for differences between mean values of Total *R* and On Host *R* in each life stage, and between On Host *R* values among different life stages and years using the jackknife resampling technique (over loci), followed by a standard unpaired *t*-test in Relatedness 5.0.8.

Spatial genetic structure

We investigated the spatial extent of genetic population structure for each of the three *B. hydrosauri* life stages using spatial autocorrelation analysis as implemented in SPAGeDi 1.2 (Hardy & Vekemans 2002). All ticks collected from the same host were assigned the capture location of their host. In this case, SPAGeDi treats all individuals collected from the same host as belonging to one 'spatial group' and permutes 'spatial group' locations to estimate the 95% confidence interval. In order to test whether the degree of relatedness among individual ticks was dependent on geographical distance, we modified one of the coordinates of each individual by altering the third decimal point of the easting such that each individual had a unique set of spatial coordinates. In this way, we permuted the locations of individual ticks and not host, that is, 'spatial group' locations. We specified distance intervals of 650 m. This interval was the smallest that had over 50% of individuals participating at least once in a pairwise comparison within an interval and a coefficient of variation < 1 for the number of times each individual participated in a pairwise comparison within an interval, conditions recommended for robust analysis by Hardy & Vekemans (2002).

We calculated for each distance interval a mean of the pairwise relatedness coefficients for all pairs of individuals within that distance interval, using Li's relationship coefficient (*L*) as a measure of pairwise relatedness (Li *et al*. 1993). We considered the genetic structure to be significantly positive (individuals more related to each other than would be expected by chance) for distance intervals where *L* (including standard errors) was positive and

exceeded the 95% confidence interval, determined from 10 000 random permutations, about the null hypothesis of random genetic structure. For this analysis, we used 374 larvae, 109 nymphs and 145 adults collected in 2005. Sample sizes from 2004 were too low for analyses.

Inbreeding

Relatedness 5.0.8 was used to calculate the mean relatedness coefficient (On Host *R*) for all pairs of opposite sex adult ticks collected from the same host (10 hosts were infested with at least one male and one female tick). This mean was compared with a Total *R* value, derived as the mean of the pairwise relatedness values for all pairs of opposite sex adults in the whole sample, using a single tick (chosen randomly) of each sex per host.

We estimated the F_{IS} coefficient within the sample of the 244 adult ticks in SPAGeDi 1.2. We tested the significance by performing 10 000 random permutations of genes among individuals within the sample. Standard error was estimated by jackknifing over loci.

Results

Testing the power of the loci to uniquely identify individuals

Preliminary analysis of the 848 multilocus tick genotypes, performed in Microsatellite Toolkit version 3.1 (Park 2001), identified 24 sets of two to six *Bothriocroton hydrosauri* individuals (14 pairs of larvae, five sets comprising a larvae and a nymph, a set comprising two larvae and a nymph, a set comprising four larvae and a nymph, a set comprising four larvae and two nymphs, a pair of adult males and a set comprising an adult male and an adult female) that had identical genotypes at all nine unlinked loci (Guzinski *et al.* 2008). One possible explanation is clonal reproduction, which has been reported in several ixodid tick species (e.g. Stone 1963; Oliver 1989), although not in *B. hydrosauri*. We explored the alternative explanation that genetically unique, but closely related individuals could not be distinguished because of the low power of the nine microsatellite loci that we used. API-CALC 1.0 (Ayres & Overall 2004) was used to estimate an average multilocus infrapopulation probability of identity (P_{ID} — this index is the probability that two randomly selected individuals that belong to a particular sample will have the same multilocus genotype; Waits *et al.* 2001) for 64 infrapopulations that comprised at least four ticks of any life stage. Following Bush *et al.* (1997), we defined an infrapopulation as all conspecific parasitic individuals infesting a particular host at the same time.

Assuming all individuals within an infrapopulation to be unrelated, the mean $P_{(ID)}$ value was 0.00175, indicating that two individuals with identical genotypes would be expected to be randomly encountered every 571 samples. When all individuals within an infrapopulation were assumed to be siblings, the mean $P_{(ID)}$ value was 0.079 indicating that two individuals with identical genotypes would be encountered roughly every 13 samples. We are not able reliably to determine the exact proportion of sibling individuals making up each infrapopulation, but in infrapopulations which comprised largely juvenile, and especially larval ticks, the proportion was likely to be substantial. Hence, the true mean within infrapopulation $P_{(ID)}$ value likely lies somewhere between these two extremes though closer to 0.079, that is, relatively large.

This result is a strong indication that some genetically distinct individuals in our sample could have been scored with identical genotypes due to a low discriminatory power of the loci. Most cases of identical genotypes were from larvae or nymphs, and all cases were either from a single host or from hosts collected less than 280 m apart. This is consistent with a sexually reproducing population following the predictions of our model because if clonal reproduction occurred in this tick species, then we would expect to encounter identical genotypes among a higher proportion of adult individuals than observed and from a wider geographical range, that is, adults with identical multilocus genotypes collected from hosts captured at distances further than a diameter of a single host range. Although the nine loci have incomplete discriminatory power, they still have sufficient power to estimate variation in *R* between the different life stages as indicated by narrow standard error estimates about this coefficient (Table 1).

Relatedness

The mean relatedness among pairs of ticks on individual hosts (On Host *R*) was consistently higher than the overall relatedness among pairs of ticks (Total *R*) (Table 1). The difference was strongly significant for each life-history stage in each year. The adult On Host *R*-value was significantly lower than for nymphs or larvae in each year, and On Host *R* for nymphs was significantly lower than for larvae in 2004 ($P < 0.001$ in all cases).

Spatial genetic structure

Larval ticks showed positive genetic structuring over the first three distance intervals. Individual larvae on hosts up to 1950 m apart were significantly more related to each other than random (Fig. 1a). For nymphs and adults, positive genetic structure was detected within the first distance interval, but not in subsequent intervals below 10 km (Fig. 1b, c). For adults, positive genetic structure was also detected for ticks sampled approximately 10 km apart (distance interval 10 400–11 050 m) although this result

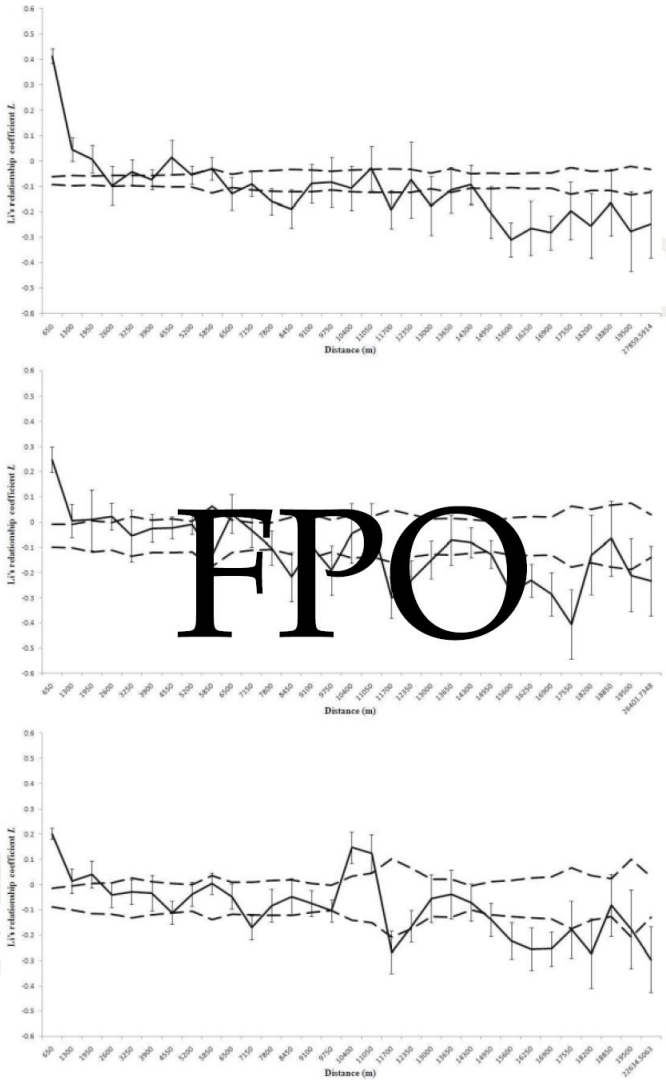

Fig. 1 Spatial autocorrelation analysis performed on (a) 374 *Bothriocroton hydrosauri* larvae (b) 109 nymphs and (c) 145 adults collected in 2005 from 46, 38 and 45 *Tiliqua rugosa* hosts, respectively. Ticks collected from the same host were assigned slightly different spatial coordinates. Significantly positive genetic structure is present within distance classes in which in which Li's relationship coefficient (*L*), including standard errors, is positive and exceeds the 95% confidence interval envelope (dashed lines) about the null hypothesis of random genetic structure.

Table 1 Estimates of mean relatedness (*R*) among pairs of ticks on the same host (On Host *R*) and among pairs of ticks on different hosts (Total *R*) for each tick life stage and for each year of the study and also for pairs of adult ticks of opposite sex (both years of the study combined)

		On host *R*		Total *R*		
Life stage	Year	*N*	*R* (SE)	*N*	*R* (SE)	*P*
Adults	2004	27	0.315 (0.025)	38	–0.021 (0.031)	< 0.001
	2005	32	0.282 (0.045)	45	–0.017 (0.013)	< 0.001
Nymphs	2004	3	0.414 (0.048)	8	0.062 (0.068)	< 0.001
	2005	28	0.439 (0.033)	38	0.002 (0.026)	< 0.001
Larvae	2004	13	0.500 (0.031)	17	0.056 (0.052)	< 0.001
	2005	38	0.454 (0.044)	46	–0.041 (0.017)	< 0.001
Male/female	2004 + 2005	10	0.280 (0.081)	83	0.026 (0.030)	< 0.001

N, number of hosts from which ticks were collected. SE, the jackknifed over loci standard error about *R*. *P*, probability of whether the On Host *R* and Total *R* values are significantly different for each category.

should be interpreted cautiously given the smaller fraction of individuals available for analysis at this distance class (Fig. 1c).

Inbreeding

Adult ticks of opposite sex infesting the same host (thus potential mating partners) were significantly more related to each other than a random pair of opposite sex ticks (Table 1). The estimated F_{IS} coefficient for the adult sample of 0.156 (SE = 0.037) was significantly positive. The F_{IS} coefficients obtained for each of the nine loci were Bohy1 0.190 [$P < 0.001$ (two-sided test)], Bohy2–0.050 ($P = 0.241$), Bohy3 0.098 ($P = 0.028$), Bohy4 0.165 ($P = 0.002$), Bohy6 0.082 ($P = 0.074$), Bohy7 0.253 ($P < 0.001$), Bohy8 0.257 ($P < 0.001$), Bohy9 0.302 ($P < 0.001$), Bohy10 0.113 ($P < 0.001$). Since the majority (seven) of the loci exhibited a significantly positive F_{IS}, a population level effect (e.g. inbreeding) is likely to be the cause rather than locus specific effects (e.g. selection, null alleles). Furthermore, we have shown that all of these loci are inherited in a Mendelian manner in six *B. hydrosauri* families (Guzinski *et al.* 2008).

Discussion

Each of the predictions of the ripple model of tick population dynamics was supported by our genetic data. Ticks on individual hosts were significantly more related to each other than ticks in the overall sample. On hosts, the relatedness was significantly higher among the juvenile stages than among adults in 2004 and 2005 and significantly higher among larvae than nymphs in 2004. For larvae and nymphs, mean relatedness values close to 0.5 were consistent with the prediction that many of the ticks that co-infested a single host were siblings. Moreover, as our observation of identical multilocus genotypes among juvenile ticks collected from the same host or nearby hosts indicated them to be full-sibs, this provided further support for the ripple model.

This genetical structure was maintained across groups of hosts collected close together. Samples of ticks from hosts located near to each other showed higher relatedness than random in the spatial autocorrelation analysis. We did not have a large enough sample for detailed fine spatial scale analysis, but larval ticks retained positive genetic structure over a larger distance interval than the other two life stages. This was predicted by our model in which 'ripples' of each successful clutch of eggs spread across the landscape, increasing the overlap from adjacent ripples, and the amount of mixing among different clutches, with later life stages. Because larvae are less mixed than nymphs or adults, they should retain genetic structure over greater distances.

Despite increased mixing at the adult stage, the ripple model still predicts that related adults are likely to co-infest the same host. This was supported by our finding of significantly high relatedness among adults on the same host. Because these ticks mate on their hosts, a consequence would be an enhanced probability of inbreeding. The genetic data showed high relatedness of potential mating partners, and a positive F_{IS} coefficient for the adult sample. The significant deficiency of heterozygote genotypes indicated by this result is consistent with the predicted inbreeding, although it could have been caused also, at least partially, by the Wahlund effect (Wahlund 1928), that is, sampling together of differentiated genetic groups. If each group exhibits different allele frequencies, then the overall heterozygosity within the pooled sample will be reduced. Thus, while interpretation of the F_{IS} coefficient remains ambiguous, nonetheless the rest of the data are consistent with the prediction of the ripple model for this tick sample.

Our genetic analysis supported the predictions of a model for population structure that was derived from ecological

and behavioural observations of one tick-host system. Can this model be generalized to other ticks? Although up to date, relatively few other studies of genetic structure within tick populations have been carried out, interestingly they do show some different patterns. Delaye *et al.* (1997) suggested that *Ixodes ricinus* was panmictic in Switzerland, and ascribed that to the large number of host species available for the ticks. Chevillon *et al.* (2007) ascertained that there was no correlation between the genetic relatedness and mating status of female–male pairs of cattle tick *Rhipicephalus microplus* in New Caledonia at both the cow and herd scales, thus indicating frequent transfers of sibling ticks between individual hosts in a cattle herd. McCoy *et al.* (2003) found that *Ixodes uriae* (seabird tick) populations of black-legged kittiwakes exhibited higher levels of genetic substructure than *I. uriae* populations of Atlantic puffins. This result concurred with their prediction that ticks infesting puffins should have greater opportunities for dispersal than ticks infesting kittiwakes due to differences in the movement patterns and social behaviour of the two host species.

In each of these examples, the factor that influences the genetic structure of the tick population is the dynamics of transmission among hosts. Where there is a relatively high rate of movement among hosts, the ticks become mixed and panmixia is more likely. Transmission dynamics may even vary within tick populations. In *I. ricinus*, de Meeûs *et al.* (2002) suggested that there were sex-specific host associations, with hosts of male ticks generating wider dispersal and greater mixing than hosts of female ticks. In our study species, we found strong genetic structure in an area where the off-host environment is stressful and where mortality of larvae is high. In more benign habitats for this species and/or where host species diversity is higher, there may be more successful female ticks. This would produce a higher density of 'ripples', and more extensive overlap of adjacent clutches, reducing the level of genetic structuring. Our study site, close to the low rainfall limit of the tick distribution, may have provided unusually harsh conditions for the ticks. Perhaps boundary populations like this one develop more genetic structure than in the centre of the distribution. This might provide conditions for accelerated micro-evolutionary change in ticks (Magalhães *et al.* 2007) that are not typically available in the rest of the range.

More broadly however, the results suggest that for any parasite species, the population structure will be intimately linked to the mode of transmission of parasites among hosts, and that parasite–host systems will provide a rich source of variability in population genetic structure for future study.

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This study formed part of Jaro Guzinski's doctoral thesis on the population genetics of an Australian reptile tick *Bothriocroton hydrosauri* under supervision of Michael Bull and Steve Donnellan. Michael Bull, from Flinders University, South Australia, studies ecological interactions between lizards and their natural parasites and aspects of the behavioural ecology of the host lizard. Steve Donnellan is a professor at the University of Adelaide and the South Australian Museum with current research interests in the population genetics and phylogeny of the Australian fauna. Mike Gardner has broad interests in applying molecular genetic techniques to ecological questions including parasite host interactions.