CHAPTER FOUR

Sequence data reflect the introduction history of the Sirex woodwasp parasitoid, *Ibalia leucospoides* (Ibaliidae, Hymenoptera)

The parasitoid wasp *Ibalia leucospoides* native to the northern hemisphere, has been introduced to the southern hemisphere as a biological control agent for the invasive woodwasp *Sirex noctilio*. Two sub-species of the parasitoid, *I. leucospoides leucospoides* (Palearctic distribution) and *I. leucospoides ensiger* (Nearctic distribution), were introduced and have subsequently hybridized. Despite extensive records of the numbers and origins of the wasps imported into the southern hemisphere, nothing is known regarding the current population diversity. In this study, the genetic variation of *I. leucospoides* in its native and introduced ranges was examined using mitochondrial (cytochrome oxidase subunit one (COI)) and nuclear (internal transcribed spacer (ITS)) markers. As expected, mitochondrial DNA diversity was higher in the native range. Introduced populations had fewer clades separated by deep divergence and greater nuclear ITS diversity than the native North American population examined. Incongruence in the clades for the ITS and mtDNA trees suggests that diversity in the southern hemisphere is intraspecific, possibly due to hybridization. The results reflect the extensive introductions of *I. leucospoides* from four continents and including two sub-species and hundreds of wasps, followed by genetic bottlenecks that fixed only a few haplotypes in these populations. The data further suggest that *I. leucospoides* populations in South America and South Africa originated in New Zealand or Australia.

Introduction

Ibalia leucospoides Hochenw. (Ibaliidae, Cynipoidea, Hymenoptera) is a parasite of siricid woodwasps (Chrystal and Myers 1928, Spradbery and Kirk 1978, Norlander *et al.* 1996), which owes its notoriety to the fact that it has been widely used for biological control of the invasive woodwasp *Sirex noctilio* F. in the southern hemisphere. The female *I. leucospoides* uses the oviposition hole made by siricid females to insert its eggs into those of the siricid host or its early instar larvae (Flanders 1925). Interestingly, *I. leucospoides* locates its siricid host by using volatiles released by the fungal symbiont of the woodwasp (Madden 1968, Martinez *et al.* 2006).

There are 19 species of Ibaliidae (Norlander *et al.* 1996). Kerrich (1973) revised the genus *Ibalia* and reduced *I. ensiger* Norton in North America and *I. suprunenkoi* Jacobson in Japan, to synonymy with *I. leucospoides*. Kerrich (1973), however, recognized the sub-species *I. leucospoides leucospoides* with a Palaearctic distribution and *I. leucospoides ensiger* with a Nearctic distribution.

Ibalia leucospoides is considered one of the most successful parasitic wasps introduced into the southern hemisphere for the control of the woodwasp *S. noctilio* (Neumann *et al.* 1987, Iede *et al.* 2000, Carnegie *et al.* 2005, Hurley *et al.* 2007). *Sirex noctilio* is native to Eurasia, but was accidentally introduced to the southern hemisphere during the course of the twentieth century (Miller and Clarke 1935, Gilbert and Miller 1952, Spradbery and Kirk 1978, Tribe 1995, Maderni 1998, Klasmer *et al.* 1998, Iede *et al.* 1998, Hurley *et al.* 2007). The pest has spread widely in the southern hemisphere and is now found in New Zealand, Australia, Uruguay, Argentina, Brazil, Chile and South Africa. It has also recently appeared as a non-

native invasive pest in North America (Hoebeke *et al.* 2005, de Groot *et al.* 2007). *Sirex noctilio*, together with its fungal symbiont *Amylostereum areolatum* Boiden, attacks and kills pine trees (Talbot 1977). Although not a pest in its native range, *S. noctilio* has become a serious pest in its introduced range in the southern hemisphere (Haugen 1990, Maderni 1998, Hurley *et al.* 2007). This has prompted the introduction of parasitic nematodes and wasps, including *I. leucospoides*, from the northern hemisphere as biological control agents (Taylor 1976, Bedding and Iede 2005, Hurley *et al.* 2007).

Both *I. leucospoides leucospoides* and *I. leucospoides ensiger* were introduced to the southern hemisphere as biological control agents. The first attempt to introduce *I. leucospoides leucospoides* was from England to New Zealand in 1931 (Nuttall 1989). This attempt failed, but later introductions from England in 1950-1951 were successful, and resulted in a breeding colony in New Zealand. In 1959-1960 *I. leucospoides leucospoides* was sent from New Zealand to Tasmania (Taylor 1967). Further introductions of *I. leucospoides leucospoides* from Europe, Turkey, Morocco and Japan, and introductions of *I. leucospoides ensiger* from USA and Canada, to Tasmania occurred from 1962-1973 (Taylor 1976). *Ibalia leucospoides ensiger* was sent from Tasmania to New Zealand and both *I. leucospoides* sub-species were sent from Tasmania to mainland Australia (Taylor 1976, Nuttall 1989). Interestingly, *I. leucospoides leucospoides* and *I. leucospoides ensiger* were reported to interbreed, with the hybrids being indistinguishable from the parent sub-species (Nuttall 1989).

In South America, *I. leucospoides* was reported as naturally introduced with *S. noctilio* and its origin was thus not known (Eskiviski *et al.* 2004). It was first detected in Uruguay in 1984 and subsequently spread to Argentina, Chile and Brazil. In 1998, *I. leucospoides* was imported into South Africa from Uruguay (Tribe and Cillié 2004).

It is not known with certainty which sub-species of *I. leucospoides* were introduced into South America, and subsequently into South Africa. *Ibalia leucospoides ensiger* together with other native siricid parasitoids was already present in North America when *S. noctilio* was first detected there (Cameron 1965, Liu and Nordlander 1992, Smith and Schiff 2002).

Almost nothing is known regarding the genetic variation within and between populations of *I. leucospoides* (Hurley *et al.* 2007). Collections of *I. leucospoides* for release in New Zealand and Australia were from a wide geographical area and involved relatively large numbers of wasps (Taylor 1967, 1976, Nuttall 1989). The origin of *I. leucospoides* introduction into South America is unknown and it is also not known how many introductions occurred. The introduction of *I. leucospoides* from Uruguay to South Africa was based on a very small number of wasps (Tribe and Cillié 2004, Hurley *et al.* 2007) which may have resulted in little or no genetic variation in this population.

The aim of this study was to consider the genetic diversity of *I. leucospoides* across the greater part of its introduced range. For comparison and based on availability, this diversity was compared with a small population from part of the natural range of the parasitoid. For this purpose sequence data for a portion of the mitochondrial cytochrome oxidase subunit one (COI) and the nuclear internal transcribed spacer (ITS) ribosomal DNA were used.

Materials and Methods

Samples and DNA extraction

Ibalia leucospoides adults were obtained from Australia, South Africa, Argentina and Chile, representing much of the introduced range of the wasp in the southern hemisphere (Table 1). Collections of the parasitoid from its native range were difficult to obtain, but included *I. leucospoides ensiger* from the USA and Canada, and two specimens of *I. leucospoides leucospoides* from Portugal (Table 1). Only two specimens were obtained from Portugal. The insect is not commonly encountered in Europe, and we were not able to obtain more wasps from this region. The majority of wasps were collected between 2006 and 2009 and stored in 70-96 % ethanol. The two specimens from Portugal were dry pinned specimens collected in 2001.

Tissue was removed from the thorax of each wasp and total genomic DNA was extracted using the PrepMan™ Ultra Sample Preparation Reagent Protocol (Applied Biosystems, USA), with 100 µl of PrepMan™ Ultra Sample Preparation Reagent used per wasp. For the two samples from Portugal, one to two legs were used and total genomic DNA was extracted using prepGEM™ insect extraction kit (ZyGem Corporation Limited, New Zealand).

Polymerase chain reaction and sequencing

The primers LCO1490 (Folmer *et al.* 1994) and C1-N-2191 (Simon *et al.* 1994) were used to amplify a portion of the mitochondria COI region. The primers

CAS18sF1 and CAS5p8sB1d (the later primer was specific for Hymenoptera) were used to amplify a portion of the nuclear ITS region that covers the ITS1 region between the 18S and 5.8S gene (Ji *et al.* 2003). The COI and ITS primers used did not amplify DNA from the dry specimens from Portugal. Thus, primers were designed using CLC Main Workbench v5.5 to amplify shorter portions within the desired DNA segment. The primer pairs DA2F (5' GGGAAACGTTTTGAGAGA) and DA2R (5' GTATGTAGGAGGAACTATGA), DA3F (5' CTTGTGACTTGTATGCGA) and DA3R (5 TTTCACGATACGGTCCTT), and DA4F (5' CGTTTTGAATGAGCCTGTG) and DA4R (5' TGCGACATCGGCAAAGAA), successfully amplified shorter portions of the ITS segment. The shorter fragments were aligned using overlapping ends to obtain one sequence per specimen. Primers could not be designed within the desired COI region, as this region was inordinately AT rich and had many variable sites. Consequently, a COI sequence was not obtained for the two Portugal specimens.

PCR reaction mixtures contained final concentrations of 1-4 µl of genomic DNA, 10 x PCR buffer (Roche Diagnostics), 0.5 mM of each dNTP, 3.5 mM $MgCl_2$, 1 U *Taq* polymerase, and 0.2 mM of each primer, and were made up with SABAX water to reach a volume of 25 µl. The PCR cycling regime for COI was 95 °C for 7 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s, and concluding with elongation at 72 °C for 10 min. The PCR cycling regime for ITS was 94 °C for 4 min, followed by 35 cycles of 95 °C for 20 s, 62 °C for 40 s and 72 °C for 20 s, and concluding with elongation at 72 °C for 2 min. PCR products were cleaned by precipitation in 3 M NaOAc (pH 4.6) and ethanol. Sequencing reactions were preformed using standard protocols and the products were cleaned using the above-

mentioned precipitation method. The ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems) was used for sequencing.

Analyses

Sequences were edited with CLC Main Workbench v5.5 and aligned with Clustal X (Thompson *et al.* 1997). Diversity estimates within localities and sequence divergence values were calculated using DNASP v4 (Rozas *et al.* 2003). TCS (Clement *et al.* 2000) was used to produce a network showing the relationship between haplotypes. Maximum likelihood trees were made with PhyLM 3.0 (Guindon and Gascuel 2003) with a bootstrap analysis of 1000 replications, and using jModelTest v0.1.1 (Posada 2008) to determine the best fit model of nucleotide substitution.

Results

For the COI region, 133 sequences of 661 bp were obtained. For the ITS region, 101 sequences of 777 bp and two sequences of 619 bp (Portugal specimens) were obtained. Due to a limited number of sequences obtained from this area, sequence data for the Chilean and Argentinean samples were combined to represent a South American population. Similarly, sequence data for the USA samples were combined with those for the Canadian specimens to represent a North American population.

Estimates of the COI data showed that the native population of *I. leucospoides ensiger* in North America had greater haplotype diversity than the introduced

populations of *I. leucospoides* in the southern hemisphere (Table 2). Fifteen haplotypes were present in the North American population, compared to one to three haplotypes in the southern hemisphere populations. However, the nucleotide diversity of the Australian and South African population was comparable to that of the North American population (Table 2). Only one mitochondrial haplotype was present in the South American population.

The diversity estimates of the ITS data showed more diversity in the southern hemisphere populations than in the native North American and European samples (Table 2). There was only one ITS haplotype in the North American population, compared to two to three haplotypes in each of the southern hemisphere populations. The two specimens from Portugal were identical in their ITS sequence and represented a haplotype different to those in the other populations.

A haplotype network was constructed to show the relationship between the haplotypes (Fig. 1). The North American haplotypes are clearly separate from the southern hemisphere haplotypes. Only one haplotype was represented in more than one region, namely Australia, South Africa and South America. The haplotypes were divided into four groups based on nucleotide differences evident in the haplotype network and also in the clades emerging from the maximum likelihood tree based on the COI data (Fig 2). The highest level of sequence divergence (2.8 %) was between haplotype groups three and four, while the lowest sequence divergence (0.2 %) was between groups one and two (Table 3).

The maximum likelihood trees for the COI and ITS data were compared to display the four mitochondrial haplotype groups and to show where the samples from these groups were represented in the ITS clades (Fig. 3). Thus, samples residing in haplotype group one and two were present in the two southern hemisphere ITS clades,

and in the sequences that didn't form a definite clade. Samples residing in haplotype group three were present only in the ITS clade that included samples from Australia, South Africa and South America. Samples residing in haplotype group four were present only in the ITS clade including samples from North America. The two samples from Portugal, representing *I. leucospoides leucospoides* resided in a separate clade.

Discussion

Results of this study based on mitochondrial and nuclear DNA markers generally reflect the introduction histories of populations of *I. leucospoides* used in biological control programmes for *S. noctilio* in southern hemisphere countries. This includes data supporting an extensive introduction programme from different continents and the effects of genetic bottlenecks. Evidence of gene flow, possibly reflecting hybridization of the two *I. leucospoides* sub-species is also shown.

The mitochondrial marker showed greater diversity in a native population of *I. leucospoides* as compared to introduced populations. Mitochondrial DNA is subject to strong genetic drift because of its maternal and haploid mode of inheritance (Avise 2000). Consequently, although many mitochondrial haplotypes of *I. leucospoides* could have been introduced from its native range in to the southern hemisphere, only the dominant haplotypes would likely be retained. In addition, the samples from Australia used in this study were only from New South Wales and parasitoids of *S. noctilio* are known to have been sent from Tasmania to Victoria and then released in to New South Wales from Victoria (Taylor 1976, Carnegie *et al.* 2005). It is possible that the introductions of *I. leucospoides* into New South Wales contained only a

portion of the genetic diversity originally introduced into Tasmania and New Zealand. Samples obtained from Tasmania and New Zealand might reveal greater diversity in the introduced range.

Although introduced populations in the southern hemisphere contained only a few mitochondrial haplotypes, the divergence between these haplotypes was deep compared to the native *I. leucospoides ensiger* population. This is shown by the comparable nucleotide diversity between these populations despite having fewer mitochondrial haplotypes in Australia and South Africa (0.00298 and 0.00322 respectively), compared to those in North America (0.00360). The deep divergence between mitochondrial haplotypes in the introduced range reflects the extensive introduction campaign to promote the biological control of *S. noctilio* (Neumann *et al.* 1987). *Ibalia leucospoides* introductions to Australia and New Zealand were from four continents, contained two sub-species and involved hundreds of wasps (Taylor 1967, 1976, Nuttall 1989). The diverse original populations of *I. leucospoides* introduced would be expected to contain divergent mitochondrial sequences due to historic geographic separation of these populations, and this divergence is still represented in the southern hemisphere populations.

The occurrence of a common mitochondrial haplotype in Australia, South Africa and South America reveals the possible origin of *S. noctilio* in South America. If *I. leucospoides* was accidentally introduced into South America together with *S. noctilio* as has been reported (Eskiviski *et al.* 2004), then the source of introduction is most likely Australia, or possibly New Zealand, as *I. leucospoides* has been moved between the two countries. This supports the findings of Slippers *et al.* (2002), where RFLP and nuclear DNA sequence data of the *S. noctilio* fungal symbiont, *A.*

areolatum, showed that *S. noctilio* most likely spread from Australia to South America and later to South Africa.

The presence of three mitochondrial haplotypes in South Africa was surprising given the limited introduction of *I. leucospoides* into this country. Only 18 female *I. leucospoides* were introduced to South Africa from Uruguay (Tribe and Cillié 2004). This indicates that Uruguay has at least three mitochondrial haplotypes of *I. leucospoides*. This is also more than the single haplotype found in the Chilean and Argentinean samples used in this study. *Sirex noctilio* and *I. leucospoides* were first reported in South America in Uruguay (Maderni 1998). It would thus be expected that the genetic diversity of *S. noctilio* and *I. leucospoides* would be higher in this country, compared to subsequently invaded regions. It is also possible that the two mitochondrial haplotypes that are present in Uruguay and not found in Chile and Argentina are present in the latter countries but that they are at low frequencies and were thus not detected in this study.

The ITS rDNA sequences representing a nuclear marker in this study showed greater diversity for *I. leucospoides* in the introduced range than in the native population, where no diversity was present. This is distinctly different from the results obtained using the mitochondrial sequences and could be due to the slower rate of evolution of the nuclear marker (Lin and Danforth 2004). Alternatively, these contradictory results might result from the fact that non-recombinant mitochondrial haplotypes are more easily retained than nuclear haplotypes, which are exposed to homogenization by gene flow (Yang and Kenagy 2009). Either way, the nuclear diversity in the southern hemisphere is expected to reflect the diversity from the different countries of the original populations, while the native population in our study is only represented by one region (eastern Canada). Differing diversity

estimates between data from nuclear and mitochondrial sequences are not uncommon. For example, Yang and Kenagy (2009), in their study on deer mice, reported that only 1.4 % of nuclear microsatellite variation was explained by mitochondrial haplotype identity.

The sequence divergence between the mitochondrial haplotype groups (0.2-2.9 %) falls within that expected for intraspecific variation, but the upper level has also been observed in some cases for interspecific diversity. Cognato (2006) reported intraspecific sequence divergence for Hymenoptera to range between 0.6-4.0 %, and between species divergence for Hymenoptera to range between 1.0-9.6 %. Unfortunately, it was not possible to obtain significant numbers of specimens of *I. leucospoides leucospoides* from Europe and there were none from Asia in this study. These data were thus insufficient to fully explore the presence and distribution of the two *I. leucospoides* sub-species and their hybrids in the introduced range. However, the incongruence between clades in trees drawn from the ITS locus compared to clades from mitochondrial sequence data, shows that there has been gene flow between populations representing even the most divergent mitochondrial clades. These results support the hypothesis that the observed mtDNA diversity is intraspecific, or alternatively represents hybrids, as was suggested previously (Nuttall 1989). Hybridization can result in greater invasion success and population vigour (Facon *et al.* 2005, Fitzpatrick and Shaffer 2007) and it would be interesting to examine the comparative spread of the two sub-species and their hybrids if more specimens of *I. leucospoides leucospoides* become available.

Results of this study illustrate the strong effect that genetic drift can have during bottlenecks imposed by importation and initial quarantine rearing of biological control agents. It also shows why mtDNA markers provide a powerful tool to reflect

demographic changes and relationships in insect populations. Despite the fact that large numbers of *I. leucospoides* were introduced into rearing programmes in Australasia, only five haplotypes were observed in these populations. These are, however, widely divergent, reflecting the wide geographic region from which *I. leucospoides* was introduced. These findings highlight the importance of considering the potential effect of such bottlenecks on the diversity in biological control populations and their potential to adapt to variable pest populations and environments.

Genetic diversity can influence an organism's ability to colonize and adapt to new environments and host types (Roderick and Navajas 2003, Crawford and Whitney 2010). Introduction of further diversity of *I. leucospoides* could be especially important if new strains of *S. noctilio* are introduced and as *S. noctilio* spreads through regions with variable environmental conditions. It is thus important to better understand the genetic diversity of *I. leucospoides*, as this could influence its present and future success.

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Table 1. Collection locations, number of samples and sample codes used in this study, showing mitochondrial haplotype for each location.

Country	Location	No. samples	Sample code	Mitochondrial haplotype/s
South Africa	Cape Town, Western Cape	14	AA, AB, AD	1, 2, 5
	Stellenbosch, Western Cape	1	AC	5
	Mixed locations, Western Cape	13	AE	1, 5
	Knysna, Western Cape	6	AF	1
	Wellington, Western Cape	2	AG	5
	Boston, Kwa-Zulu Natal	4	BA, BB, BC	1, 5
Chile	unknown	1	CA	1
Argentina	El Bolson	15	CB	1
Australia	Greenhills SF, New South Wales (NSW)	19	DA	1, 3
	Margle SF, NSW	2	DB	1
	Riamu, NSW	3	DC	1
	Nowendoc, NSW	1	DD	1
	Pennsylvania, NSW	9	DE	1, 4
	Green Hills, NSW	2	DG	1
USA	Oswego, New York	2	EA	7, 20
	Syracruse, New York	3	EB	6, 7
Canada	Linda work area, Ontario	6	EC, ED	9, 12, 14, 18, 20
	Edward work area, Ontario	6	EE, EF	8, 12, 19, 20
	Hugh work area, Ontario	4	EG, EH	6, 9, 12, 15,
	Sauble, Ontario	5	EI	11, 12, 14, 16
	Sandbanks, Ontario	5	EJ	9, 10, 12, 20
	Midhurst, Ontario	5	EK	12, 17
	Tottenham / Orangeville / Hendrie, Ontario	5	EL	9, 12, 13, 20
Portugal	Moncao	2	P	NA

Table 2. Diversity estimates of *I. leucospoides* for different locations, from the COI and ITS sequence data. The first value given is calculated from the COI data and the second value from the ITS data.

Locality	N	NH	H	S	Pi
Australia	40 / 34	3 / 3	0.512 / 0.599	5 / 4	0.00298 / 0.00152
S. Africa	36 / 19	3 / 2	0.375 / 0.515	8 / 1	0.00322 / 0.00068
S. America	16 / 11	1 / 2	0 / 0.509	0 / 1	0 / 0.00067
N. America	41 / 37	15 / 1	0.855 / 0	14 / 0	0.00360 / 0

Note: There were only two sequences from Portugal which were identical, so these data were not included in the table. N = sample size; NH = number of haplotypes; H = haplotype diversity; S = number of polymorphic sites; Pi = nucleotide diversity

Table 3. Sequence divergence (%) between CO1 haplotype groups.

	Group 1	Group 2	Group 3	Group 4
Group 1	-	0.2	1.1	1.6
Group 2		-	1.5	1.9
Group 3			-	2.8
Group 4				-

Figure 1. Haplotype network showing the relationship between the 20 mitochondrial haplotypes of *I. leucospoides*. The colour of the circle indicates the geographic region where that haplotype is present and the size of the circle indicates the number of samples in each haplotype, as indicated by the scale provided. Solid black circles indicate hypothesized intermediate haplotypes. Lines between haplotypes indicate a one step mutational change. The haplotype number is indicated inside the circles. The haplotype numbers correlate with those in Table 1 and the haplotype groups with those in Fig. 2.

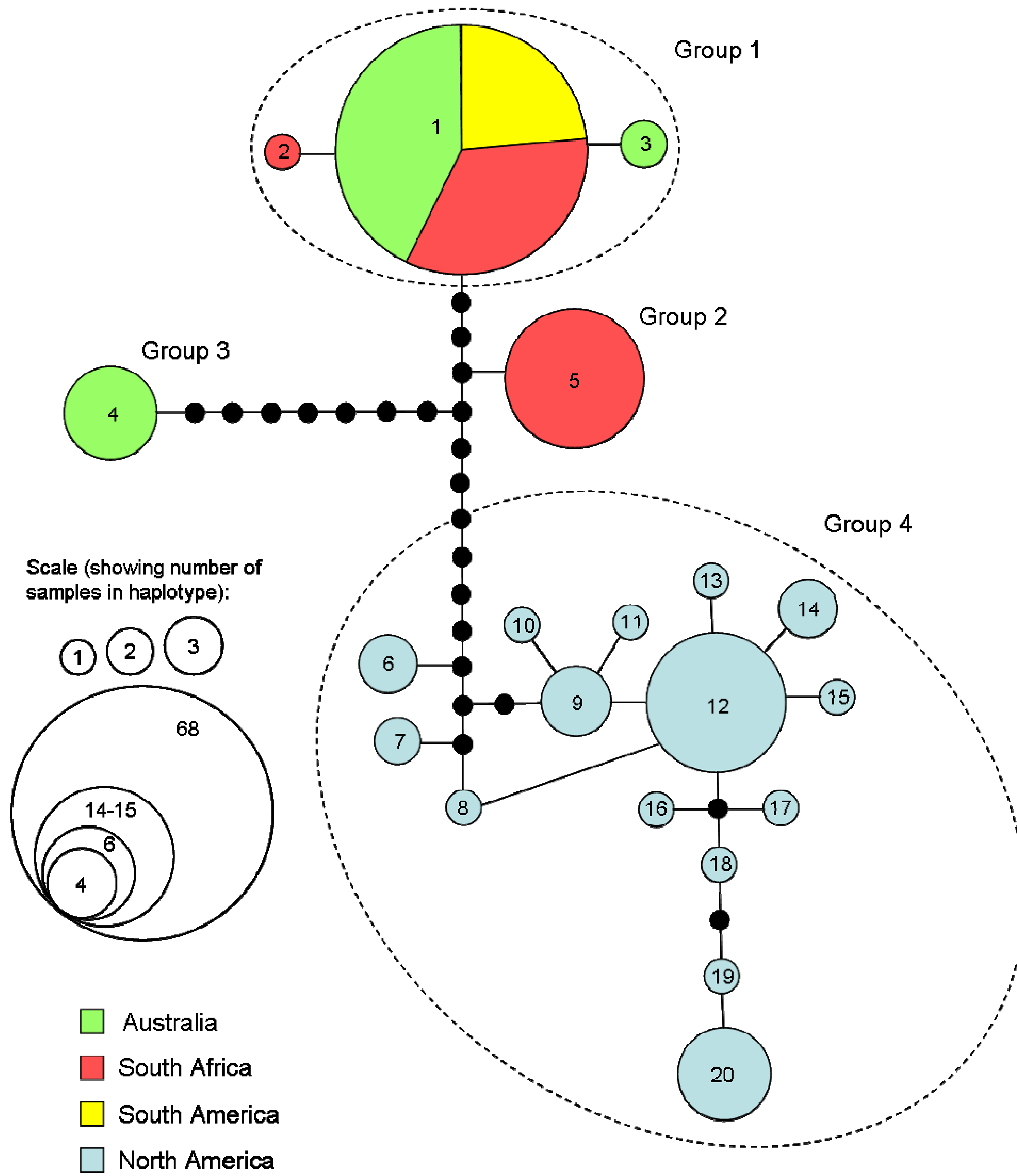
Group 1
1
2
3
Group 2
5
Group 3
4
Group 4
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Scale (showing number of samples in haplotype):
1
2
3
68
14-15
6
4
Australia
South Africa
South America
North America

Figure 2. Maximum likelihood trees for the COI gene region (left) and ITS gene region (right). Mitochondrial haplotype groups from Fig. 1 are shown on both trees. Sample names correlate to those in Table 1. Bootstrap values for major branches are indicated at the nodes.

70
60
87
100
100
Group 1:
Group 2:
Group 3:
Group 4:
0.005
60
Australia
South Africa
South America
South Africa
94
94
Portugal
62
North America
0.0005