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

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Abstract

1. The parasitoid wasp *Ibalia leucospoides* is native to the northern hemisphere and 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 are reported to have hybridized.

2. Despite extensive records of the numbers and origins of the wasps imported into the southern hemisphere, nothing is known regarding their current population diversity. We investigated the genetic variation of *I. leucospoides* in its native and introduced ranges using mitochondrial (COI) and nuclear (ITS) markers.

3. Mitochondrial DNA diversity in the introduced range was limited, with only five haplotypes, but sequence divergence between these haplotypes was high. Similarly, the ITS rDNA sequences revealed multiple clades present in the introduced range.

4. These results reflect introductions from a wide geographical range, but where genetic bottlenecks have possibly reduced the genetic diversity. The data further reflect the origin of the *I. leucospoides* populations in South America and South Africa from New Zealand or Australia. We found no evidence of hybridization between the two sub-species of the parasitoid in its introduced range, and no evidence that *I. leucospoides ensiger* has established outside its native range.

Key words: genetic diversity, hybrid, biological control, parasitoid

Introduction

Ibalia leucospoides Hochenw. (Ibaliidae, Cynipoidea, Hymenoptera) is a parasitoid of siricid woodwasps (Chrystal & Myers, 1928; Spradbery & Kirk, 1978; Norlander *et al.,* 1996). The wasp has been widely used for biological control of the invasive woodwasp *Sirex noctilio* F. in the southern hemisphere. Female *I. leucospoides* utilise the oviposition holes of females to insert eggs into those of the host or its early instar larvae (Chrystal & Myers, 1928). Interestingly, *I. leucospoides* locates its siricid host 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 *S. noctilio* (Neumann *et al.,* 1987; Iede *et al.,* 2000; Carnegie *et al.,* 2005; Hurley *et al.,* 2007; Fischbein & Corley, 2015). *Sirex noctilio* is native to Eurasia, but was accidentally introduced into the southern hemisphere during the course of the twentieth century (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) and China (Li *et al.*, 2015).

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 (Bain *et al.*, 2012; Carnegie & Bashford, 2012; Hurley *et al.,* 2012; Corley *et al.*, 2018). This has prompted the introduction of parasitic nematodes and wasps, including *I. leucospoides*, from the northern hemisphere as biological control agents (Taylor, 1976; Bedding & Iede, 2005; Hurley *et al.,* 2007; Cameron, 2012).

Both subspecies of *I. leucospoides* have been introduced into 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). Both subspecies were reported to interbreed (Nuttall, 1989).

In South America, *I. leucospoides* was reported as naturally introduced with *S. noctilio* and its origin was thus not known (Fischbein & Corley, 2015). 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 & Cillié, 2004). It is not known with certainty which sub-species of *I. leucospoides* was 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 (Liu & Nordlander, 1992; Smith & Schiff, 2002; Long *et al.,* 2009).

Nothing is known regarding the genetic variation within or between populations of *I. leucospoides*. 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 were made. The introduction of *I. leucospoides* from Uruguay to South Africa was based on a very small number of wasps (Tribe & Cillié, 2004), which may have resulted in little genetic variation in this founding 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 samples from the native ranges of *I. leucospoides ensiger* in North America, and *I. leucospoides leucospoides* in Spain and Portugal. 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.

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	5, 6, 7
	Stellenbosch, Western Cape	1	AC	7
	Mixed locations, Western Cape	13	AE	6, 7
	Knysna, Western Cape	6	AF	6
	Wellington, Western Cape	2	AG	7
	Boston, Kwa-Zulu Natal	4	BA, BB, BC	6, 7
Chile	unknown	1	CA	6
Argentina	El Bolson	15	CB	6
Australia	Greenhills SF, New South Wales (NSW)	19	DA	3, 6
	Margle SF, NSW	2	DB	6
	Riamu, NSW	3	DC	6
	Nowendoc, NSW	1	DD	6
	Pennsylvania, NSW	9	DE	1, 6
	Green Hills, NSW	2	DG	6
USA	Oswego, New York	2	EA	11, 24
	Syracruse, New York	3	EB	8, 11
		19	US	9, 10, 11, 13, 22
Canada	Douglastown, Ontario	1	EC	22
	Goderich, Ontario	5	ED	13, 14, 17, 24
	Colborne, Ontario	3	EF	13, 23
	Guelph, Ontario	2	EG	8, 14
	Hanover, Ontario	2	EH	13, 19
	Sauble, Ontario	5	EI	13, 15, 17, 21
	Sandbanks, Ontario	8	EE, EJ	12, 13, 14, 16, 24
	Midhurst, Ontario	5	EK	13, 20
	Tottenham / Orangeville / Hendrie, Ontario	5	EL	13, 14, 18, 24
Portugal	Moncao	2	P	NA
Spain	Galicia	19	SP	2, 4

Materials and Methods

Samples and DNA extraction

Ibalia leucospoides adults were obtained from Australia, South Africa, Argentina and Chile, representng a large proportion 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 *I. leucospoides leucospoides* from Portugal and Spain (Table 1). The majority of wasps were collected between 2006 and 2015 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 (PCR) 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 mitochondrial 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^{TM} 3100 Genetic Analyzer (Applied Biosystems) was used for sequencing.

Statistical analyses

Sequences were edited in CLC Main Workbench v5.5 and aligned with Clustal X (Thompson *et al.,* 1997) and MAFFT v7 (Katoh & Standley, 2014). NETWORK v4.6.1.3 (Brandelt *et al.,* 1999) was applied to the CO1 dataset to produce a network, based on the median-joining algorithm, showing the relationship between haplotypes. Maximum likelihood trees (ML) were made with PhyML v3.1 (Guindon & Gascuel, 2003) with a bootstrap analysis of 1000 replications, and using jModelTest v2.1.3 (Posada, 2008; Darriba *et al.*, 2012) to determine the best-fit model of nucleotide substitution. Maximum parsimony tree (MP) analysis was performed using PAUP v4.0b10 (Swofford, 2002) with a bootstrap analysis of 1000 replications. Tree length (TL), consistency index (CI), rescaled consistency index (RC) and the retention index (RI) were obtained.

Results

For the COI region, 171 sequences of 633 bp were obtained. Of the 633 bp, 31 were parsimony-informative and 602 constant characters. The 90 most parsimonious trees were identified (TL = 41, CI = 0.854, RI = 0.994, and RC = 0.849). For the ITS region, 140 sequences of 676 bp and two sequences of 619 bp (Portugal specimens) were obtained. Seven of the 676 bp were parsimony-informative and the rest constant characters. 1000 most parsimonious trees were identified (TL = 7, CI = 1.00, RI = 1.00,

and RC = 1.00). 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.

24 CO1 haplotypes were obtained from the sequences (Fig. 1). The haplotypes were divided into five groups based on nucleotide differences evident in the haplotype network and from the clades emerging from the maximum likelihood tree based on the COI data (Fig 2). The native population of *I. leucospoides ensiger* in North America (haplotype group 4) contained more CO1 haplotypes than the introduced populations of *I. leucospoides* in the southern hemisphere and the population sampled from the native range of *I. leucospoides leucospoides* (Spain) (Fig. 1). Seventeen haplotypes were present in the North American population, compared to one to three haplotypes in the southern hemisphere and Spain populations. Only one haplotype (haplotype 6) was represented in more than one region, namely in Australia, South Africa and South America. The South American population had the lowest CO1 diversity, with only one haplotype present.

The North American haplotypes, representing *I. leucospoides ensiger*, were clearly distinct from the haplotypes in Spain, representing *I. leucospoides leucospoides*, and the haplotypes in the southern hemisphere, representing introduced populations of *I. leucospoides* (Fig. 1). Sequence divergence was 2.2-4.4% between haplotypes in North America and Spain and 1.7-4.1% between haplotypes in North America and the southern hemisphere. Although there were no shared haplotypes between the southern hemisphere and Spain populations, sequence divergence between these haplotypes was generally low (Fig. 1). Sequence divergence between Spain haplotype 4 and four of the five southern hemisphere haplotypes was 0.3-0.9%. Spain haplotype 2 was further separated from those same southern hemisphere haplotypes, with sequence divergence of 2.1-2.5%. Haplotype 2 was closer to Australia haplotype 1 (sequence divergence 0.8%), which was itself the most distant from the other southern hemisphere haplotypes (1.9-2.4%).
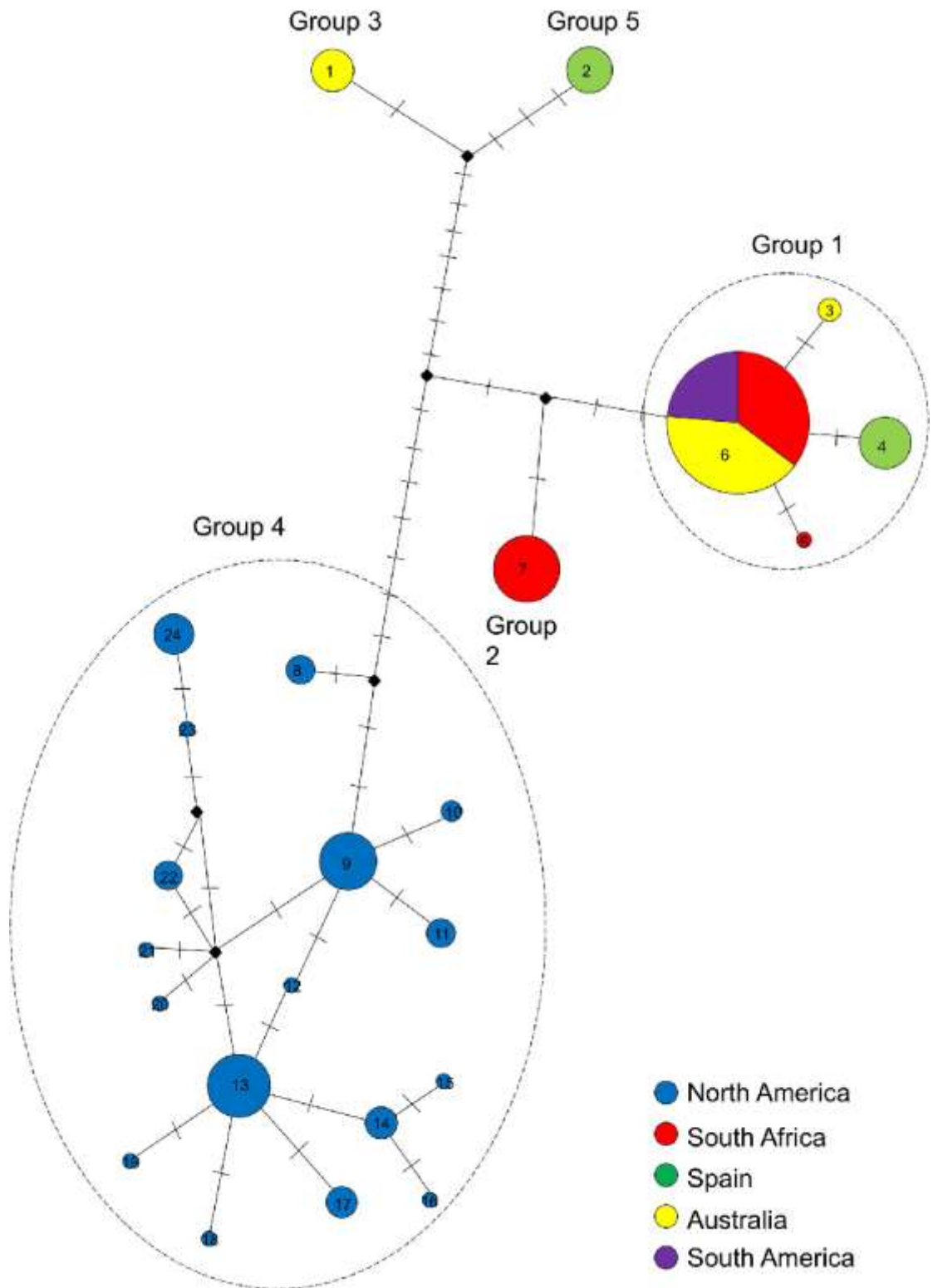
Figure 1. Haplotype network showing the relationship between the 24 mitochondrial haplotypes of *I. leucospoides*. The colour of the circle indicates the geographic region where that haplotype is present and the size of the circle indicates the number of samples in each haplotype. Squares indicate hypothesized 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 Figure 2.
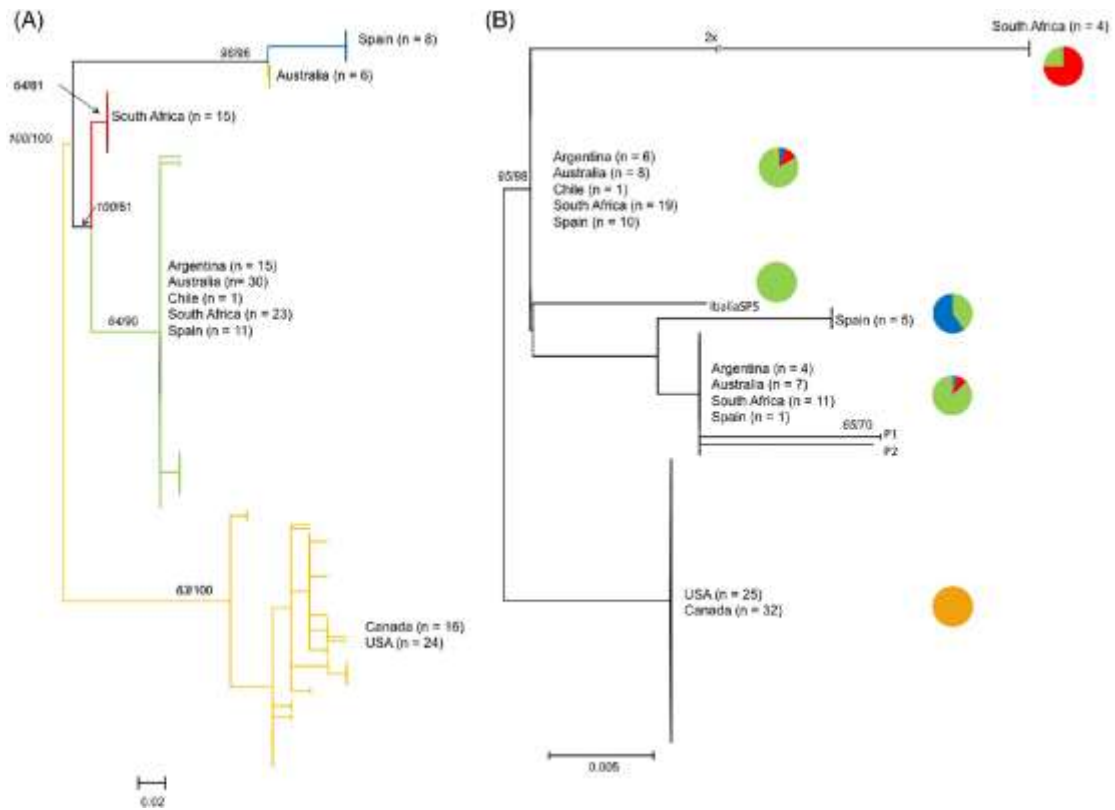
Figure 2. Maximum likelihood trees for the A. COI gene region and B. ITS gene region. The pies indicate the mitochondrial haplotype groups represented in the different ITS clades, where the colours in the pie correspond to the colour of the mitochondrial group. The number of samples in each group / clade are shown in brackets. A 1000 bootstrap replicates were run for statistical support and all the bootstrap values above 70% are indicated for ML (roman) and Maximum Parsimony (italics) at the nodes

The maximum likelihood trees for the COI and ITS data were compared to investigate the representation of the different populations in the clades and to indicate where samples from the five mitochondrial haplotype groups where represented in the ITS clades (Fig. 2). Samples from three of the southern hemisphere CO1 clades (blue, green and red) were represented in more than one ITS clade. This included the ITS clade with only South African samples, the clade with only Spanish samples, the clade with specimens from both the introduced range in the southern hemisphere and the native range (Spain and Portugal), and the group of specimens that did not form a definite clade (including sequences from the southern hemisphere and Spain). Samples residing in haplotype group four, which included all the samples from North America, were present only in one ITS clade.

Discussion

The results of this study revealed some of the long-term outcomes of the introduction history on the populations of a biological control wasp, *Ibalia leucospoides*, used in control programmes for *S. noctilio* in southern hemisphere countries. The data showed that introductions from different regions of the world have led to deep genetic divergences in the introduced populations. Yet, despite fairly large numbers of introductions, the introduced populations had relatively low number of haplotypes from these populations. The results confirmed the establishment of the European subspecies, *I. leucospoides leucospoides*, in the introduced range, but did not confirm the establishment of the North American subspecies, *I. leucospoides ensiger*. Contrary to previous assertions, there was no evidence of hybridization between the two subspecies in the introduced range.

The mitochondrial marker used in this study revealed only five haplotypes in the introduced populations of *I. leucospoides*. Mitochondrial DNA is subject to strong genetic drift due to 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 into the southern hemisphere, only the dominant haplotypes would likely be retained if drift had affected the populations. In addition, the samples from Australia used in this study were only from New South Wales. Parasitoids of *S. noctilio* were originally introduced into Tasmania, and then later from Tasmania to Victoria, and from Victoria to New South Wales (Taylor, 1976; Carnegie *et al.,* 2005). It is consequently likely that the introductions of *I. leucospoides* into New South Wales included only a portion of the genetic diversity originally introduced into Tasmania and New Zealand.

Limited haplotype diversity was expected from samples of *I. leucospoides* collected in South America and South Africa. The natural introduction of the wasp into South America most likely consisted of only a small number of individuals (Fischbein & Corley, 2015). Releases in South Africa were also based on a very small number of wasps (Tribe & Cillié, 2004; Hurley *et al.,* 2007). The limited haplotype diversity from the native range of *I. leucospoides leucospoides* is likely because the samples were from only one site in both Portugal and Spain.

The sequence divergence between the mitochondrial haplotype groups representing North America and the southern hemisphere (1.7-4.1%) fell 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 sequence divergence for Hymenoptera to range between 1.0-9.6%. The sequence divergence between the southern hemisphere haplotypes and the native population of *I. leucospoides leucospoides* (Spain) was generally low (0.3-0.9%) and comparable with the sequence divergence between haplotypes represented by the southern hemisphere samples. This result confirms that the European sub-species *I. leucospoides leucospoides* has established in Australia, South Africa and South America.

Although introduced populations of *I. leucospoides* from the southern hemisphere revealed only a few mitochondrial haplotypes, the divergence between these haplotypes was deep. For example, sequence divergence between haplotype 1 and the other southern hemisphere haplotypes was 2.1-2.5%. The deep divergence between these mitochondrial haplotypes in the introduced range of the wasp is most likely due to the extensive introduction campaign to promote the biological control of *S. noctilio* (Neumann *et al.,* 1987; Cameron, 2012). *Ibalia leucospoides* introductions into Australia and New Zealand were from a wide geographic area 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. This divergence is evidently still represented in the southern hemisphere populations. Similarly, the ITS rDNA sequences, representing nuclear sequence diversity, indicated multiple clades of *I. leucospoides* in the introduced range. This also reflects historical geographic separation of the populations of origin.

The main clades emerging from analyses using the nuclear marker did not correspond with those revealed by the mitochondrial marker. This suggests possible admixture between the populations representing the mitochondrial haplotypes prior to and /or after their introduction into the invasive range of the target host *S. noctilio*. No samples from North America, the native range of *I. leucospoides ensiger,* were represented in the clades comprised of samples from the introduced range. Consequently, our data did not confirm that hybridization has occurred between the two sub-species, *I. leucospoides leucospoides* and *I. leucospoides ensiger* in the introduced range. This is despite the fact that these sub-species were reported to have hybridized in captivity during rearing programs in Australia (Nuttall, 1989).

The occurrence of a common mitochondrial haplotype of *I. leucospoides* in Australia, South Africa and South America indicates 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 suggested (Fischbein & Corley, 2015), then the source of introduction was most likely Australia, or possibly New Zealand. New Zealand populations were not sampled in the present study but *I. leucospoides* were shared between Australia and New Zealand.

An Australia/ New Zealand origin for the *S. noctilio* South American introduction supports the results of previous studies. Slippers *et al.* (2002) used RFLP and nuclear DNA sequence data of the *S. noctilio* fungal symbiont, *A. areolatum*, and showed that *S. noctilio* most likely spread from Australia to South America and later to South Africa. A later study using microsatellites and mitochondrial sequence data of *S. noctilio* suggested more than one introduction of the wasp into South America, but also supported an Australian origin (Boissin *et al.,* 2012). This information is important for any future reintroductions of *I. leucospoides* amongst these three countries to increase genetic diversity within the different populations.

The presence of three mitochondrial haplotypes from the collections of *I. leucospoides* in South Africa was surprising. This is because a very small number of wasps (18 females) were introduced into the country from Uruguay (Tribe & Cillié, 2004). Carnegie & Bashford (2012) reported that *I. leucospoides* was sent from Australia to South Africa to supplement field releases, however those wasps were only used for research purposes and were not released in the field. This suggests that either the South American population has more genetic diversity than was sampled in the present study, or alternatively that the South African population originated from more than the small recorded introduction from Uruguay. The latter possibility would be supported by the finding that *S. noctilio* itself might have been introduced into South Africa more than once (Boissin *et al.,* 2012). Another possibility is that the haplotypes of *I. leucospoides* found in South Africa are present in Uruguay, but not in subsequently invaded areas of Argentina and Chile (Fischbein & Corley, 2015), which were sampled in the present study.

The results of this study demonstrate two contrasting effects that (intentional) human introduction of organisms can have as part of biological control programs. One of these effects is that genetic bottlenecks can arise from collection, transport and captive breeding that increase the impact of genetic drift and potentially increase

inbreeding (Baker *et al.*, 2003). Alternatively, admixture could arise from collections for introductions that are made in divergent areas (Rius & Darling, 2014). These contrasting effects are reflected by the five, but widely divergent, haplotypes of *I. leucospoides* reported in this study from the introduced regions, and that nuclear and mitochondrial lineages were recombined. The findings highlight the importance of considering the potential of such effects on the diversity in biological control populations.

Genetic diversity can influence the ability of organisms to colonize and adapt to new environments and host types (Roderick & Navajas, 2003; Crawford & Whitney, 2010). Consequently, introductions of *I. leucospoides* representing a broader base of genetic diversity in the future could be especially important given the diversity of *S. noctilio* in introduced areas from multiple introductions (Boissin *et al*., 2012). This should also serve to account for the divergent environmental regions in which *S. noctilio* is already established and into which it is gradually spreading (Carnegie & Bashford, 2012). Such a broader base of genetic diversity should include *I. leucospoides ensiger,* which based on samples included in this study, was not confirmed to have established in the introduced range.

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