BIODIVERSITY RESEARCH

Inferring historical introduction pathways with mitochondrial DNA: the case of introduced Argentine ants (*Linepithema humile*) into New Zealand

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ABSTRACT

The threat imposed by invasive species and difficulties associated with control and management places more impetus on trying to prevent their introduction. The identification of introduction pathways is a vital component towards this goal. In this study, we use a genetic marker-based approach to retrospectively investigate the pathway of origin of the invasive Argentine ant (*Linepithema humile*) into New Zealand. We intensively sample the mitochondrial gene cytochrome *b*, from the entire known range of Argentine ants in New Zealand. No genetic variation was found in New Zealand. In order to identify likely introduction pathways, we use two alternative genetic analyses and suggest that a TCS approach that collapses identical haplotypes and calculates the probability of parsimony is superior to standard phylo-genetic tree-building algorithms. A minimum spanning network allowed relationships to be examined among sequences collated from previous international studies. The cytochrome *b* sequence, when compared to a global database, matched that from an Australian population. That Australia is the potential source of Argentine ants is in agreement with the New Zealand interception record, as goods from Australia have the highest number of interception records of Argentine ants. Our approach can easily be duplicated for other organisms and the methodology can be more widely applied to help aid further efforts to identify the routes of transmission for other invasive species and allow us to efficiently direct our biosecurity monitoring effort.

Keywords
Biological invasions, Formicidae, source populations, invasion history, molecular markers, population genetics.

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INTRODUCTION

Given the difficulties and costs of eradicating and controlling invasive species (Myers *et al.*, 2000; Simberloff, 2001), many authors now suggest that a more effective strategy would be to focus on preventive measures, with systems for early detection and rapid response (IUCN, 2000; Leung *et al.*, 2002; Meyerson & Reaser, 2002). A vital component of this prevention is the identification of pathways of introduction into new locations. This information can be used to calculate risk trade-offs and to determine the optimal allocation of limited biosecurity resources to particular trade routes and facilities such as ports and airports. For example, New Zealand's biosecurity authority partially bases its assessment of the risk of uncleared goods on their country of origin (New Zealand Biosecurity Council, 2003). However, reconstructing introduction pathways of species accidentally introduced is generally a difficult task. Introduction events may

occur over large temporal and spatial scales (Puth & Post, 2005) and are characterized at least initially by small population sizes that lack immediately recognizable impacts. Consequently, invasive species often go undetected until some time after their arrival and there is often a lack of relevant records to provide a meaningful resolution of their invasion history. However, recent advances in molecular markers allow us to retrospectively construct individual invasion histories (for example Durka *et al.*, 2005).

The invasive Argentine ant (*Linepithema humile* (Mayr)) has successfully established in many countries around the globe (Suarez *et al.*, 2001; Hartley *et al.*, 2006). Genetic studies on *L. humile* principally employ highly variable microsatellite markers to yield valuable information on inbreeding (Keller & Fournier, 2002), mating frequency (Krieger & Keller, 2000), and small-scale dispersal patterns (Ingram & Gordon, 2003). However, at the global level there have been few systematic attempts to

address larger scale patterns of dispersal events. The allele frequency-based approach required for microsatellites at the global level faces problems given that microsatellites exhibit a high mutation rate and are polymorphic within a nest and colony and novel alleles could potentially arise in introduced populations. Additionally, bottlenecks experienced by introduced populations of Argentine ants (Tsutsui *et al.*, 2000; Tsutsui & Case, 2001) may lead to high divergence rates between the source and the introduced population as shown for other species (Hinten *et al.*, 2003; Pruett & Winker, 2005). These bottlenecks make inferring commonality by descent with microsatellites much more difficult. In such circumstances it may be useful to use a character-state-based approach with a more conserved marker. Character states allow us to maintain information regarding a gene's evolutionary history and a conserved marker will reduce the influence of mutation. DNA sequence data of a mitochondrial gene would fulfil both these requirements. Additionally, mtDNA lacks recombination and is maternally inherited, making it an ideal candidate for investigating the historical spread of ants that require a founding queen. In Argentine ants, the use of mtDNA as a tool for investigating dispersal dynamics was pioneered by Tsutsui *et al.* (2001), who constructed an eight-nation database using the mitochondrial gene, cytochrome *b*. The low mutation rate of cytochrome *b* (Simmons & Weller, 2001) coupled with the recent phenomenon of Argentine ant invasions (the first recorded introduction internationally was in 1882, Suarez *et al.*, 2001) allows us to be confident that genetic correspondence of introduced populations from around the world to those in the native range are associations caused by dispersal between the genetically matching populations.

This study focuses on Argentine ants in New Zealand, providing the most comprehensive sampling regime in a nation to date. We collate previous published Argentine ant sequences and reanalyse the data set using an alternative methodology to that in Tsutsui *et al.* (2001). Such an approach has many benefits; however, caution is required when relying on such a database. As with DNA barcoding, tight procedures for the verification of data would be of benefit, such as the recording of authors, dates, georeference coordinates, and taxonomic verification. In our study we addressed the following questions: (1) How much haplotypic variation is found in New Zealand and around the world, and what does this variation imply as to the number of likely source populations? (2) What is the most applicable technique for analysing the data set? (3) How do the haplotype(s) present in the New Zealand population relate to those found worldwide and how does this relate to colony structure? (4) Do the genetic data and border security interception record border data pinpoint a likely source population(s) of New Zealand Argentine ants?

METHODS

Sampling and genetic analysis

We collected Argentine ants from 14 urban centres around New Zealand in January 2005. Sites were spaced over a 900-km area
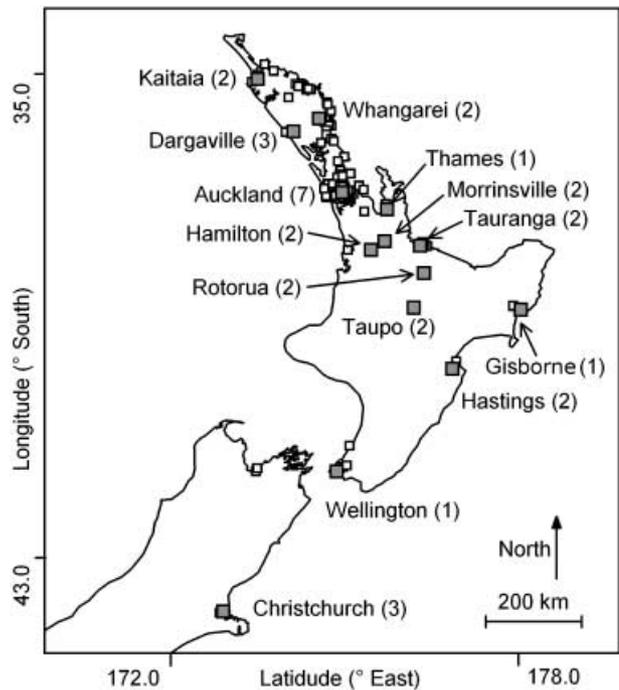

Figure 1 The 14 locations throughout New Zealand from which Argentine ants were collected in January 2005 (grey squares). Numbers in parentheses refer to the number of nests collected from each urban centre. The smaller open squares represent the distribution records for Argentine ants in New Zealand.

and encompassed the longitudinal and latitudinal extremes of the known extent of Argentine ant distribution in New Zealand at that time (Fig. 1). At each location, between one and seven nests were sampled for ants with nests spatially separated by a minimum distance of 500 m. Alternatively, if the infestation was too small to allow this, we either took only one sample or the two nests as far apart as conditions would allow. For the purposes of this study, we consider a nest to be an aggregation of queens, workers, and brood that usually consist of chambers and galleries. These nests might then be organized into colonies, or a series of nests among which aggression was absent. We intensively sampled Auckland as it is the location of the first collection of Argentine ants in New Zealand, the most likely source of other populations in New Zealand (Green, 1990). It is also the most likely to display the highest genetic variation. At each nest, approximately 30 individual workers were collected from the nests and immediately stored in at least 70% ethanol, at 4 °C until genetically analysed. To asses intranest genetic variation, we multiply the sampled three nests (three workers in one nest and two in the other two nests) with one worker genetically analysed in each of the remaining 29 nests. This resulted in a total of 36 DNA sequences being obtained from 32 locations throughout New Zealand.

DNA was extracted from individual Argentine ants using a Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was used to amplify a 805-bp partial sequence (calculated from the honey bee genome accession

number: L06178) from the mitochondrial gene cytochrome *b*, using the primers CB1 and tRs2 (Chiotis *et al.*, 2000). Each 25 µL reaction consisted of 1 µL of template DNA, 0.4 mg mL^{-1} of bovine serum albumin (BSA) 1.5 mM MgCl$_2$, 200 µM of each of the four dNTPs and 0.4 µM of each primer, and 0.2 U of BioTherm DNA Polymerase (GeneCraft, Münster, Germany). PCRs were conducted on a GeneAmp 2700 (Applied Biosystems, Foster City, CA, USA) with a thermal regime of an initial 2-min denaturing step at 94 °C followed by 35 cycles of 25 s at 94 °C, 25 s at 42 °C, 90 s at 72 °C, and a final extension step of 90 s at 72 °C.

PCR products were purified using High Pure PCR product Purification columns (Roche, Basel, Switzerland) and then sequenced directly using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI 3730 Genetic Analyser. After manual editing, cytochrome *b* sequences were aligned using the CLUSTALW algorithm (Thompson *et al.*, 1997). Haplotype diversity was calculated with DNASP version 4.10.3 (Rozas *et al.*, 2003) and genetic distances were calculated in MEGA version 3.1 (Kumar *et al.*, 2004) using the Tamura–Nei model (Tamura & Nei, 1993) to correct for unequal base composition.

Comparison of methods for analysis

The software packages MEGA version 3.1 (Kumar *et al.*, 2004) and TCS version 1.21 (Clement *et al.*, 2000) were used for analysis. MEGA implements standard phylogenetic tree building algorithms such as neighbour-joining, maximum parsimony, and minimum evolution. Alternatively, TCS collapses identical haplotypes and calculates the probability of parsimony (Clement *et al.*, 2000). Based on a statistical assessment of mutations, minimum connections inferring genealogical relationships are made in TCS using a 95% confidence assessment based on the conditional probability of the change of more than one nucleotide at a particular site (Crandall *et al.*, 2006). Identical sequences from New Zealand were collapsed into a single sequence for analysis in TCS to prevent the biased sampling regime causing statistical artefacts in the results. Note that the network analysis merely only displays difference between haplotypes. The lines between haplotypes do not infer a pathway history for invasions, but rather show mutational differences likely to have arisen over in evolution over tens of thousands of years.

We compared the results obtained from each method. Explicitly, this analysis consisted of determining their ability to distinguish valid mutational differences among samples and haplotypes. We also used both forms of analysis to visualize relationships between identical haplotypes from the database and how these were arranged spatially across the globe.

Source of Argentine ants into New Zealand

New Zealand's border security agencies have maintained records of Argentine ant interceptions at both New Zealand airports and maritime ports. Information was gathered and analysed from two discrete time periods, 1966 to 1982 (Richardson, 1979; Keall, 1981; Townsend, 1984) and 1997 to 2004 (Ministry of Agriculture and Forestry, unpublished data). A positive interception was recorded for every ant identified as *L. humile* (formerly *Iridomyrmex humilis* (Mayr)). There were in total 37 recorded interceptions. These data were then collated to determine the countries that have been recorded as the source for Argentine ants transported into New Zealand, and the frequency at which these interceptions have occurred among countries. Further detail on this data set is given by Lester (2005) and Ward *et al.* (2006).

RESULTS

Haplotype variation within New Zealand and around the world

The 36 DNA sequences obtained from 32 locations throughout New Zealand were found to be identical among the 720 bp of readable sequence (GenBank accession number EF363097). That New Zealand has only one haplotype present could indicate that one Argentine ant population colonized New Zealand, though we cannot determine the number of introduction events from this source location. We collapsed the New Zealand sequences into one and reduced it to 405 bp in length to match previously published sequences (Chiotis *et al.*, 2000; Tsutsui *et al.*, 2001) or *L. humile* from other locations around the world. These sequences were then pooled and analysed collectively ($n = 29$).

From the pooled data we found a significant A-T skew in nucleotide base composition ($f_A = 32.8$, $f_T = 39.7$, $f_C = 17.8$ & $f_G = 9.7$) that was corrected for using the Tamura–Nei model. Overall, haplotype diversity was high [$h = 0.92$ ($n = 29$ SD = 0.001)] with a total of 18 haplotypes, differing among 31 variable nucleotide sites. Nucleotide diversity (the mean sequence divergence among haplotypes, ð) among all individuals was 2.1% ($n = 29$ SE = 0.5%). In contrast to New Zealand, multiple haplotypes have been found within a nest in both the introduced range [average genetic distance = 0.5% ($n = 2$) Sweetwater Reservoir California] and the native range [average genetic distance = 3.3% ($n = 3$ SE = 0.7%) Ocampo Argentina].

Comparison of methods for analysis

A phylogenetic tree-based analysis generally under-represented the differences among groups, and tended to cluster samples together despite genetic differences (Fig. 2). For example, South Africa (SA1) clusters identically with New Zealand despite the presence of a single mutational difference. Network analysis, using a method designed for population-level data (Clement *et al.*, 2000), revealed much finer scale differences with only identical samples clustering together (Fig. 3). Additionally, the relationships displayed in the network analysis were significant ($P < 0.05$), with network topology remaining stable up to a level of 98% statistical parsimony. Given that traditional phylogenetic tree-building methods require relatively large levels of variation, it has been suggested that such an analysis may only elucidate broad genetic patterns (Hoffman & Blouin, 2003) and may make invalid assumptions at the population level (Clement *et al.*, 2000). We suggest that the network analysis presented in Fig. 3 provides a more useful basis for the purpose of identifying source

Figure 2 MEGA neighbour-joining phylogeny depicting the relationship between native and introduced populations of the Argentine ant using 405 bp of the mitochondrial gene, cytochrome *b*. Locations in uppercase refer to ants from the native range, lowercase the introduced range. Location numbers in parentheses refer to ants from the same geographical location and the same colony; location numbers not in parentheses refer to ants from the same geographical location but from a different colony. An asterisk at any location denotes ants from the same geographical location from which behavioural data required to assign them to a colony are lacking.

populations, and we use this form of analysis to examine the potential site of introduction into New Zealand.

Relationships among sampled populations and the link with colony structure

The TCS statistical parsimony network shows that there appears to be enough resolution in the sampled mitochondrial DNA region to distinguish among a variety of populations around the globe with 95% confidence (Fig. 3). Another aspect clearly illustrated in Fig. 3 is that each introduced population does not necessarily have a matching haplotype in the native range. Given the recent nature of introductions of Argentine ants, this result is likely due to inadequate sampling of the genetic diversity present in the native range.

Many of the sampled populations have been tested behaviourally to determine colony structure. These tests are commonly thought in many ant species to indicate the genetic relationship between members of the same colony (those that are not aggressive) and those from different colonies (those that are aggressive) (e.g. Abbott *et al.*, 2007). In our study, behaviourally aggressive colonies

periodically clustered together that shared the same haplotype, or behaviourally non-aggressive colonies were separately clustered. For example, members of the large Californian colony that are non-aggressive among one another, and represented genetically by the locations La Jolla and Los Angeles (Tsutsui *et al.*, 2001), do not cluster together on the haplotype network (Fig. 3). In fact, Los Angeles clusters with a nearby location, which is not part of the colony — Lake Skinner. Alternatively, clear splits in haplotypes are found between some behaviourally aggressive colonies of *L. humile*, for example, Hawaii, which can be divided into two separate colonies (Tsutsui *et al.*, 2001). In this case the colonies designated by behavioural work cluster independently with a large genetic distance between the two (Fig. 3).

We also used this information to infer the source of an introduced population of Argentine ants in varying locations. In South Africa and Australia we can distinguish two clear haplotypes. In the case of South Africa, those sampled in Betty's Bay and those sampled from the rest of South Africa differ by 10 nucleotides. In Australia, 18 differing nucleotide sites distinguish samples taken from Victoria and those from Perth. This result
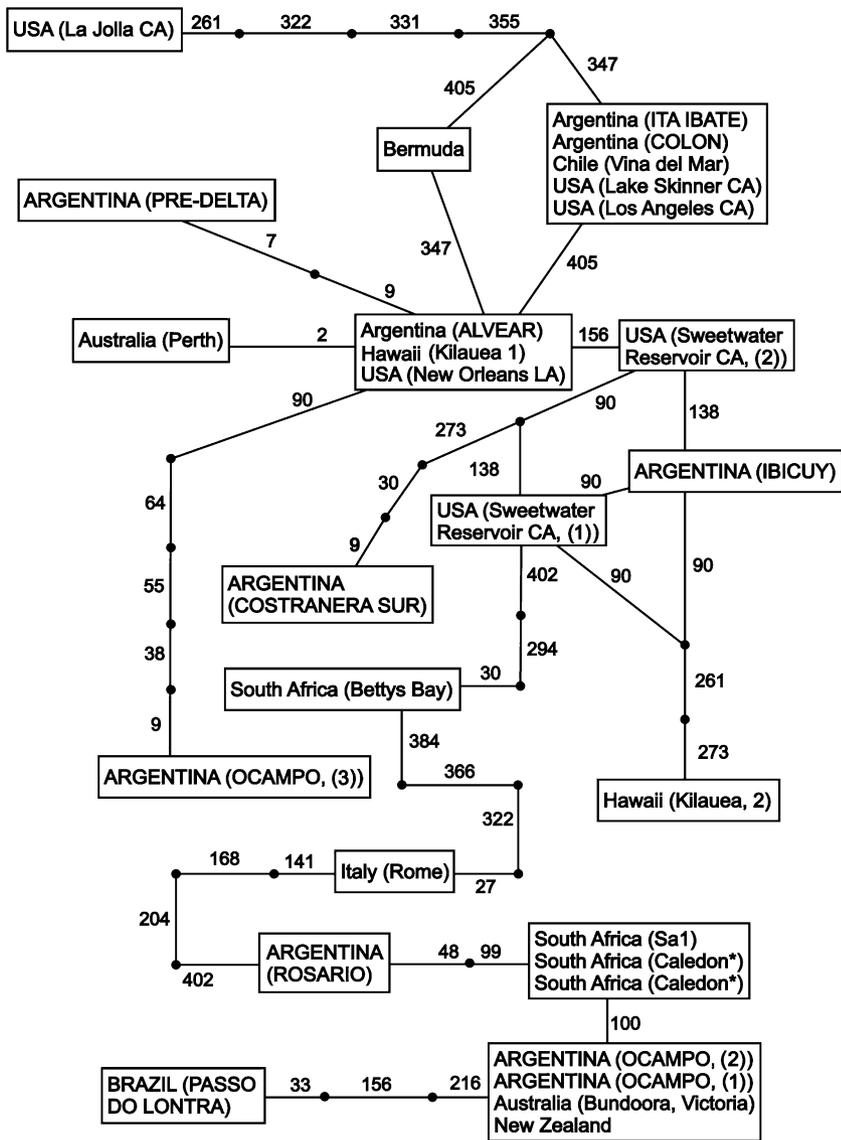
Figure 3 TCS statistical parsimony network analysis depicting the relationship between native and introduced populations of the Argentine ant using 405 bp of the mitochondrial gene, cytochrome *b*. Locations surrounded by a box denotes those that share a common haplotype. These are separated by lines with the numbers representing parsimonious mutational differences and their locations on the sequence profile. The outgroup has been removed for clarity and ease of reading. Locations in uppercase refer to samples taken from the native range, lower case the introduced range. Location numbers in parentheses refer to ants from the same geographical location and the same colony; location numbers not in parentheses refer to ants from the same geographical location but from a different colony. An asterisk at any location denotes ants from the same geographical location from which behavioural data required to assign them to a colony are lacking.

could indicate at least two separate introduction events from different source populations in both cases.

Source of Argentine ants in New Zealand

The New Zealand populations of Argentine ants are genetically identical to sequences obtained from a nest in Ocampo Argentina and a nest in eastern Australia (Fig. 3). This result suggests that both the eastern Australian and the New Zealand populations share a common source population, most similar to that found in Ocampo Argentina. We also augmented our genetic data with border interception data. These data unsurprisingly shows that New Zealand's closest neighbour Australia has accounted for the majority of Argentine ant interceptions (38% of all recorded intercepts) (Fig. 4). Though other countries have been responsible for interceptions, the only other locale pinpointed through genetic means (Argentina) has not been recorded in the known New Zealand interception record.

DISCUSSION

This study investigated the use of a mitochondrial DNA marker for inferring the source populations of an invasive ant in introduced populations from nine nations. We found that a network analysis with a population-based program was a more informative approach than a phylogenetic tree-based method for using cytochrome *b* as a genetic marker to investigate large-scale dispersal dynamics. From the available sequence data our findings suggest that Argentine ant populations in New Zealand originated from eastern Australia.

Haplotype variation within New Zealand and around the world

To our knowledge, this sampling of New Zealand's unicolonial population represents the first study to look in detail at genetic variation within a colony. Previously, the maximum number of
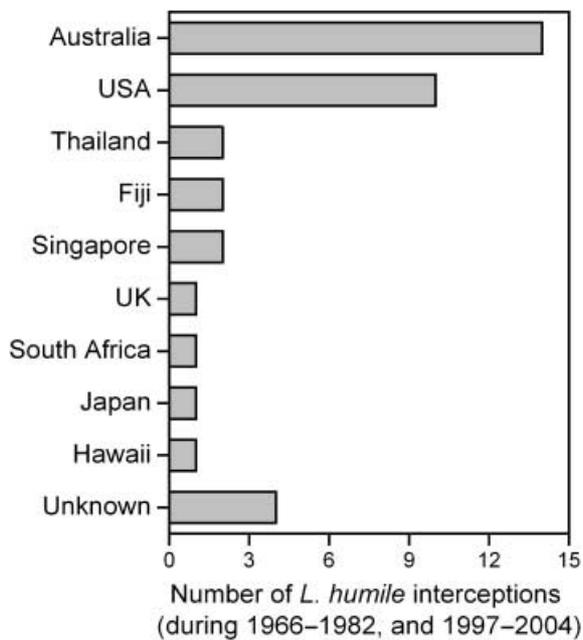
Figure 4 A summary of the Argentine ant interception record for New Zealand. The data were combined from two discrete time periods, 1966 to 1982 (Richardson, 1979; Keall, 1981; Townsend, 1984) and 1997 to 2004 (Ministry of Agriculture and Fisheries, unpublished data).

ants sampled from a single nest was three (Tsutsui *et al.*, 2001). Therefore, prior work has relied on the assumption that there is no or little meaningful mitochondrial variation among nests in a colony. Our finding of a single haplotype across an entire colony supports the applicability of such an assumption. It also suggests that, at least within New Zealand, there is only one mitochondrial cytochrome *b* haplotype, which is homogeneous throughout a nest. Our reanalysis has shown that mitochondrial variation is sometimes found within both a nest and a colony. This suggests that further efforts are required to determine the amount of mitochondrial variability within nests and/or colonies to accurately determine the extent of genetic variation before we can confidently infer commonality through shared haplotypes.

Comparison of methods for analysis

Our results indicate that the use of a standard phylogenetic construction technique such as the neighbour-joining tree-building algorithm may ignore much of the valuable variation at the population level (Posada & Crandall, 2001). Another problem with such a method is that it will treat all populations as the tips of evolutionary branches, though in actual fact persisting populations that have given rise to others and should in fact be represented as internal nodes on a tree. The analysis of intraspecific phylogenies, characterized by low genetic divergence may benefit from the use of a network approach in comparison to that of a bifurcating tree (Posada & Crandall, 2001). Where the purpose is to investigate these population level differences and applying

them to the question of determining source populations we believe that a network approach provides the better choice.

Relationships among sampled populations and the link with colony structure

Tsutsui *et al.* (2001) found nuclear microsatellite data to indicate Rosario, Argentina, as the most likely source of many introduced populations. Using mitochondrial data, our results indicate that there is no specific source population that accounts for the majority of introductions. The majority of the possible sources are clustered in the north-eastern region of Argentina; a finding not dissimilar to fire ants, with the suspected origin of the US population likely to be from northern Argentina (Mescher *et al.*, 2003). The lack of congruence between mitochondrial DNA and nuclear DNA is not an unusual finding and has been reported by a number of investigators who have used both nuclear and mitochondrial markers to investigate population structuring in ants (Ross *et al.*, 1997; Doums *et al.*, 2002; Gyllenstrand & Seppä, 2003; Sanetra & Crozier, 2003). Such genetic differences may occur in organisms with sedentary females and dispersing males (Avise, 1994). Only the female mitochondrial information will be passed to following generations, whereas the nuclear genome is wrought from both female and male genetic material. In polygynous Argentine ants, females have limited dispersal, losing their wings after mating and either remain in the nest or bud off from an existing nest travelling a short distance away (Newell & Barber, 1913; Suarez *et al.*, 2001; Heller *et al.*, 2006), whereas winged males are free to disperse further distances. Additionally, there is evidence to suggest that substantial local gene flow is mediated by males (and so far not female sexuals) (Passera & Keller, 1994; Krieger & Keller, 2000; though see Pedersen *et al.*, 2006).

Given this disparity, it is important to remember that the mitochondrial genome allows us to follow matriarchal lineages, an important fact as queens are required to found new introduced populations. Therefore, we suggest that more weight should be placed on mitochondrial DNA for introduction pathway analysis.

Numerous studies have suggested that behaviour is influenced by genetic relatedness (as measured by nuclear microsatellites) in Argentine ants, with more related ants forming non-aggressive colonies and exhibiting aggressiveness against less-related ants (Suarez *et al.*, 1999; Tsutsui *et al.*, 2001, 2003). If this were the case, we would expect to see colonies clustering together genetically irrespective of geographical location, a pattern which we found mixed support for. This result is not surprising, given the importance environmental factors such as diet can have on recognition interactions among Argentine ants, as shown in laboratory experiments (Liang & Silverman, 2000; Liang *et al.*, 2001; Silverman & Liang, 2001; Corin, 2007). Perhaps a more encompassing hypothesis would be that both genetic and environmental factors influence kinship recognition, as ants constantly use their resident colony environment to form a fluid recognition template. Under such a hypothesis we might be able to explain the lack of aggression in some parts of the native range (Heller, 2004), as too much variation forming too wide a template for

other ants to be always recognized as non-kin. Inklings of this hypothesis were raised by Tsutsui *et al.* (2003), who noted that less genetically diverse nests were often the instigators of aggressive interactions. Furthermore, we might be able to use this hypothesis to explain why aggressive interactions are so polarized (Tsutsui *et al.*, 2003) in the introduced range where low genetic diversity is the norm.

Finally, it is important to note that the lack of matching between some introduced populations and any native population suggests inadequate sampling in the native range.

Source of Argentine ants in New Zealand

Both the genetic and the interception record data implicate eastern Australia as the most likely source of Argentine ants into New Zealand. Though the genetic sample was obtained from Victoria, Argentine ants in this area form part of the greater Melbourne supercolony (Suhr, 2004), making it a possibility that the introduction occurred somewhere from this colony, rather than specifically Victoria. Interestingly, Suhr (2004) noted that the Australian interception record contained a high number of interceptions from New Zealand. This is far from surprising given the close proximity and trade flows between the two countries. Furthermore, Australia has had a longer history of Argentine ants [first located in Australia in 1939 (Jenkins, 1961)] as compared to New Zealand, with the initial discovery in 1990 (Green, 1990), making it more likely that *L. humile* has come from Australia to New Zealand rather than vice versa.

Interestingly, the interception record registered samples from Fiji, Singapore, and Thailand. Currently, there is no known Argentine population established in either of these countries and climatic modelling suggests that in some of these localities Argentine ants may be unable to establish (Hartley *et al.*, 2006). This result raises two possibilities. First, ephemeral populations might mediate the spread from one location to another. Second, Fiji and other countries might play a part as transit sites through which many alien species pass.

CONCLUSIONS

Given the importance of trying to prevent the spread of unwanted species around the world, growing emphasis will be placed on identifying introduction pathways. Our study has shown that genetic methods can help us retrospectively infer such pathways. Our results suggest that a variation in the mitochondrial marker cytochrome *b* provided sufficient signal to allow a tcs analysis to define distinct haplotypes of Argentine ants around the world (with 95% confidence). Such a methodology can be more widely applied to help aid our identification of routes of transmission of invasive species and allow us to efficiently direct our biosecurity monitoring effort.

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