

Introduction Pathway Analysis Into New Zealand Highlights a Source Population ‘Hotspot’ in the Native Range of the Red Imported Fire Ant (*Solenopsis invicta*)

by

Steve E. Corin¹, Peter A. Ritchie² & Philip J. Lester²

ABSTRACT

Threats posed by invasive species and the difficulties associated with their control and management places impetus on trying to prevent their spread. The identification of introduction pathways is a vital component towards this goal. We use a genetic marker-based approach to retrospectively investigate the pathway of origin of the invasive red imported fire ant (*Solenopsis invicta* Buren) into New Zealand. In addition we determine colony structure by analysing the nuclear locus *GP-9*. The mitochondrial gene cytochrome oxidase subunit I was sampled, from known incipient nests of *S. invicta* in New Zealand. A polygyne and a monogyne nest was found. No genetic variation was found within any of the nests, but there was a high level of divergence between the nests suggesting separate incursion events and source populations. We combined both species level and population level genetic analyses in an attempt to identify likely introduction pathways. Our source population approach indicated that was no single introduction pathway for this invasive ant in New Zealand that can be specifically targeted by biosecurity authorities. We compared our sequences information to available international studies and found that despite high levels of divergence, geographically the sequences cluster very closely together within their native range. The sequence data suggested that the New Zealand nests ultimately originated in north east and north central Argentina, a finding mirrored in China and possibly the United States.

Keywords: biological invasions, Formicidae, source populations, invasion history, molecular markers

¹ERMA New Zealand, PO Box 131, Wellington, New Zealand

²School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand.

*Author to whom correspondence should be addressed (email: steven.corin@erमानz.govt.nz,

INTRODUCTION

There is a growing understanding that we need to shift the focus away from simply managing invasive species to predicting which species will become invasive and preventing them from spreading (Leung *et al.* 2002). The red imported fire ant (*Solenopsis invicta* Buren) is an important invasive species that can have major ecological effects (Porter & Savignano 1990; Wojcik *et al.* 2001; Allen *et al.* 2004), be extremely costly to the economy (Lard *et al.* 2002), and place human life at risk (deShazo *et al.* 1999). It has a wide native distribution in South America, which encompasses southern Brazil, Paraguay, Uruguay, Bolivia and northern Argentina (Buren *et al.* 1974; Pitts 2002). *Solenopsis invicta* has recently spread across many parts of the globe, including the West Indies (Davis *et al.* 2001), Puerto Rico (Callcott & Collins 1996), Taiwan (Chen *et al.* 2006), Australia (Natrass & Vanderwoude 2001) and North America, where the ant has spread prodigiously despite the best efforts of quarantine regulations (Callcott & Collins 1996). This ant has been observed in New Zealand on three occasions (2001, 2004 and 2006) all from unknown sources (Harris 2001; Pascoe 2002). Here, we attempt to reconstruct the invasion pathways of *S. invicta* in New Zealand.

Reconstructing introduction pathways of species accidentally introduced is a difficult task. Introduction events may occur over large temporal and spatial scales (Puth & Post 2005) and are characterized by small population sizes and lack of immediately recognizable impacts. Consequently, invasive species often go undetected for significant time periods after arrival. Henshaw *et al.* (2005) used microsatellite data to compare a number of North and South American populations to those in Australia. Here, we rely on mitochondrial DNA (mtDNA) instead of nuclear DNA to elucidate these relationships. The major benefit of mtDNA is that there is no male contribution. In *S. invicta* males appear to mediate gene flow in the native and introduced range (even between the two different social forms) partly homogenizing the genetic differences amongst nests and therefore losing much of the nuclear genetic signal (Ross & Shoemaker 1993; Ross *et al.* 2007; Shoemaker & Ross 1996). mtDNA is maternally inherited and therefore an ideal candidate for investigating the historical spread of ants that require a founding queen and mtDNA has been

successfully used to ascertain the likely source population of Argentine ants in New Zealand (Corin *et al.* 2007).

Solenopsis invicta nests may be monogyne (nests with a single reproductive queen) or polygyne (nests with multiple reproductive queens). The two social forms differ in many respects including their modes of dispersal (Banks *et al.* 1973; Vargo & Porter 1989) and the density at which nests are present (Porter *et al.* 1991). The identification of social form is essential for control programs to be tailored for maximum effect. Molecular techniques are now available for the determination of social form of *S. invicta*, using specific variants of the gene *GP-9* (Krieger & Ross 2002). *GP-9* has two main alleles, *B* and *b*, of which the monogyne social form is exclusively *B* and the polygyne form, is *Bb* (Ross & Keller 1998; Krieger & Ross 2002). An observation that different social forms of *S. invicta* were present in New Zealand would also provide evidence for multiple, rather than a single, invasion event.

We conducted genetic analyses on collected *S. invicta* samples from all of New Zealand's established nests to: (a), identify their social form with the locus *GP-9* analyses (Krieger & Ross 2002); (b), determine the relatedness between nests found in New Zealand; and (c), determine the relationship of New Zealand nests to those from samples gathered around the world and thereby try to determine the source population of New Zealand nests using the mtDNA gene cytochrome oxidase subunit I (COI).

MATERIALS AND METHODS

Sample collections

There have been three occurrences in New Zealand where *S. invicta* has been observed outside quarantine conditions (Fig. 1.). The 2001 infestation was a single mature nest located at Auckland International Airport that appeared to have been present for at least 9 months but no more than 2 years (Pascoe 2002). After its identification the nest was treated and no additional nests have since been found. This nest was represented only by a number of dried samples, from which we attempted to extract DNA from 3 workers. None of these extractions proved successful. The second detected nest was found in 2004 at the Port of Napier in Napier. The nest is represented by a small number of dried samples from which we extracted DNA from one worker. The third and latest (2006) established nest was found in Whirinaki on a forestry

site near a major highway. Workers were collected from this nest, preserved in 95% ethanol and stored at 4°C until analysis. We extracted DNA from 2 worker ants from this nest. New Zealand's biosecurity agency, the Ministry of Agriculture and Fisheries (MAF), provided all the samples of worker(s) from each of these nests. DNA was extracted using a modified 5% w/v chelex resin solution extraction protocol (Sepp *et al.* 1994).

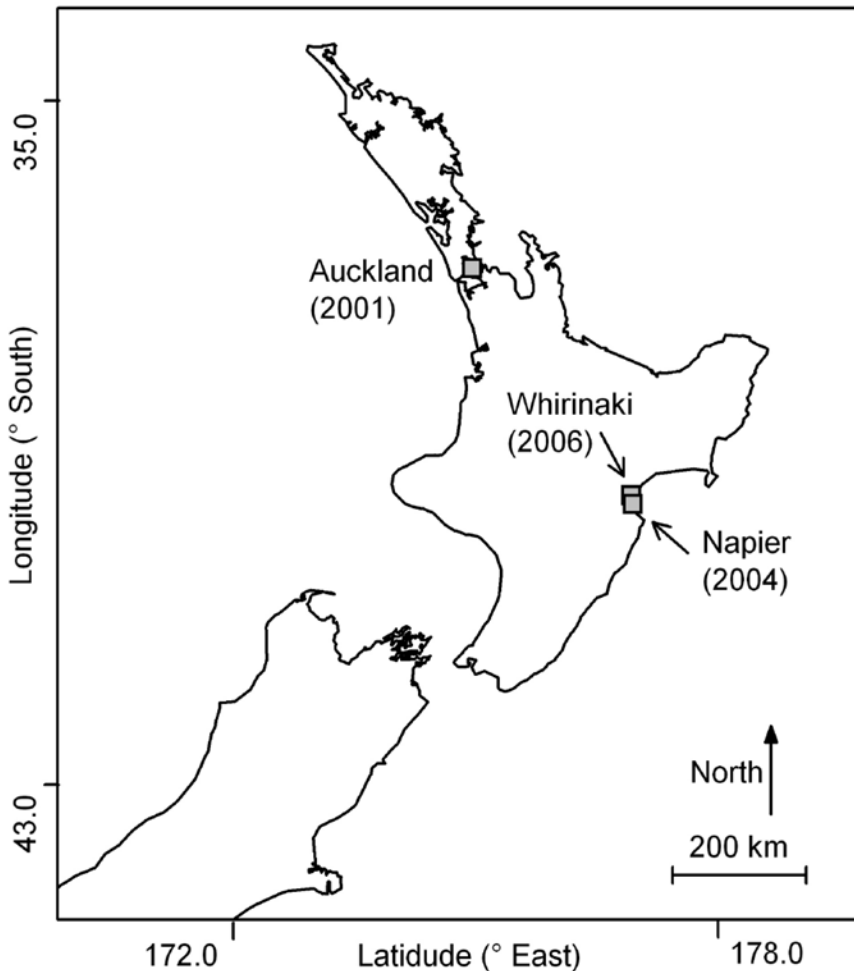


Fig.1. Map of New Zealand depicting the three known *S. invicta* infestations and their year of detection.

Genetic analysis of social form

A single gene (named general protein-9 (*Gp-9*)) has been implicated in coding for odorant binding proteins, which serve as the basis of nest social behaviour in *S. invicta* (Krieger & Ross 2002). There are two allele families so far identified (*B*, *b*), with monogyne queens having a *Gp-9^{Bb}* genotype, and polygyne queens having a *Gp-9^{Bb}* genotype (*Gp-9^{bb}* individuals die due to a lethal recessive allele). We followed the methods of Krieger & Ross (2002), using the primers *Gp-9_169F* and *Gp-9_490R* to amplify part of the *GP-9* coding region under a PCR amplification protocol of an initial 1 minute denaturing step at 92°C followed by 35 cycles of 20 seconds at 92°C, 30 seconds at 56°C, 1 minute at 72°C and a final extension step of 5 minutes at 72°C on a Eppendorf2700 thermocycler (Applied Biosystems). These products were then digested for 3 hours at 37°C with the enzyme *Bsa*I, and visualized on an agarose gel stained with ethidium bromide under UV light. The *B* alleles yield two fragments (545 and 283 bp), whereas the *b*-like alleles yield three (428, 283, and 117 bp). Samples were then scored as either monogyne or polygyne based on the alleles present.

Genetic analysis of nest relatedness

PCR was used to amplify a ~950 bp sequence to obtain a partial sequence of the mitochondrial gene cytochrome oxidase subunit I (COI) as well as the coding regions for tRNA_{LEU} and a small section of the gene cytochrome oxidase subunit II (COII). 0.4 µM of each of the primers C1-J-2195 and DDS-COII-4 (Ahrens *et al.* 2005) were combined in a total PCR reaction of 25 µl with 1 µl of template DNA, 0.4 mg/mL of bovine serum albumin (BSA), 1.5 mM MgCl₂, 200 µM of each of the four dNTP's and 0.2 Unit of BioTherm DNA Polymerase (GeneCraft). PCR's were conducted on an Eppendorf2700 (Applied Biosystems) with a thermal regime consisting of an initial 1 minute denaturing step at 94°C, followed by 35 cycles of 30 seconds at 94°C, 1 minute at 47°C, 2 minutes at 68°C and a final extension step of 5 minutes at 72°C. PCR products were purified using High Pure PCR product Purification columns (Roche) and were sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI 3730 Genetic Analyzer.

The sequences were manually edited, combined and trimmed to 785 base pairs in order to match the 101 available *S. invicta* sequences on Genbank

(DQ831670-DQ831672; Ross & Shoemaker 2005; Shoemaker *et al.* 2006) and the outgroup *S. interrupta* (Shoemaker *et al.* 2006) included. This total data set of 104 sequences were then aligned using the ClustalW algorithm (Thompson *et al.* 1997) and analysed.

Basic statistics and genetic distances amongst samples were gathered using the LogDet nucleotide correction in the software package MEGA v3.1 (Kumar *et al.* 2004). As previously suggested (Corin *et al.* 2007) we used the population level software TCS v1.21 (Clement *et al.* 2000) to elucidate relationships amongst the sequences. In this study TCS failed to distinguish differences among haplotypes, even those widely divergent. Instead TCS identified all haplotypes as outgroups. Given this failure, we instead opted for a mixed analysis, using standard phylogenetic tree building algorithms to identify the larger relationships (i.e. which immediate clade the New Zealand samples clustered with) and then attempted to use TCS to investigate the small levels of divergence between these haplotypes within the clade. For our phylogenetic tree we used FINDMODEL (Tao 2005) to determine the best model of nucleotide substitution. Both a similar study (Shoemaker *et al.* 2006) and the results of FINDMODEL suggested we use the HKY model +I + Γ distances. This analysis was implemented in the package SplitsTree 4.6 (Huson & Bryant 2006). Similar runs were conducted using maximum likelihood in the package PHYML v2.4.4 (Guindon & Gascuel 2003) and N-J and MP algorithms in MEGA v3.1 (Kumar *et al.* 2004), these methods all produced the same results, though we show only the SplitsTree network.

RESULTS

Genetic analysis of social form

Both polygyne and monogyne nests were detected in New Zealand on the basis of our *GP-9* assay. The two workers from the 2006 nest were both clearly polygyne and the 2004 nest was monogyne. Queens in polygyne nests carry both the *B* and *b* allele and can give rise to monogyne nests, though not vice versa (Ross & Keller 1998). This is important for two reasons; surveillance methods for detecting any residual nests from this population need to be wide enough to detect a monogyne nest dispersed by wing yet intensive enough to pick up nests spread by budding. Secondly, the polygyne 2006 nest is unlikely to be directly related to the monogyne 2004 incursion.

Genetic analysis of nest relatedness

We found a large skew in nucleotide frequencies in the mitochondrial DNA ($f_A = 31.4, f_T = 37.6, f_C = 18.9$ & $f_G = 12.1$). Many other insects including ants also appear to have an A + T bias (Simon *et al.* 1994; Smith *et al.* 2005). In order to correct for any influence this nucleotide bias might have on our results all genetic distances cited are LogDet corrected. In addition to this skew in nucleotides there was a particularly high level of variability amongst the sequences with, out of the 785bp, there being 169 variable sites, 110 of which were parsimonially informative. This variation is evident upon comparing the average genetic distance between haplotypes which is 4.26% (SE = 0.5%). These mutations were predominantly transitions, which were 5.7 times more common than transversions. As identified by Shoemaker *et al.* (2006) the *S. invicta* sequences can be divided into 7 clades (Fig. 2.), with levels of genetic distance commonly associated with different species (Table 1).

Table 1. Average genetic distance (as a percentage) between each of the seven *S. invicta* clades and their outgroup.

	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Outgroup
Clade 1	3.4	6.7	7.3	6.9	6.5	6.6	8.9
Clade 2		5.8	6.5	6.1	6.1	6.0	7.8
Clade 3			3.1	4.1	3.5	3.8	8.0
Clade 4				4.5	3.9	4.0	8.9
Clade 5					3.5	3.5	8.9
Clade 6						2.5	9.1
Clade 7							8.9

In New Zealand there were no sequence differences within the 2006 nest (Genbank accession number EU290756). We were unable to test for divergence within the 2004 nest, as we were only able to extract DNA from one individual but given the nest was monogyne or descended from a single queen we would not expect there to be any divergence (Genbank accession number EU290757). We were unable to retrieve mtDNA from the older 2001 nest. Relatedness between the 2004 and 2006 nests was very low, with a genetic distance of 5.98% (SE = 0.8%). This is an amount of genetic divergence typical of that between two species.

Source population identification

Both New Zealand sequences cluster into separate clades (Fig. 2.). Each different clade was on the same branch as a separate clades from China. As noted by Shoemaker *et al.* (2006) each of the seven clades contain samples that come from geographically similar regions across the native range. This strong relationship gives us confidence that we can identify the native origin of the nests that have been detected in New Zealand. Both New Zealand samples cluster with samples obtained from Argentina. The 2006 nest sequences fall into Clade 2, from north-central Argentina, the 2004 nest sequence clusters with Clade 4, which generally contains samples from north-eastern Argentina. Our statistical parsimony network analysis refined these relationships. By looking only at closely related sequences we found that the 2006 nest sequences match those from Santa Fe, Argentina. The sequence is also very similar, separated by only one mutation, to six other sequences, five of which are from Corrientes in Argentina and the last also from Santa Fe (Fig. 3.). The sequence obtained from the 2004 New Zealand nest did not exactly match any from the native range but was, however, closely related to a number of sequences found in Formosa, Argentina (Fig. 3.). Interestingly, both these clades contain haplotypes found in the introduced population in China. The only additional Chinese sequence clustered within Clade 3, which contains a range of samples from north-central Argentina (Fig. 2.). We found the strength of

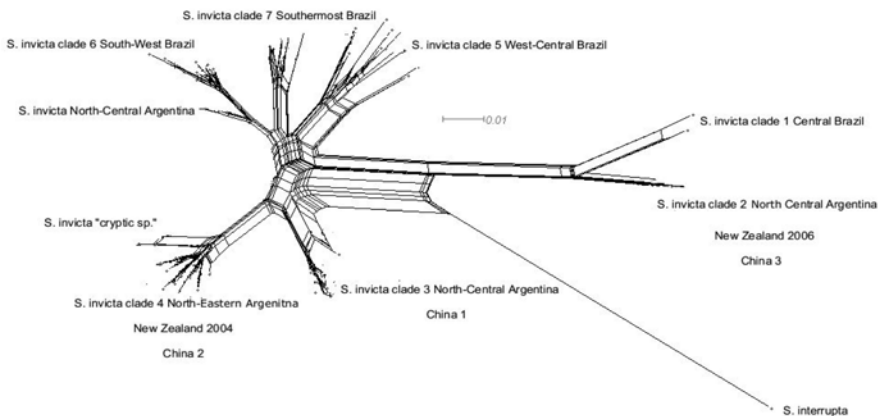


Fig. 2. Non-rooted SplitsTree network depicting the relationships amongst *S. invicta* clades. The seven *S. invicta* clades are labelled along with the predominant location of the samples (n=103). Scale equals genetic distance

this geographical relationship surprising. All 5 introduced populations, from New Zealand and China, appeared to originate within the native range from either north-eastern or north-central Argentina, despite *S. invicta* having a large native range, encompassing southern Brazil, Paraguay, Uruguay, Bolivia and Argentina.

DISCUSSION

A number of recent studies suggest that New Zealand is potentially at risk from successful invasion by *S. invicta* (Morrison *et al.* 2004; Lester 2005). Given this theoretical possibility and the presence of a number of nests previously found in New Zealand, it is important that efforts are undertaken to prevent further nests from establishing. One way to proactively prevent this is to determine if these past nests were related and to try and locate the source population of these nests. We found that these nests did not share the same social form. In addition, the nests previously detected are not closely related and are likely to be from different populations. However, we did find

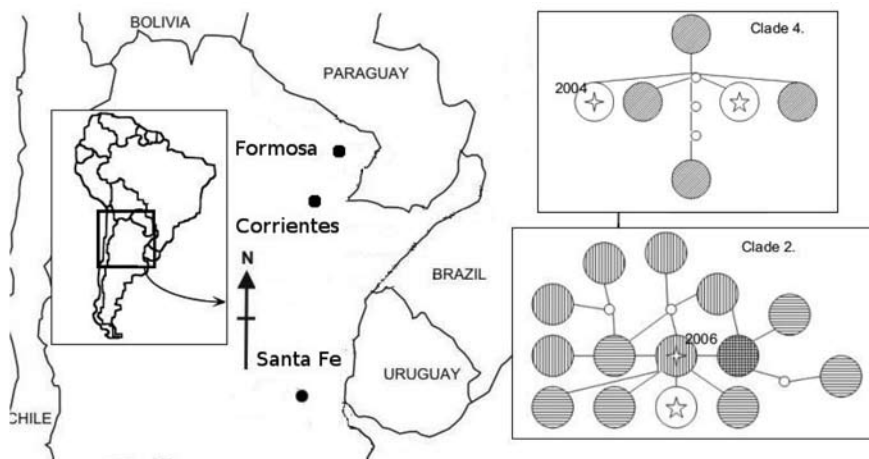


Fig. 3. Map of Argentina and TCS statistical parsimony network analyses. Those sequences most immediately related to New Zealand sequences (as determined from Fig. 2. and undisplayed phylogenetic trees) are analysed in a network analysis to circumvent problems associated with high divergence levels. These two separate analyses are shown in each box labelled Clade 4. and Clade 2. Filled circles each represent a haplotype, which are interconnected by lines representing mutations. Diagonal hatching refers to haplotypes from Formosa, Argentina, vertical hatching Santa Fe, Argentina and horizontal hatching Corrientes, Argentina. Sequences from New Zealand are represented with four pointed stars, and Chinese sequences with five pointed stars.

evidence to suggest that these populations share similar geographic origins from the native range with there being links to the north-eastern or north-central region of Argentina.

Both polygyne and monogyne nests of *S. invicta* were observed in New Zealand. These are unlikely to be directly related, given that the polygyne nest was observed 2 years after the monogyne colony. For management purposes, it is important to quickly identify the social form of any *S. invicta* invasive populations or nests. There are a number of differences between the two social forms that influence the management protocols needed to be put in place to eradicate or control a *S. invicta* infestation. For example dispersal is achieved in different ways with polygyne nests budding, whereby an inseminated queen walks or forages for a new nest a number of metres away with a pre-existing worker stock in tow (Vargo & Porter 1989). In the case of monogyne nests, queens will disperse on the wing alone, find a suitable nesting spot, shed their wings and start to lay the eggs that will develop into the worker population. Although most monogyne queens probably nest within 5 km of their parent mound (Vogt *et al.* 2000), dispersal includes a period of flight so there is greater potential for them to spread further when conditions are windy, for example distances of 8 - 32km have been recorded (Markin *et al.* 1971; Banks *et al.* 1973; Wojcik 1983). The two different social forms are also found nesting at significantly different densities (Porter *et al.* 1991), meaning that their potential impacts differ, which necessitates different methods of control and bait application.

Another important reason to genetically test social form is to determine the risk of establishment. Previous work suggests that the establishment risk of the red imported fire ant in NZ is low and that much of New Zealand is unsuitable habitat due to its cold temperatures (Morrison *et al.* 2004; Lester 2005). One of the important differences between the two social forms is the size of their nests. Polygyne nests are generally larger than monogyne, which could provide them with two benefits in the New Zealand environment. Their larger size may buffer them from starvation in the winter months (Kaspari & Vargo 1995) and the additional density of ants may provide more metabolic heat and raise nest temperatures, protecting them against the cold (as has been found in termites, Korb & Linsenmair 2000). We do not know how long each of the nests have been present in New Zealand, but from the

size of the polygyne nest it would appear to have been present for at least two years (surviving two winters)(Gunawardana & Sarty 2006). Overall, we suggest that polygyne nests are much more of a threat to New Zealand's biosecurity given the density of their nests, their greater ability to do harmful damage, their potential ability to sire both polygyne and monogyne nests, the presence of multiple queens and the close association of diffuse nests in the polygyne social form that may allow this social form to occupy habitats in which monogyne forms may not persist.

Our mitochondrial DNA analysis indicated that at least two of the three *S. invicta* nests in New Zealand were not related, with wide sequence divergence between the two sequenced nests. From our search of available mtDNA databases of *S. invicta*, the New Zealand incursions were genetically similar to two separate populations in China. This result does not necessarily imply that the incursions originated in China, as there is currently limited worldwide mtDNA sampling of *S. invicta*. Once more sampling has been undertaken, this method will become more useful in the identification of invasion pathways. This result seems not uncommon, both Australia (Henshaw *et al.* 2005) and China (Fig. 2.) have had multiple introductions of different strains of *S. invicta*. Our source population approach indicated that there is no single introduction pathway that can be specifically targeted by biosecurity authorities for this invasive ant in New Zealand. Instead, these interceptions indicate distinct incursions of both forms of *S. invicta*, which in this case does little to aid biosecurity monitoring.

Our analysis suggests that the ultimate native source population of the New Zealand 2006 nest is the Santa Fe or Corrientes region of Argentina. In addition, the 2004 nest was traced to the Formosa region of Argentina. Previous studies corroborate these findings. Populations in the United States population are believed by some to have originated from the Southern half of their native range (but most probably north-eastern Argentina, Mescher *et al.* 2003). That *S. invicta* populations in their global introduced range come from such a small area is surprising, given that their native range encompasses southern Brazil, Paraguay, Uruguay, Bolivia and north-eastern Argentina (Buren *et al.* 1974; Pitts 2002). Likewise, our results of work with Argentine ants also suggests that their source populations cluster around north-eastern Argentina (Corin *et al.* 2007). *Solenopsis richteri* Forel is another damaging

ant that also likely originated from northern Argentina or southern Brazil. These results suggest that the north-eastern region of Argentina has disproportionately sourced a number of invasive populations around the world.

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