Geographical origin of an introduced insect pest, *Listronotus bonariensis* (Kuschel), determined by RAPD analysis

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The Argentine stem weevil, *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae), is an important introduced pasture pest in New Zealand. In this study geographical populations of this species were analysed using polymerase chain reaction-based randomly amplified polymorphic DNA (RAPD), in an attempt to determine the geographical origin of the pest. Morphologically indistinguishable individuals were collected from nine South American, five New Zealand and one Australian populations. Ten primers were screened for usefulness, two of which revealed significant, scorable polymorphisms between these populations. The results indicated that the sampled New Zealand *L. bonariensis* populations originated from the east coast of South America.

Keywords: genetic variation, introduced species, *Listronotus bonariensis*, populations, RAPD.

Introduction

The Argentine stem weevil *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae) is an introduced pest (Goldson & Emberson, 1980) attacking improved graminaceous pasture throughout New Zealand (Prestidge *et al.*, 1991). The damage caused by this pest is estimated to range from \$NZ 78–251 million annually (Prestidge *et al.*, 1991). Native to South America, *L. bonariensis* is thought to have been accidentally introduced to New Zealand and Australia; it was first reported in New Zealand in 1927 (Marshall, 1937) and, with the advent of pastoral grazing, quickly achieved pest status.

Elucidation of genetic variation in geographical populations can be an important aspect of pest studies (Kambhampati *et al.*, 1990; Roehrdanz & Johnson, 1988), providing insight into the geographical origin of colonized populations (Kambhampati *et al.*, 1991). Within the native habitat, the extent of genetic variation between geographical populations depends on several factors, including gene flow between populations and time since separation (Hartl, 1980; Templeton *et al.*, 1990). Genetic differences within the introduced population can be the result of the genetic variation in the founder population, the number of

founding events and selection pressure (Baker & Stebbins, 1965).

It is probable that *L. bonariensis* arrived in New Zealand (and Australia) less than 100 years ago, and given that 2–3 generations a year are possible (Kelsey, 1958) only about 200–250 generations can have elapsed since its establishment. Given that the New Zealand environment resembles that of South America, it is thought that selection pressures in the native and colonized habitats would be comparable. That, together with the short time since introduction, suggests genetic similarity between the introduced population and the population of origin might be high. However, founder effect, random genetic drift and/or recurrent introductions could have altered the colonized populations' genetic make-up.

In order to define the origin of pest populations it is necessary to demonstrate genetic similarities between colonized and potential source populations. This type of study can use numerous techniques including phenotypic variation, allozyme data, DNA data (restriction fragment length polymorphisms, sequence analysis), morphometrical analysis, or behavioural differences. With small insects, one common difficulty encountered with DNA analysis is lack of material. In this study, this problem was overcome using polymerase chain reaction-based randomly amplified polymorphic DNA (RAPDs) (Welsh & McClelland, 1990; Williams, *et al.*, 1990).

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This relatively new technique offers advantages with respect to the amount of material needed and the fact that nothing need be known about the genomic organization of the organism. The technique has generated useful results in the identification of unique individuals (Smith *et al.*, 1992), detection of genetic variability (Chalmers *et al.*, 1992; Landry *et al.*, 1993), race identification (Crowhurst *et al.*, 1991) and generation of linkage maps (Paran *et al.*, 1991). The technique is becoming widely used (see Hedrick 1992 for a review of various current applications), and a review of potential applications has been given by Hadrys *et al.* (1992).

Materials and methods

Sample collection

Adult *L. bonariensis* were collected from nine climatically diverse localities in South America between September 1989 and February 1990 (Fig. 1). Methods of collection and transportation have been discussed elsewhere (Goldson *et al.*, 1990). Samples were then frozen at -100°C until use. Adult weevils were also collected from five localities in New Zealand in January and April 1992 (Fig. 2) and one locality in Australia (Flaxley, South Australia) in December 1991.

Fig. 1 South American *Listronotus bonariensis* collection sites.

DNA isolation

DNA was isolated from individual weevils. Each weevil was ground under liquid nitrogen and incubated in isolation buffer (8.5 mM Tris pH 8, 25 mM EDTA pH 8, 0.5 per cent SDS, 25 mM β -mercaptoethanol and 0.5 mg ml^{-1} Proteinase K) at 50°C for 1 h with occasional inverting. Following phenol:chloroform washes, the DNA was precipitated with 100 per cent ethanol and washed with 70 per cent ice-cold ethanol, then air dried and suspended in TE (10 mM Tris pH 8.0, 0.1 mM EDTA) containing $25 \text{ } \mu\text{g ml}^{-1}$ RNase A. The samples were incubated at 37°C for 30 min, then diluted with $690 \text{ } \mu\text{l H}_2\text{O}$. $5 \text{ } \mu\text{l}$ of this DNA (approximately 30 ng) was used as a template for RAPD reactions. Each weevil yielded enough DNA for approximately 145 RAPD reactions.

Primer selection

The primers used were 10-bp oligonucleotides of random sequence (Oligos Etc., Inc., Gullford, Connecticut). Ten primers were initially screened using pooled samples of DNA from each population, as follows. $20 \text{ } \mu\text{l}$ of DNA from each individual weevil within each population were pooled. $5 \text{ } \mu\text{l}$ (30 ng) were

Fig. 2 New Zealand *Listronotus bonariensis* collection sites.

then taken from each pooled sample as templates for the RAPD reaction. This resulted in one reaction for each population and made it possible to select useful primers. Primers were designated as useful if they generated few, distinct bands which showed polymorphisms between populations. Primers that generated many bands (> 15 per sample) were designated as unscorable and not pursued. Of the 10 primers initially screened, two were selected as potentially useful. These were primer 267/2 (5'-ACATAGACGG-3') and primer GT09 (5'-TCTGCCGTGA-3').

RAPD reactions and electrophoresis

RAPD reactions were carried out based on the methods developed by Williams *et al.* (1990) and Welsh and McClelland (1990). The 25- μ l reactions contained 0.4 mM dNTPs, 1x Taq polymerase buffer, 1 unit Taq polymerase, 30 ng DNA and either primer 267/2 (200 nM) or primer GT09 (600 nM). Each reaction was overlaid with a drop of silicon oil.

The reaction profile was 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min. The samples were then held at 72°C for 5 min. Amplifications were carried out on a HybaidTM thermal reactor and were all carried out on the same thermocycler to eliminate the possibility of machine to machine variation. Ali-

quots (5 μ l) of the amplification products were then electrophoresed at 65 V for 2 h through 1.4 per cent SeaKemTM LE Agarose in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 0.25 μ g ml⁻¹ ethidium bromide and visualized using UV light.

Based on this protocol, the DNA from 12 individuals from each of the nine populations (except Brazil, in which case six individuals were used) was amplified by the two useful primers. This resulted in a banding pattern for each individual and also resulted in a population profile for each population. A negative control (without template DNA) was included in each set of reactions amplified. The same sample amplified with the same primer and under the same reaction conditions in different tubes on the same day or on different days resulted in the same bands (not shown). Amplification profiles were similar to those of DNA extracted from individual *L. bonariensis* wing muscles (not shown).

Scoring of bands

Only distinct bands were included in the data set. Each individual was scored for the presence (1) or absence (0) of every such band. This was done by initially scoring against a molecular weight marker (BRL 1 kb ladder) and assigning tentative identification numbers

Fig. 3 Population profiles generated by primer GT09. The amplifications and electrophoresis were carried out as described in Materials and methods. In each lane are the amplification products of one individual from each of the labelled populations. The lanes marked (-) are the negative controls. The molecular weight marker (MW) is the BRL 1 kb ladder. Population profiles from (a) Ruakura, (b) Uruguay, (c) Lincoln, and (d) Bariloche.

Fig. 4 Population profiles generated by primer 267/2. The amplification and electrophoresis were carried out as described in Materials and methods. In each lane are the amplification products of one individual from each of the labelled populations. The lanes marked (–) are the negative controls. The molecular weight marker (MW) is the BRL 1 kb ladder. Population profiles from (a) Ruakura, (b) Uruguay, (c) Lincoln, and (d) Bariloche.

to each band based on approximate molecular weight. These band identification numbers were verified by running an aliquot of representative individuals from each gel that contained the band of interest side by side on a subsequent gel (verification gels not shown). Bands that ran at the same molecular weight were scored as the same band.

Data analysis

The presence/absence data matrix was analysed using the SAS procedure DISCRIM (Parker, 1987) (similar to Kambhampati *et al.*, 1992). This procedure generated ordered canonical discriminant functions for the variables (bands). The means of three canonical discriminant functions for each of the sampled populations were plotted. The first two canonical discriminant functions were selected as the *x* and *y* axes. The third canonical discriminant function was selected as the *z* axis, perpendicular to the plane of the page, and is represented in Fig. 5 by relative size of the letters representing the plotted values.

The data were also analysed by cluster analysis and principal coordinate analysis to verify that the results did not depend on the type of analysis used. Similarity matrices were calculated from the data on band presence or absence for each individual. Genetic distance or similarity were determined by Jacquard similarity, genetic distance (Gower, 1985), band sharing (Hedrick, 1985, and references therein), and

Nei and Li band sharing (Nei & Li, 1979), again to provide cross-checks on interpretation. These individual similarity coefficients were reduced to population similarities by averaging individuals within populations. The similarity coefficients presented in Table 1 used the band-sharing measure of similarity (Hedrick, 1985, and references therein). The Nei and Li measure of band sharing was calculated from population frequencies to give the similarity between two populations. This measure was determined in two ways, using positive frequencies only, and using both positive and negative frequencies.

Dendrograms were constructed from the similarity matrices by hierarchical cluster analysis with an average link option using the GENSTAT program (Genstat 5 committee, 1987). The similarity matrices were also analysed in Genstat by principal coordinate analysis to generate a simpler display of the relationship between populations than the dendrogram.

Results

Primer GT09 generated 28 bands and primer 267/2 generated 34 bands from the 174 *L. bonariensis* studied. The number of bands generated for each individual varied from 1 to 12. All distinct bands were included in the analysis.

Primer GT09 resulted in one prominent band of approximately 800 bp in all the New Zealand populations, along with many other bands detectable at

Fig. 5 A 3D distribution of sampled *Listrionotus bonariensis* populations generated by the SAS procedure DISCRIM. The abbreviations used are: AS (Australia), BA (Buenos Aires), BB (Bahia Blanca), BL (Bariloche), BR (Brazil), G (Gore), K (Ruakura), L (Lincoln), LS (La Serena), P (Reporoa), RN (Rio Negro), SC (South Chile), U (Uruguay), W (Wellsford), Z (Mendoza).

various molecular weights (e.g. Fig. 3, a and c). The Australian population also generated a similar banding pattern (not shown). Conversely, the South American populations generated quite diverse population profiles (e.g. Fig. 3, b and d). Using GT09, there was an apparent similarity between the New Zealand, the Australian and the Uruguayan profiles (Fig. 3, a and c, compared to b). The Buenos Aires profile was also similar to these (not shown).

Primer 267/2 resulted in two prominent bands (1600 bp and 700 bp) in the New Zealand (Fig. 4, a and c) and Australian (not shown) populations. Other fragments were also generated, some individual-specific and others present in several individuals. Again, the South American population profiles were quite distinct from one another (e.g. Fig. 4, b and d). As with primer GT09, primer 267/2 generated population profiles for the Uruguay and Buenos Aires populations, which closely resembled the New Zealand and Australian profiles (Fig. 4, a and c, compared to b).

The mean canonical variate scores for each population from the discriminant analysis (using the SAS procedure DISCRIM) of the presence/absence data matrix are plotted in Fig. 5. The mean scores for the New Zealand and Australian populations clustered in the vicinity of the means for the Uruguay and Buenos Aires populations (Fig. 5), supporting the initial impression based on visual examination of the gels. The population from Gore was slightly displaced from this cluster. In terms of the third canonical discriminant function (the z axis)

Buenos Aires was slightly displaced from the New Zealand/Australia/Uruguay grouping. South Chile was split from the rest of the sampled populations by canonical discriminant function 2 and Bariloche was split from the rest of the populations by canonical discriminant functions 1 and 2.

The population similarity matrix calculated by reducing the full shared fragment similarity matrix between individuals is given in Table 1. The New Zealand and Australian populations are most similar to each other and the Uruguayan population and to a slightly lesser degree to the Buenos Aires population. On the leading diagonal are values for within population similarity, which range from 0.388 to 0.647. Four of the six introduced populations (Lincoln, Ruakura, Wellsford and Australia) exhibit a higher degree of within population similarity than the native populations. This reduction in intra-population variability is consistent with some sort of genetic 'bottleneck', such as a colonization event.

An example of the dendrograms generated using the band frequencies for each population is given in Fig. 6. All five populations from New Zealand, the Australian population and Uruguay and Buenos Aires cluster as a distinct and separate group. This cluster was observed in all five dendrograms generated, with a minor amount of branch swapping noted. The clustering of the South American populations in some cases reflected the geographical distance separating the populations. For example, the Buenos Aires and Uruguay populations

Table 1 Similarity matrix generated by shared fragments as described in Materials and methods. The abbreviations used are: L (Lincoln), W (Wellsford), K (Ruakura), P (Reporoa), G (Gore), AS (Australia), BA (Bahia Blanca), BB (Bariolche), BR (Brazil), LS (La Serena), RN (Rio Negro), SC (South Chile), U (Uruguay), Z (Mendoza)

	L	W	K	P	G	AS	BA	BB	BL	BR	LS	RN	SC	U	Z
Lincoln	0.647														
Wellsford	0.634	0.624													
Ruakura	0.617	0.592	0.647												
Reporoa	0.504	0.488	0.493	0.411											
Gore	0.485	0.448	0.465	0.395	0.489										
Australia	0.508	0.525	0.484	0.413	0.449	0.577									
Buenos Aires	0.436	0.446	0.453	0.378	0.402	0.438	0.404								
Bahia Blanca	0.389	0.341	0.367	0.321	0.388	0.360	0.358	0.435							
Bariolche	0.338	0.338	0.295	0.283	0.282	0.308	0.293	0.229	0.537						
Brazil	0.373	0.361	0.352	0.323	0.316	0.382	0.346	0.319	0.264	0.406					
La Serena	0.357	0.332	0.398	0.324	0.372	0.350	0.323	0.293	0.273	0.255	0.476				
Rio Negro	0.424	0.361	0.408	0.363	0.383	0.372	0.377	0.426	0.327	0.380	0.291	0.474			
South Chile	0.313	0.305	0.342	0.291	0.359	0.341	0.339	0.328	0.238	0.349	0.330	0.327	0.400		
Uruguay	0.552	0.549	0.538	0.437	0.426	0.513	0.430	0.347	0.303	0.390	0.334	0.387	0.328	0.488	
Mendoza	0.340	0.327	0.366	0.297	0.332	0.339	0.352	0.305	0.362	0.300	0.311	0.372	0.299	0.347	0.388

Fig. 6 Dendrogram of *Listrionotus bonariensis* populations generated by average linkage cluster analysis using Nei and Li similarity based on shared fragments. For this dendrogram, only positive matches were counted.

cluster together and are only about 60 km apart. The populations from Bahia Blanca and Rio Negro also cluster together and are less than 100 km apart. There also were clusters of populations that were separated by fairly large distances. For example, the population from Brazil clustered with the population from South Chile.

Discussion

The bands generated using the RAPD technique are clearly genetic markers, albeit anonymous. The specific interpretation of these markers is open to discussion; however, for the purposes of this study they have proved useful.

The profiles generated by these primers indicated that the populations in South America are distinct from each other. This result suggests that (within the limits of the technique) most of these populations are to some extent genetically isolated, consistent with the geographical and climatic barriers that separate them.

Within New Zealand, most of the populations sampled generated very similar profiles. This suggests that within the limits of sampling, there is a high degree of genetic similarity between populations throughout the country.

The population from Australia generated a very similar profile to the New Zealand populations, indicating a strong genetic similarity between the weevils

sampled in these countries. This suggests either that one of these countries was colonized by *L. bonariensis* from the other, or that both countries were colonized from a single source.

The New Zealand and Australian profiles closely resembled the profiles from the east coast of South America (Uruguay and Buenos Aires), indicating that there was significant genetic similarity between these populations. It should be noted that the Buenos Aires and Uruguay collection sites were only about 60 km across the Rio de la Plata from each other.

The populations from Brazil and South Chile clustered together in all of the dendrograms generated (e.g. Fig. 6). This clustering suggested that, despite the vast distance separating them, these populations were genetically similar (although not to the extent of the New Zealand group). To our knowledge, this is the first reported study on the population genetics of *L. bonariensis*, and the data suggest that there may have been some migration or colonizing events between these two populations. Further interpretation of the relationships between the South American populations may be possible using these data, but is beyond the focus of this study.

The dendrograms clearly showed that the South American populations clustering with the New Zealand/Australian populations are Buenos Aires and Uruguay. Although there was some branch swapping noted for the different dendrograms, this cluster was always present. The degree of similarity within this cluster is higher than between this cluster and the other South American populations. There is a lower degree of similarity between the rest of the South American populations, with the exception of Rio Negro and Bahia Blanca. Based on these results, the most likely geographical source of the introduced populations of *L. bonariensis* sampled in New Zealand and Australia is the Rio de la Plata region of South America.

The emphasis of this contribution has been to answer the question of where this pest originated and to make an approximation of when this occurred. Due to the geographical isolation of New Zealand and the limited flight capacity of *L. bonariensis* it is unlikely that *L. bonariensis* arrived in New Zealand prior to the onset of concerted trade and travel, about 150 years ago. It is also unlikely that any *L. bonariensis* have been introduced into New Zealand in the past 50 years, due to the strict importation and quarantine standards imposed. Thus, introduction has been limited to a finite time span. Exactly how this introduction occurred will never be known, but it is likely that *L. bonariensis*, being a grassland species, was present in the grasses or hay used as stockfeed on trading ships.

As an initial screen of the genetic makeup of the sampled *L. bonariensis* populations, RAPDs have provided new and useful information which will assist further research on this important agricultural pest. The results presented here demonstrate the use of the RAPD technique, which may have wide application in studies of other populations.

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