Investigating genetic diversity to improve the biological control process

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Summary Prickly acacia, identified as a Weed of National Significance, now covers over seven million hectares in Australia. Biological control of this and many other weed species has not been achieved to date and there is now evidence that changes in the genetic diversity and fitness of biological control agents may be reducing the success of classical biological control efforts. This project uses a prickly acacia biological control agent as a test system to demonstrate that genetic impediments may reduce the effectiveness of current biocontrol methods. An approved host specific biological control agent, the geometrid moth *Chiasmia assimilis* (Warren) (Lepidoptera: Geometridae), was imported from South Africa and the genetic diversity and fecundity resulting from various rearing regimes were monitored over multiple generations. We measured changes in allele frequencies between group-reared lines of insects (the usual breeding practice) and isofemale line rearing methods. We provide evidence that there are deleterious effects from inbreeding on the biocontrol agent of prickly acacia. The genetic basis for selection, laboratory maintenance and release of insect biological control agents should receive considerable attention when developing biological control programs.

Keywords Biological control, *Acacia nilotica*, prickly acacia, genetic impediments, isofemale lines, genetic diversity, *Chiasmia assimilis*.

INTRODUCTION

Biocontrol programs in Australia have historically had varying success with their use of insects as biocontrol agents (McFadyen 1998). As such, biocontrol scientists are looking to improve every stage of the process from insect selection through to breeding and release of agents. Microsatellites are a powerful technology for population genetic analysis, and will assist in the development and maintenance of genetically 'healthy' cultures of biological control agents. The importance of maintaining genetic diversity within the laboratory cultures of biological control agents has been frequently emphasised (Bartlett 1985, Hopper *et al.* 1993, Roush and Hopper 1995, Spafford Jacob and Briese 2003). To date, little research has determined the best practices for the collection, breeding and genetic management of insects being reared for release and establishment as biological control agents.

Chiasmia assimilis (Warren) is a geometrid moth that has been previously collected from Kenya and more recently from South Africa to control prickly acacia, *Acacia nilotica* subsp. *indica* (Benth.) Brenan (Mimosaceae). Kenyan collections of *C. assimilis* were reared using standard group rearing methodology and were not observed to establish in northern and central Queensland, Australia after three years of a mass release program. However South African collections have established in three coastal north Queensland sites. It is suspected that inbreeding during the rearing process, differences in local predacious fauna, or differences in climate may have been responsible for the failure of establishment of the Kenyan collections. The extremely host specific nature of *C. assimilis* and its ability to defoliate mature trees prompted us to recollect. Recent CLIMEX modelling suggested South Africa was also a suitable climate match to Australia (data not shown).

Our aim was to collect *C. assimilis* from South Africa and maintain the maximum genetic diversity through to release using isofemale lines and hybridisation between these lines immediately prior to release. At the same time we aim to replicate a standard group rearing methodology used in many biological control programs. Over multiple generations we monitored fecundity and population genetic diversity using five microsatellite markers (Wardill *et al.* 2004) to quantify changes in the allele frequencies between group and isofemale line rearing methodology.

MATERIALS AND METHODS

Field collections of larvae were made in February 2002 from four South African sites; Hoedspruit (Limpopo Province, 24.4666°S, 31.1025°E), Mkuzi (KwaZulu Natal Province, 27.6197°S, 32.1808°E), Songimvelo (Mpumalanga Province, 26.0408°S, 30.9258°E) and Ohrigstad (Northern Province 24.8133°S, 30.5536°E). Specimens were reared to moths, identified and used to start isofemale lines. At Ohrigstad, *C. assimilis* was not abundant. Only one isofemale line produced greater

than 100 healthy larvae prior to importation as pupae to Australia. An average of 43 lines for Hoedspruit, Mkuzi and Songimvelo sites were initially established. Only 17, 12 and 15 lines per Hoedspruit, Mkuzi and Songimvelo site respectively produced greater than 100 healthy larvae prior to importation as pupae to Australia. Once in Australian quarantine, lines were randomly reduced to 10 per Hoedspruit, Mkuzi and Songimvelo site, in addition to the single Ohrigstad line. Isofemale lines were each maintained with 10 interbreeding randomly chosen pairs of healthy moths emerging at the same time, per line for each generation. Larvae were reared in quarantine in isolated plastic cages with cut foliage until generation five where culturing was transferred from quarantine and larvae reared on potted plants in separate cages in glasshouse conditions.

'Control lines' were initiated from generation-three-moths using a two generational mixing process. Three control lines were established, one for each site. We used three randomly chosen pairs of moths from ten isofemale lines per site (60 interbreeding moths). The Mkuzi control line used only nine isofemale lines, which had emerged in time, and for one of these lines only three females had emerged (51 parental moths). The Hoedspruit control line included the Ohrigstad line as it emerged at the same time and Ohrigstad was the closest site to Hoedspruit (66 parental moths). The Songimvelo control line lacked three males from one line (57 parental moths). In the next generation (gen. 4), ten randomly selected pairs of moths from each control line (within site mixes) were used to establish three replicate control lines (60 moths). This process allowed all control lines to have alleles from 30 isofemale lines. Control lines were maintained with 30 randomly chosen pairs of moths emerging at the same time, per line for each generation. Control lines represented the standard group rearing technique, where all selected adults interbreed as one large population, or a few subpopulations, and juveniles are generally raised as one large population.

'Mixed lines' were initiated at generation eight from the remaining isofemale lines that had not failed due to low fecundity. Mixed lines were created using the same two-generation mixing process used to create the control lines in generation three. Three replicates per site were established, making a total of nine mixed lines. Due to a loss in fecundity in some isofemale lines, insufficient numbers of moths were available for all mixed lines. An average of nine, seven and seven isofemale lines had sufficient moths to establish the three replicate 'mixed lines' from Mkuzi, Hoedspruit and Songimvelo respectively. Three 'hybridised' line replicates were created at generation nine with the same method as the control lines in generation four.

Table 1. Number of isofemale lines tested for fecundity analysis.

Site	Gen. 8	Gen. 9	Gen. 10
Mkuzi	9	8	4
Hoedspruit	8	5	4
Songimvelo	7	6	4

Fecundity was measured in generations 8 to 11 by establishing separate breeding experiments with four pairs of moths representing one replicate and an average of three replicates per isofemale line and five replicates per control being tested for each generation. The number of isofemale lines tested for fecundity analysis is shown in Table 1. The total number of eggs produced over the first four days from commencement for each breeding replicate was counted using a customised macro with ImageJ (Version 1.28, National Institutes of Health, http://rsb.info.nih.gov/ij/) and a sub-sample taken of 300 eggs per replicate to estimate numbers of neonates produced.

DNA for microsatellite analysis was extracted from isofemale parents using a single leg with a Chelex DNA extraction method (Walsh *et al.* 1991) and from a head/thorax segment of generation 1 to 9 moths using a 96-well modification of the Miller *et al*. (1988) protocol. Five microsatellite loci were chosen (CA07, CA08, CA15, CA21 and CA30) from Wardill *et al.* (2004) using the same published amplification conditions. Microsatellite scoring was on a 0.6xTris-Borate-EDTA buffer (TBE), 6% native acrylamide gel (100 μm thick, 18 cm well to laser read) in a GS2000 Genetic Analyser (Corbett Research, Mortlake, NSW) using ethidium bromide (~4 ng mL^{-1}) for detecting polymerase chain reaction products. Alleles were scored for each loci using optical density analysis with OneDScan 2.05 (Scanalytics, Inc., Fairfax, Virginia) and databased in Excel. The number of individuals screened is indicated in Figure 1. Genepop data files were created in Excel using GenAlEx 5.1 (Peakall and Smouse 2001). Genepop files were then converted in FSTAT version 2.9.3 (Goudet 2001) and genetic diversities calculated using Nei's (1987) statistic.

RESULTS

Estimated fecundity differed between the isofemale lines and the control, mixed and hybridised lines (Figure 1). For all generations tested there was variation among generations, with generation nine showing obvious increases in fecundity when compared with others.

Average genetic diversities calculated per loci across each culture type (Figure 2) show clear reductions in isofemale lines in generation eight. After mixing between isofemale lines in generation 9, 10 and 11 genetic diversity again increases to levels seen in generation one. In comparison the control lines appeared to have maintained a genetic diversity as high as the initial population through to generation eight.

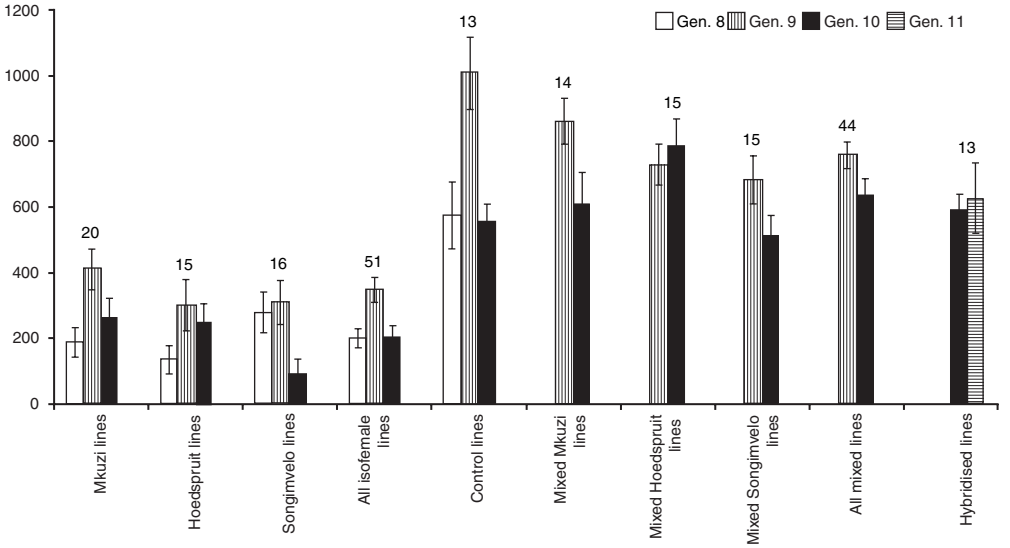

Figure 1. Fecundity – number of neonates per replicate averaged for each type of culture of *Chiasmia assimilis* from generations 8 to 11. Error bars represent the standard error and numbers above error bars represent the average number of replicates per culture tested per generation.

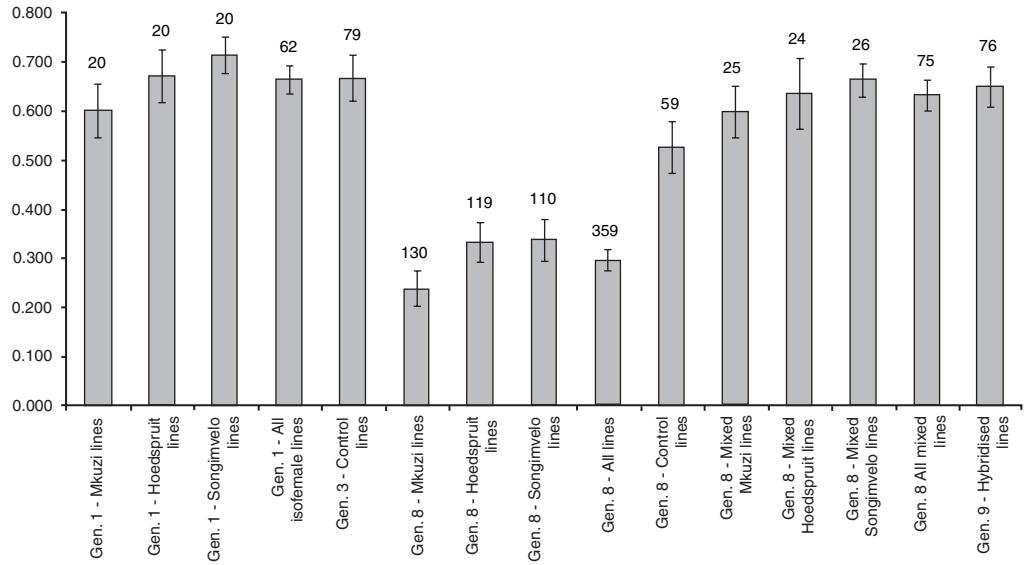

Figure 2. Gene diversity – Nei's genetic diversity averaged across loci for each type of culture of *Chiasmia assimilis* from generations 1 to 9. Error bars represent the standard error and numbers above error bars represent the average number of individuals screened across five loci.

DISCUSSION

Roush and Hopper (1995) predicted that single-family lines or isofemale lines could maintain genetic diversity in laboratory cultures. Inbreeding in isofemale lines has resulted in obvious fixation of alleles (Figure 2). There is a reduction in fecundity for isofemale lines, possibly as a result of the loss of alleles and the fixation of rare deleterious alleles (Figure 1). Following the hybridising of these lines, genetic diversity was restored to levels similar to the founder populations. Fecundity was dramatically increased, to levels similar to those found in generation 1, although not tested in this study. Differences in fecundity between generations seems to be due to variation in breeding success for the four pairs of moths per replicate. Most likely, this was due to the unnatural breeding conditions experienced by moths when using typical rearing containers.

Clearly, biocontrol scientists should consider the use of isofemale lines as a method for maintaining genetic diversity over many generations. Immediately prior to release, isofemale lines can be hybridised and the subsequent increase in fecundity should produce a far superior biological control agent than current rearing practices can produce, especially for insects prone to inbreeding. More analysis of our data is required to estimate how many lines we would recommend and what rearing techniques should be employed. At the very least biocontrol scientists should maintain many replicate (but genetically separate) cultures to preserve genetic diversity over many generations of culturing.

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