Improving the effectiveness of the release strategy for the array of *Dactylopius tomentosus* biotypes for the biocontrol of *Cylindropuntia* species by using DNA analysis

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Summary Six biotypes of *Dactylopius tomentosus* (Lamarck) (Hemiptera: Dactylopiidae) have been imported into Australia as biological control agents to combat the eight naturalised invasive *Cylindropuntia* species. Each *D. tomentosus* biotype tested exhibited significant differences in host specificity, host preference and performance across the *Cylindropuntia* species. Biocontrol success is dependent on the correct identification of the targeted plant species and the correct matching of an insect or pathogen to that plant. An optimal release strategy is to match the most effective biotype to the targeted *Cylindropuntia* species. Biotype matching to *Cylindropuntia* species was enhanced through the use of molecular phylogenetic tools and more thorough morphological observations of the cacti species. Molecular studies demonstrated that using two plastid markers (Rpl36-Rps8 spacer region and TrnH-PsbA spacer) and a nuclear region (phosphoenolpyruvate carboxylase) allowed for differentiation of the eight *Cylindropuntia* species. Molecular analyses using three markers enable the identification of unknown *Cylindropuntia* species when species identification is not possible using traditional phenological and morphological tools. The genetic identification of the *Cylindropuntia* provides a time efficient match of target host species and biotype within days rather than the traditional long term host trials (three month life cycle) previously required. Utilising DNA analyses will markedly improve the chance of containment of *Cylindropuntia* infestations in Australia through better matching of biocontrol agents with their most preferred host.

Keywords Host specificity, biotypes, molecular analysis, biocontrol.

INTRODUCTION

The genus *Cylindropuntia* is native to southern USA and Mexico and comprises 33 species, eight of which have naturalised in Australia. These eight species: *C. fulgida* var. *mamillata* (DC.) Backeb., *C. imbricata* (Haw.) F.M.Knuth, *C. kleiniae* (DC.) F.M.Knuth, *C. leptocaulis* (DC.) F.M.Knuth, *C. prolifera* (Engelm.) F.M.Knuth, *C. rosea* (DC.) Backeb., *C. spinosior* (Engelm.) F.M.Knuth and *C. tunicata* (Lehm.) F.M.Knuth are shrubs ranging in height from 60 cm to 3 m (Holtkamp 2012b, Botanic Gardens Trust 2013). These plants have succulent segments growing end-to-end that are armoured with large barbed spines and tiny bristles known as glochids. Collectively these weeds pose serious environmental, agricultural and recreational problems through restricting access to recreational areas, reduced productivity of agricultural land and injury to livestock and native animals.

Variation within species has been a major obstacle for correct identification and the wide variability in morphological traits is increased by environmental conditions that ultimately influence the growth habit of the plants – leading to confusion in correct identification of some species.

Two cochineal insects, *Dactylopius tomentosus* 'imbricata' biotype and *D. tomentosus* 'cholla' biotype have been released to control *C. imbricata* and *C. fulgida* respectively. There are another four *D. tomentosus* biotypes in quarantine awaiting approval for field release. These six biotypes collectively attack the majority of *Cylindropuntia* species found in Australia. However, they all show a significant difference in performance and preference between the eight *Cylindropuntia* species.

Successful biological control of these *Cylindropuntia* species is dependent on releasing the most effective biotype to control each individual *Cylindropuntia* species. Ultimately, the success of the release strategy is thus dependent on the correct identification of the infestation species.

This paper describes the molecular techniques on how to distinguish the eight naturalized *Cylindropuntia* species in Australia and the impact this has on the future success of the *Cylindropuntia* biological control program.

MATERIALS AND METHODS

Plant specimens All *Cylindropuntia* species were identified by recognized taxonomists and Table 1 details the species and localities of plants used in the trial. Identified plant specimens from the US, Mexico and Spain were also used to validate our results.

Table 1. *Cylindropuntia* specimens and the locality collected.

Species	Locality
C. fulgida var. *mamillata*	Longreach, Qld
C. fulgida var. *mamillata*	Burakin, WA
C. fulgida var. *mamillata*	Booligar, Qld
C. fulgida var. *mamillata*	Charleville, Qld
C. fulgida var. *mamillata*	Augathella, Qld
C. fulgida var. *mamillata*	Moama, Qld
C. imbricata	Boggabri, NSW
C. kleiniae	Lightning Ridge, NSW
C. leptocaulis	Curlewis, NSW
C. prolifera	Lightning Ridge, NSW
C. prolifera	Longreach, Qld
C. rosea	Grawin, NSW
C. rosea	Lorne Station, NSW
C. rosea	Willows, Qld
C. rosea	Quilberry Creek, Qld
C. rosea	Charleville, Qld
C. spinosior	Bexley, Qld
C. spinosior	Grawin, NSW
C. tunicata	Glengarry, NSW
C. tunicata	Grawin, NSW
C. tunicata	Cracow, Qld
C. tunicata	Mundubbera, Qld
C. tunicata	Kalkada, Qld

DNA extraction, amplification and sequencing Two plastid markers (Rpl36-Rps8 spacer region and TrnH-PsbA spacer) and one partial nuclear region (phosphoenolpyruvate carboxylase, ppc) were evaluated to differentiate the eight *Cylindropuntia* species. DNA was extracted using a commercial kit and three loci were amplified by PCR. Nucleotide sequences were determined for each amplicon. When needed, further discrimination was achieved using High Resolution Melt PCR of a partial region of the ppc locus.

RESULTS

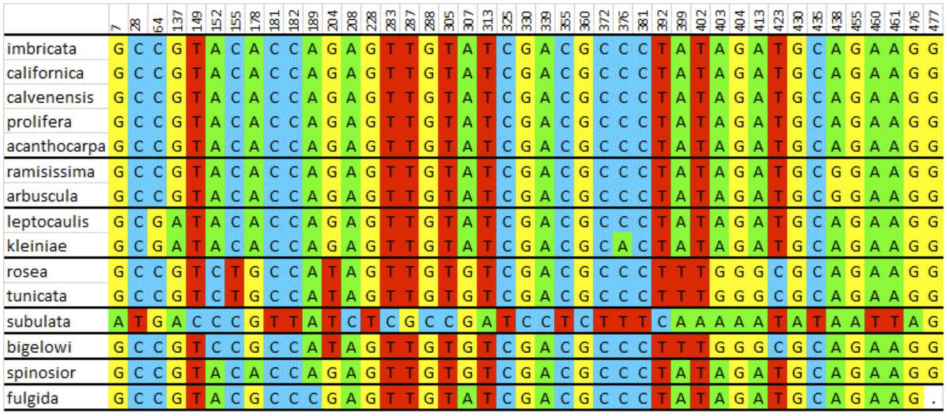
The eight naturalised *Cylindropuntia* species could be differentiated from each other by amplicon sequence (Figure 1). A High Resolution Melt (HRM) PCR using partial phosphoenolpyruvate carboxylase amplicon was required to distinguish *C. rosea* from *C. tunicata* (Figure 2), as differentiation using sequence alone was not always conclusive due to heterozygosity in the *C. rosea* ppc gene (Figure 1). The HRM curve characteristics were consistent for all individuals tested of these two species.

DISCUSSION

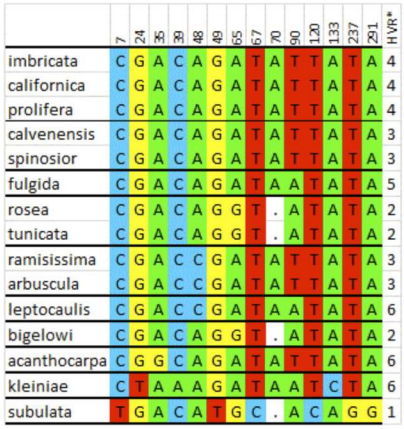
The current biocontrol project has six *Dactylopius tomentosus* biotypes targeting the *Cylindropuntia* species. Two cochineal insects, *Dactylopius tomentosus* 'imbricata' biotype and *D. tomentosus* 'cholla' biotype have been released to control *C. imbricata* and *C. fulgida* respectively and another four *D. tomentosus* have biotypes undergone host specificity testing in quarantine and are awaiting approval for release. A recent survey in the US and Mexico collected another 16 *Dactylopius tomentosus* biotypes to target the two *Cylindropuntia* species, *C. spinosior* and *C. prolifera*, that the current suite of biotypes do not attack. Mathenge *et al.* (2009), Mathenge *et al.* (2010) and Jones *et al.* (2015, 2016) showed that populations of *D. tomentosus* displayed distinct host preferences when collected from different *Cylindropuntia* species within their native ranges. These populations (biotypes) display significant differences in feeding and development preferences and ultimately their impact on different *Cylindropuntia* species. To date, a biotype most suited to controlling each *Cylindropuntia* species has been identified through a series of host specificity, efficacy and fitness index trails.

The use of the three molecular markers can reliably identify the eight naturalised *Cylindropuntia* species and can be confirmed within a couple of working days. As a result, samples of an undetermined species recently discovered in the field can be identified and the most suitable biotype to target this species can be confirmed and released promptly at the infestation site.

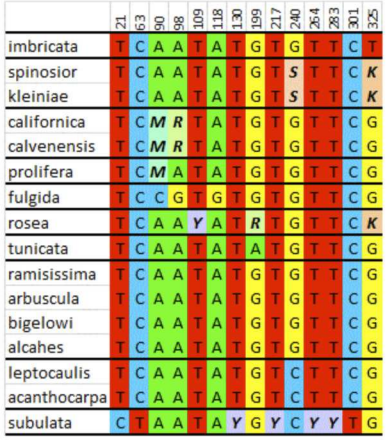
Previously, without a confirmed identification, a series of host tests and feeding impact trials would be required to determine the most suitable biotype. These trials would take up to three months to complete. There are multiple benefits in adopting this molecular technique for species identification. First and most important, correct identification will lead to matching the most effective biotype. Second, time efficient confirmation allows rapid and appropriate response to new infestations. Third, these *Dactylopius tomentosus* biotypes can interbreed and the progeny display a



Rpl36-Rps8 spacer region

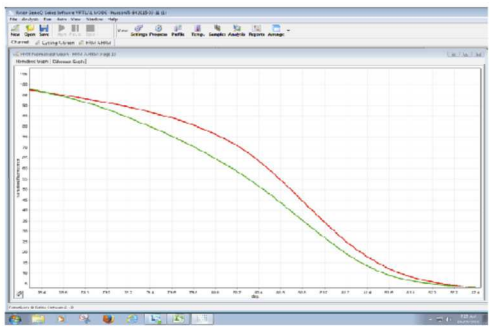


TrnH-PsbA spacer region

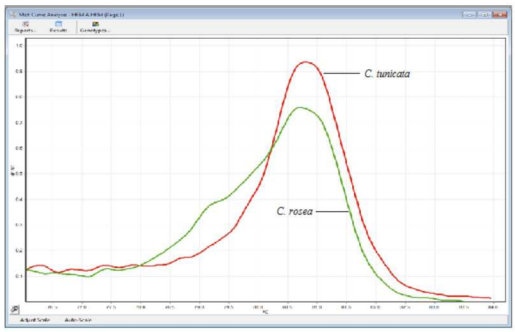


phosphoenolpyruvate carboxylase, ppc

Figure 1. Differentiation sites for the three sets of primers to distinguish the eight naturalized *Cylindropuntia* species in Australia, US, Mexico and Spain. *hypervariable region between sites 140-225 for the TrnH-PsbA spacer appears to have specific patterns that may be recognisable.



HRM Curve



HRM dF/dT Peak

Figure 2. High Resolution Melt to distinguish *C. rosea* (green) seen as heterozygous for a selected partial region of ppc gene and *C. tunicata* (red) which is homozygous at the corresponding site.

difference in host range and host performance, which could result in a reduction of the virulence and impact on the target species. Correct identification will assist in reducing cross contaminating the biotypes at an infestation site.

ACKNOWLEDGMENTS

This project was funded by Qld. Department of Agriculture Fisheries, NSW Department of Primary Industries, Western Catchment Management Authority and Rural Industries Research and Development Corporation. Authors thank Tony Pople for their advice and comments on the manuscript.

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