Biology, host specificity and DNA barcoding of cryptic *Eueupithecia* species (Lepidoptera: Geometridae), and implications for biological control of *Parkinsonia aculeata* (Fabaceae) in Australia

Michelle A Rafter,1* Fernando McKay,2 Marcelo Parisi,2 Alejandro Sosa,2,3 Tim A Heard,1 Andrew White,1 Gio Fichera,1 Dean Brookes,4 Kumaran Nagalingam,1 Lauren Kaye1 and SRagh1

1CSIRO, GPO Box 2583, Brisbane, Qld 4001, Australia.
2Fundación para el Estudio de Especies Invasivas, Bolívar 1559, Hurlingham, Buenos Aires 1686, Argentina.
3Consejo Nacional de Investigaciones Científicas y Técnicas, Godoy Cruz, Buenos Aires 2290 (C1425FQB), Argentina.
4School of Biological Sciences, The University of Queensland, Brisbane, Qld 4072, Australia.

**Abstract**

*Parkinsonia aculeata* L. (Fabaceae: Caesalpinioideae), native to the Americas, is a designated Weed of National Significance in Australia. The leaf-feeding geometrid moth species, *Eueupithecia cisplatensis* Prout (Lepidoptera: Geometridae), was identified as a potential biological control agent of *P. aculeata* following native range surveys in Argentina. After importation into quarantine for host specificity assessment, this species was identified as a cryptic species complex of two morphologically similar species. The new species, *Eueupithecia vollonoides* Hausmann (Lepidoptera: Geometridae), was subsequently described. The biology and host range of both moth species were examined to determine their suitability as biological control agents. Host specificity was evaluated in the native range, by surveying insects found on closely related plant species, and in the laboratory, by no-choice larval development tests. Native range surveys and laboratory assays revealed a high level of specificity to *P. aculeata* by both *Eueupithecia* species. Of the 71 plant species from the Fabaceae family that were tested, *E. cisplatensis* completed development on only one non-target species, *Parkinsonia praecox*. This plant species is not present in Australia, and no *Eueupithecia* species were found to use *P. praecox* in the field in its native range. Hence, the risk of non-target effects if these species were to be released in Australia was considered to be low. One mitochondrial (COI) gene region and two nuclear (28S and CAD) gene regions were tested for their ability to differentiate between the two cryptic species. COI haplotypes from *E. cisplatensis* and *E. vollonoides* belong to two divergent haplotype groups and can reliably distinguish either species from the other. Some 28S and CAD gene haplotypes are shared at low frequencies, perhaps because of rare or historical hybridisation or incomplete lineage sorting, thus cannot reliably discriminate between the species. This pattern of mito-nuclear discordance requires further elucidation using population genetics studies to determine its biological significance. Based on the results of host specificity tests, *E. cisplatensis* and *E. vollonoides* were approved for release in Australia in May 2013 and May 2014, respectively.

**Key words** native range survey, natural enemy, no-choice test, species delimitation.

**INTRODUCTION**

*Parkinsonia aculeata* L. (Fabaceae: Caesalpinioideae) (palo verde, Parkinsonia, Jerusalem thorn) is a tree species native to the Americas. Globally, it was introduced as an ornamental, hedging, fodder and shade tree leading to a pantropical distribution where it established in areas with arid, seasonally flooded climates (Hawkins et al. 2007). In northern Australia, the species occupies over 8000 km² where it forms dense thickets in floodplains, grasslands, along water courses and bore drains. It negatively impacts the pastoral industry and rangeland production systems as thickets limit pasture growth, restrict stock access to water and impede mustering (van Klinken 2006). For these reasons, the species is recognised as a Weed of National Significance (Thorpe & Lynch 2000); it is a declared weed in all Australian states and territories.

Control techniques available to manage *P. aculeata* include the use of herbicides, machinery, fire and grazing and classical biological control (van Klinken et al. 2009). Biological control efforts began in the 1980s and resulted in the release of three insect species: *Rhinacloa callicrates* Herring (a sap-sucking mirid), *Mimosestes ulkei* (Horn) (retama weevil) and *Penthobruchus germaini* Pic. (seed-feeding bruchids) (Flanagan et al. 1996; Julien & Griffiths 1998; Donnelly 2000; Briano et al. 2002; van Klinken 2005; van Klinken & Flack 2008). Of these three insect species that have been released in Australia, only populations of the seed-feeding bruchid,
Eueupithecia species in preparation for monitoring these two cryptic species in their introduced geographical range.

**MATERIALS AND METHODS**

**Biology**

*Eueupithecia cisplatensis* cultures were established in the laboratory from 50 larvae collected in February 2012 on *Parkinsonia aculeata* plants growing near La Plata, Buenos Aires Province (S34.5516°; W57.5716°). Experiments were conducted in controlled environment chambers at 25 ± 1°C and 60 ± 5% relative humidity, with a 14:10 light-dark (L:D) photoperiod. Newly hatched larvae were fed bouquets of freshly excised leaves of *P. aculeata* and reared individually in 0.5 L plastic jars with perforated lids and moist tissue paper. The petioles of leaves of *P. aculeata* were inserted into small floral tubes filled with water. Bouquets were replaced every 48 h. Head capsule widths of individual larvae were measured to determine the number and the duration of developmental instars. Adult longevity and fecundity were estimated from eight pairs of newly emerged adults. Each pair was placed in a 3 L ventilated plastic container and reared as above. One replicate was terminated when the female died; males were replaced if they died before the female. For each pair, we recorded pre-oviposition period, number of eggs laid and longevity of females. The duration of the egg, larval and pupal stage were recorded while rearing the agent.

For *Eueupithecia volumnoides*, cultures were established from larvae collected from *P. aculeata* plants growing near Santa Fe, Reconquista (S29.17355°; W59.67059°), and Villa Ocampo (S28.48962°; W59.32394°), Argentina. Data for this species were collected while rearing the agent in the quarantine facility, located in Brisbane, QLD, Australia. Colonies of the agent were held in controlled environment chambers at temperatures of 25 ± 1°C and 60 ± 5% relative humidity, with a 14:10 L:D photoperiod. Newly hatched larvae were reared on potted plants of *P. aculeata*. The duration of the egg, larval and pupal stage were recorded along with head capsule widths of individual larvae.

**Natural enemies of Eueupithecia species**

To determine abundance and identity of parasitoids, larvae of *Eueupithecia* species were collected and reared for the emergence of either adults or parasitoids. A total of 486 larvae were collected (3–197 larvae per batch) from 12 sites in two field trips across northern Argentina in December 2012 and March 2013.

**Native range surveys of host use**

*Parkinsonia aculeata* and six co-occurring legume species (*Vachellia caven* (Molina) Seigler & Ebinger, *Vachellia aroma* (Gillies ex Hook. & Am.) Seigler & Ebinger, *Geoffroea decorticans* (Gillies ex Hook. & Am.) Burkart, *Parkinsonia praecox* (Ruiz & Pav. ex Hook.) Hawkins, *Prosopis affinis* Spreng. and *Prosopis ruscifolia* Griseb.) were sampled for

*P. germainii*, have established and are widely dispersed. However, seed consumption rates were found to be low, and this agent is unlikely to be causing population-level impacts (van Klinken 2005; van Klinken & Flack 2008). These agents were discovered during native range explorations focused in the USA and northern Mexico (Woods 1992; Bell et al. 2014). Recent genetic studies using AFLP markers have identified that *P. aculeata* populations in South America are the likely source of introductions into Australia (Hawkins et al. 2007) and highlighted the need to search for additional natural enemies in this area. Native range surveys conducted in Argentina between 2008 and 2013 revealed the presence of a leaf-feeding moth identified as *Eueupithecia cisplatensis* Prout (Lepidoptera: Geometridae).

*Eueupithecia* was historically considered a monotypic genus, with only *E. cisplatensis* described (Siivonen et al. 2011). Specimens collected on *P. aculeata* from different localities of Argentina were initially identified as *E. cisplatensis* by Axel Hausmann (Bavarian State Collection of Zoology). Difficulties maintaining cultures with moths collected from both the northern and southern parts of the moth’s distribution in Argentina suggested that a cryptic species may be present. Further taxonomic examination has resulted in the description of a new species (*Eueupithecia volumnoides* Hausmann) based on reproductive anatomy and COI barcoding (Hausmann et al. 2016). Although there are striking differences in female and male genitalia and 4.1% difference in COI gene sequence between the two *Eueupithecia* species, significant and consistent differential external features in colour or pattern of adults or larvae have not been found (Hausmann et al. 2016).

Several Geometridae have been used in weed biological control programs. *Comostolopsis germana* Prout (bitou tip moth) has been released as a control agent against *Chrysanthemoides monolifera* (L.) Norlindh (boneseed), in Australia causing reduction in seed set of up to 70% (Adair & Edwards 1996). *Aplocera plagiata* L. (St John’s Wort inchworm) is variably effective in controlling *St John’s Wort* (*Hypericum perforatum* L.) in Canada and the USA, but not in Argentina (Julien & Griffiths 1998). *Chiasmia inconstipica* (Warren) and *Chiasmia assimilis* (Warren) from Kenya were released in 2000 for biological control of *Vachellia nilotica* (L.) Willd. Ex Delile (prickly acacia) in Queensland (QLD). *Chiasmia assimilis*, but not *C. inconstipica*, established quickly and spread rapidly in coastal areas of QLD (Palmer et al. 2007). *Digrammia pallidata* (Packard 1873) and *Leuciris fimbraria* (Stoll, 1781) were released in Australia for control of *Mimosa pigra* L. (giant sensitive tree). Both have established, with the former inflicting significant damage on the target plant (Heard et al. 2010). Given this history of successful use of geometrids in weed biological control, *E. cisplatensis* and *E. volumnoides* were investigated as potential control agents for parkinsonia.

In this paper, we present the results of investigations on the biology and host specificity of both *E. cisplatensis* and *E. volumnoides*. We also report the results of DNA barcoding of COI and nuclear (28S and CAD) gene regions for each
Eueupithecia species. Nine sites were sampled across five provinces in Argentina (Chaco, Corrientes, Entre Ríos, Formosa and Salta) on three field trips between 2009 and 2013. Insects were sampled by beating foliage over a 1 m² sheet. The plant species surveyed and the number of collected larvae were recorded. Immature insects were transported to the laboratory and held in plastic containers with fresh leaves until adult emergence. Adult insects were identified, and voucher specimens of the plants and insects collected were deposited at the Fundación para el Estudio de Especies Invasivas (FuEDEI) in Hurlingham, Argentina.

Host-test list
Seventy-one species from the Fabaceae family, in addition to P. aculeata, were screened to examine the host range of Eueupithecia species. Tests were conducted both in Argentina and Australia (see Table S1 for a detailed list of species tested and their phylogenetic relationship to P. aculeata). Plants were sourced from the field or purchased at nurseries. Test species identification was confirmed by members of the team with skills in Fabaceae plant taxonomy. The test-plant list was compiled following contemporary methods (Sheppard et al. 2005) based on their known phylogenetic relationship to P. aculeata (Bruneau et al. 2008 and references therein), their economic importance, biogeographic overlap with P. aculeata in Australia, morphological similarity and availability. The host-test list was developed based on the most up-to-date phylogenetic information available at the time. The relationship between Caesalpinioideae and Mimosoideae has been recently revised as part of a major taxonomic revision of Fabaceae (Azani et al. 2017). The mimosoid species are now considered a distinct clade nested within Caesalpinioideae (Azani et al. 2017). Australia’s Acacia species are, therefore, more closely related to P. aculeata than was previously understood. The impact of these systemic revisions on our study is minimal, however, because our original test list was extensive and had good coverage within Mimosoideae (Table S1). Besides P. aculeata, which is the only Parkinsonia species known to have naturalised in Australia, P. praecox, a species native to Argentina, was also tested for its suitability as a host for the Eueupithecia species.

Host specificity testing
The host specificity of Eueupithecia species was examined in the laboratory by no-choice larval development assays on cut plant material in Argentina and no-choice larval development assays on whole plant species in Brisbane, QLD, Australia. Cut plant material was used in Argentina as this was an efficient way to screen the host use of the insects prior to exporting to Australian quarantine. Whole plants were then used in Australian testing to ensure that insects were exposed to the full complement of plant volatiles and secondary compounds. Larval development tests were employed as female moths proved to be indiscriminate ovipositing while confined to experimental cages, as has been the case with other Lepidopteran agents in no-choice testing (e.g. Macaria pallidata (Lepidoptera: Geometridae) (Heard et al. 2010)).

No-choice early larval development tests on cut plant material
Experiments in Argentina were carried out in controlled environmental chambers (25 ± 2°C; 60–80% relative humidity; 16:8 L:D). In each replicate, 10 newly emerged E. cisplatensis or E. vollonoides larvae were placed in 0.7 L plastic containers with perforated lids and moist tissue paper. The larvae were fed bouquets of freshly excised leaves with their petioles inserted in small floral tubes filled with water. The bouquets were replaced every 48–72 h as needed. Feeding damage and larval mortality were recorded daily until adult emergence. Typically, 10 replicates of each plant species were tested, although fewer replicates were done for some plant species (see Table S1).

No-choice larval development tests on living plants
To test the survival and development of Eueupithecia species on whole plants, 50 neonate larvae were placed on the foliage of an individual test plant species and the feeding damage and larval mortality until adult emergence was scored. Individual potted test plants were held in an aluminium framed cage lined with gauze and measuring 250 × 250 × 800 mm or 250 × 250 × 500 mm, depending on the size of the plant. Plants in cages were kept in the quarantine glasshouse (located in Brisbane, Australia) under natural illumination, or, when day lengths decreased, in quarantine-controlled environment rooms under artificial lighting. Plants were monitored regularly, and extra plants of the same species were added if the larval feeding depleted the original plant (this only occurred on the target plant, P. aculeata). Plants were held for an average of 47 days (range 28 to 69 days), by which time all adults had emerged from the P. aculeata control plant. One P. aculeata control plant and two to four test plant species, depending on the availability of larvae, were used in each trial. The inclusion of one P. aculeata control plant in each trial ensured that the larvae and environmental conditions were suitable for development to adult. A total of 27 trials for E. cisplatensis, with four replicates for each test species, and 18 trials for E. vollonoides, with two replicates for each test species, were completed. Fewer replicates for two test species were completed when plant material was not available (see Table S1). For each plant species, different individual plants were used in each replicate in all trials. Initial studies showed that leaves of P. aculeata of all ages are suitable for larval development, which precluded the need to control for plant age.

DNA barcoding
A total of 102 samples of E. cisplatensis (n = 45) and E. vollonoides (n = 57) from laboratory colonies maintained at CSIRO quarantine facilities, from Argentinian native range and from the introduced range in Australia were used in the study (see Table S2). Samples from Argentina were collected by beating P. aculeata foliage over a 1 m² sheet, while individuals from
Australia were trapped using delta sticky traps in Burketown, QLD, where E. vollonoides was released as part of the biological control program.

DNA was extracted from E. cisplatensis and E. vollonoides samples that had been stored at −20°C using the spin column DNA extraction protocol described by Ridley et al. (2016). Briefly, either 3 legs or ‘head + thorax’ were homogenised in 200 µL of lysis buffer and 5 µL of Proteinase K and then digested overnight at 55°C. Lysate, binding buffer (4 M GuHCl) and ethanol were combined in equal parts and added to an EconoSpin mini spin column (Epoch Life Science, Texas, USA). The spin column was washed twice with 500 µL of 70% ethanol, and then the DNA was eluted using 100 µL of 10 mM Tris. Eluted DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer’s protocol.

The gene regions COI, 28S and CAD were PCR amplified using the primers listed in Table S3. A 20 µL reaction containing 1 × MyTaq HS buffer, 0.2 µm of forward primer, 0.2 µm of reverse primer, 1 unit of MyTaq HS DNA polymerase (Bioline, UK) and 3.0 µL of DNA template were amplified using the following PCR conditions. One cycle of 95°C for 2 min and then 40 cycles of the following: 95°C for 30 s, annealing for 45 s and 72°C for 1 min 15 s. A final cycle of 72°C for 10 min was used. Annealing temperatures were 51°C for COI and 48°C for 28S and CAD. All PCR products were cleaned by adding 1 unit of Exonuclease I and 1 unit of Antarctic Phosphatase (New England Biolabs, USA) and heating at 37°C for 30 min. PCR products were sequenced in both directions using Sanger sequencing by Macrogen (South Korea).

Sequence data were trimmed, aligned and checked using CodonCode Aligner version 4.1.1 (CodonCode Corporation, Centerville, MA, USA), and priming sites were removed. Sequences were checked manually for problems typical of each gene region, including the presence of pseudogenes, including stop codons, large numbers of protein coding changes and GC bias, then submitted to GenBank (Table S4). Nuclear DNA haplotypes were reconstructed using the PHASE algorithm as implemented in DNAsp 6.12.01 using 10 000 interactions with 1000 burn-in, an output probability of 0.9 and both the hybrid and recombination models (Rozas et al. 2017). Popart was used to create haplotype networks using the TCS network model (Clement et al. 2002; Leigh & Bryant 2015).

RESULTS

Biology

Eueupithecia cisplatensis

Females laid 78.8 ± 58.7 (mean ± SD; n = 6) brown, club-shaped eggs, singly or one upon another in strings (3–8) on the leaflets. Eggs were laid 1.8 ± 0.6 days (n = 6) after adult emergence. Egg incubation period was 6.5 ± 0.6 days (n = 20), and this species has four larval instars (Table 1). No overlap was found in head capsule width ranges; therefore, this trait can be used to distinguish the instars (Fig. 1). Body colour of larvae changes progressively from light brown-greenish in the early instars to green-purple in the later instars, mimicking Parkinsonia aculeata leaf rachises and young shoots. As larvae develop, they eat most of the leaflets and parts of the rachises. Larval mortality was greater during the first and second instars and the survival to

| Table 1 | Life stage duration and mortality of Eueupithecia cisplatensis and E. vollonoides reared on Parkinsonia aculeata |
|---------|----------------------------------------------------------------------------------------------------------|
| Stage   | n       | Life stage duration (days) | Mortality (%) | Cumulative survival (%) |
|         |         | Mean ± SD | Range      |                                  |                           |
| Eueupithecia cisplatensis | | | | | |
| Egg incubation | 20      | 6.5 ± 0.6 | 5–7        | –                                 | –                           |
| Total larval duration | 21      | 16.9 ± 3.1 | 13–27      | 61                  | 49                           |
| Adult emergence | 18      | 5.7 ± 2.8 | 1–13       | –                                 | 42                           |
| Eueupithecia vollonoides | | | | | |
| Egg incubation | 19      | 4.9 ± 1.2 | 3–7        | –                                 | –                           |
| Total larval duration | 19      | 14.1 ± 1.8 | 12–16      | –                                 | –                           |
| Adult emergence | 19      | 18.1 ± 1.5 | 16–20      | –                                 | 52                           |

| Fig. 1. Larval head capsules width (mm; mean ± SE) of E. cisplatensis (circle, larval instar 1 (n = 16), larval instar 2 (n = 25), larval instar 3 (n = 17), larval instar 4 (n = 25)) and E. vollonoides (triangle, larval instar 1 (n = 15), larval instar 2 (n = 15), larval instar 3 (n = 42), larval instar 4 (n = 15)). |
the adult stage was 42% (Table 1). The larval stage lasted 16.9 ± 3.1 days \((n = 21)\).

**Eueupithecia vollonoides**

The morphology of the clutches of eggs laid by *Eueupithecia vollonoides* is similar to that of *Eueupithecia cisplatensis*. However, *E. vollonoides* females are far more fecund and laid 136.8 ± 38.1 eggs \((n = 6)\) and all females initiated oviposition 1 day after adult emergence \((n = 6)\). The average duration of egg incubation was 4.4 ± 0.9 days \((n = 19)\) (Table 1). There were four larval instars, and head capsule width is smaller than *E. cisplatensis* in instar 1 but is larger than *E. cisplatensis* by instar 4 (Fig. 1). The larval stage lasted 14.1 ± 1.8 days \((n = 12)\), and adults begin to emerge an average of 18 days from egg hatch \((n = 19,\ \text{range}\ 16–20\ \text{days})\) (Table 1). The majority of emergence occurs within the first few days and continues for as long as 37 days. A tendency to enter diapause in the pupal stage was noticed when day length decreased.

**Natural enemies**

Two species of *Conura* (Hymenoptera: Chalcidoidea) were reared from both *Eueupithecia* species pupae. From the 370 *E. cisplatensis* larvae collected across seven sites (52.9 ± 64.8 larvae per site), 71 parasitoids emerged, with an effective parasitism rate of 20.9 ± 5.7% per site. From the 116 *E. vollonoides* larvae collected across five sites (23.2 ± 13.0 larvae per site), 46 parasitoids emerged, with an effective parasitism rate of 38.2 ± 13.1% per site.

**Native range surveys of host use**

At the nine sites surveyed, a total of 427 larvae of *Eueupithecia* species were collected on *P. aculeata* and reared to adult. No *Eueupithecia* species larvae were collected on any of the other surveyed legume species (Table 2). It is particularly instructive that *E. vollonoides* was not found on *Parkinsonia praecox* even though this species was consistently collected on *P. aculeata* at the same sites.

**Host range testing**

**No-choice early larval development tests on cut plant material**

When 10 larvae were introduced to cut test plant material, a mean of 6.1 ± 1.8 *E. cisplatensis* \((n = 21)\) and 6.7 ± 0.3 *E. vollonoides* \((n = 10)\) larvae completed development on the target weed, *P. aculeata*. The only other species on which *E. cisplatensis* larvae completed development was *P. praecox* (0.3 ± 0.5, \(n = 10\)). No feeding by either *Eueupithecia* species occurred on any other non-target species, with larval mortality observed within 2–4 days of the start of the test, likely from starvation.

**No-choice larval development tests on living plants**

A mean of 27.7 ± 7.2 *E. cisplatensis* \((n = 29)\) and 20.8 ± 7.7 *E. vollonoides* \((n = 18)\) survived and completed development on whole *P. aculeata* plants, from an initial introduction of 50 neonate larvae. No feeding by either *Eueupithecia* species occurred.

**Table 2**  Number of *Eueupithecia* species and other geometrids found on various legume plants species from surveys of plant use under natural condition in Argentina

| Locality and date            | Surveyed plant species | Beats | *Eueupithecia cisplatensis* | *Eueupithecia vollonoides* | Other Geometridae/Noctuidae |
|-----------------------------|------------------------|-------|-----------------------------|-----------------------------|-----------------------------|
| Pucheta, Corrientes         | *Parkinsonia aculeata*  | 50    | 44                          | 0                           | 0                           |
| 3 December 2009             | *Prosopis affinis*      | 2     | 0                           | 0                           | 2                           |
|                             | *Vachellia caven*       | 10    | 0                           | 0                           | 9                           |
| Cuatro Bocas, Corrientes    | *P. aculeata*           | 32    | 43                          | 0                           | 0                           |
| 3 December 2009             | *P. affinis*            | 8     | 0                           | 0                           | 5                           |
| Mocoretá, Corrientes        | *P. aculeata*           | 17    | 13                          | 0                           | 0                           |
| 3 December 2009             | *V. caven*              | 5     | 0                           | 0                           | 1                           |
| Chajarí, Entre Ríos         | *P. aculeata*           | 46    | 195                         | 0                           | 0                           |
| 3 December 2009             | *V. caven*              | 10    | 0                           | 0                           | 1                           |
| Concepción del Uruguay, Entre Ríos | *P. aculeata*     | 30    | 35                          | 0                           | 0                           |
| 4 December 2009             | *P. affinis*            | 4     | 0                           | 0                           | 0                           |
|                             | *V. caven*              | 5     | 0                           | 0                           | 0                           |
| 60 km NW Juárez, Formosa    | *P. aculeata*           | 10    | 0                           | 24                          | 5                           |
| 20 March 2010               | *Parkinsonia praecox*   | 3     | 0                           | 0                           | 12                          |
| 60 km NW Juárez, Formosa    | *P. aculeata*           | 15    | 0                           | 2                           | 20                          |
| 26 September 2010           | *P. praecox*            | 10    | 0                           | 0                           | 15                          |
| Pozo del Mortero, Formosa   | *P. praecox*            | 10    | 0                           | 0                           | 29                          |
| 19 March 2010               | *P. aculeata*           | 10    | 0                           | 35                          | 0                           |
| Fortín Lavalle, Chaco        | *Prosopis ruscifolia*   | 10    | 0                           | 0                           | 0                           |
| 23 March 2010               | *V. caven*              | 10    | 0                           | 0                           | 0                           |
| Fontana, Formosa            | *P. aculeata*           | 62    | 0                           | 25                          | 0                           |
| 8 March 2013                | *Vachellia aroma*       | 10    | 0                           | 0                           | 0                           |
|                             | *Geoffroea decorticans* | 10    | 0                           | 0                           | 1                           |
|                             | *P. ruscifolia*         | 10    | 0                           | 0                           | 0                           |
on any non-target test plant species, and hence, no larval development was observed on non-target species.

**DNA barcoding**

*Eueupithecia cisplatensis* and *E. vollonoides* collectively had 5, 13 and 18 haplotypes, respectively, at a 658 base pair (bp) fragment of the COI mtDNA gene, a 702–705 bp fragment of the 28S rDNA gene and a 685 bp fragment of the CAD nuDNA gene (Fig. 2). Haplotypes were obtained from 100, 91 and 90 individuals for COI, 28S and CAD, respectively (Table S4). Phased haplotypes for the 28S gene were identical whether the hybrid or recombination model was used. Phased haplotypes for the CAD gene differed for two individuals, ECAJu13n6 and EVAJ13n6 (Table S4). The hybrid PHASE model produced the most appropriate result for the CAD gene, as it more appropriately resolved the position of haplotype CAD-hap14 as an intermediate haplotype of the two main haplotype groups.

The COI haplotype network shows two divergent haplotype groups, and each haplotype group is associated with one *Eueupithecia* species, in both the native range and laboratory culture samples (Fig. 2a). The field collected *E. vollonoides* individuals from the Australian range shared COI haplotypes with the *E. vollonoides* haplotype group, those from the *E. vollonoides* laboratory culture and *E. vollonoides* from the native range (Fig. 2a). The minimum pairwise identity (pairwise ID) is 4.0% between *E. cisplatensis* and *E. vollonoides* using all available COI sequences, those from this study and those of Hausmann et al. (2016). The haplotype associations of individuals are indicated in Table S4.

The CAD gene region had two haplotype groups (Fig. 2b) and each was mostly associated with a single species, but also the alternate species at a low frequency. A few individuals from the *E. cisplatensis* laboratory culture shared a CAD haplotype with *E. vollonoides* of laboratory origin, and the reverse was true, with a few individuals from the *E. vollonoides* culture sharing a CAD haplotype with *E. cisplatensis* of native and laboratory origin (Fig. 2b). Of the haplotypes assigned to a species atypical for the haplotype group, 60% (3/5) were unique to that species (CAD-Hap13, CAD-Hap16 and CAD-Hap17 in Fig. S1). Two haplotypes were intermediate between the two groups. All Australian field collected individuals shared CAD haplotypes with *E. vollonoides* (Fig. 2b).

The 28S gene had three high frequency haplotypes that were differentially associated with the two *Eueupithecia* species, but the haplotypes were poorly differentiated overall (Fig. 2c). Two of the three 28S haplotypes found at a high frequency were shared by the laboratory *E. cisplatensis*, native *E. cisplatensis* and the laboratory *E. vollonoides* (Fig. 2c). The third 28S haplotype found at a high frequency was shared by both *E. cisplatensis* and *E. vollonoides* of laboratory and native origin, and all individuals which were trapped in the introduced range (Fig. 2c). Overall, the 28S haplotype network was similar to the CAD haplotype network, but with no identifiable haplotype groups.

**DISCUSSION**

Aspects of the biology of the *Eueupithecia* species indicate they have potential as biological control agents of *Parkinsonia*...
aculeata in Australia. The females produce many offspring which are very damaging to P. aculeata plants in the laboratory, the larval developmental period is short and both species produce several overlapping generations per year. These attributes are desirable in a biocontrol agent which may result in the rapid build-up of populations in the field (Crawley 1989). We expect that the extensive foliar damage by the Eueupithecia larvae could reduce the total photosynthetic area of P. aculeata plants, resulting in a reduction in vigour, growth rate and seed production.

Both Eueupithecia species feed on vegetative tissue, and thus, it is unlikely that they will interact with the existing agent. The two Eueupithecia species may interact with each other, as they both feed on leaf material. However, the geographic distribution of the two species does not overlap in their native range in Argentina (with Eueupithecia cisplatensis and Eueupithecia vollonoides occurring in southern and northern Argentina, respectively) (Hausmann et al. 2016), likely due to differential adaptation to climatic conditions (Mukherjee et al. 2021). We anticipate that the two species will occupy different climatic zones after introduction to Australia and, therefore, that the geographic distribution of the two species will be complementary in the context of biological control (Mukherjee et al. 2021).

The native range surveys and no-choice tests used to evaluate the specificity of Eueupithecia species delivered a consistent result: strong specificity to one plant species, P. aculeata. Surveying closely related species growing sympatrically in the native range of the target is informative in determining the host specificity of the proposed biological control agent (Witt 2004; Goolsby et al. 2006). Although the field survey in the native range could only be conducted on a small number of legume species, the fact that Eueupithecia species were not found on the co-occurring congenic Parkinsonia praecox constitutes strong evidence of a restricted ecological host range.

Laboratory no-choice testing on both cut and living plant material revealed that Eueupithecia species larvae did not feed on the non-target plant species tested, except for P. praecox. Eueupithecia cisplatensis recorded a very low rate of survival and development to the adult stage on the closely related P. praecox; only 3% of larvae completed development on this non-target species in contrast to over 60% on P. aculeata. Under restricted conditions, many herbivorous insect species display extended host ranges (Sheppard et al. 2005; Rafter et al. 2021). That is, they feed and develop on food sources upon which they typically would not in nature (Heard & van Klinken 1998; Heard 2000). The development of E. cisplatensis on P. praecox could be a result of the artificial conditions in cages, considering that this host was not found to be used by Eueupithecia species in the field. The inability of both Eueupithecia species to survive or develop on the 70 plant species tested provides strong evidence that both species are sufficiently host specific and of low risk to non-target plants in the field.

The COI gene mitochondrial haplotypes are entirely species specific and so post-release identification of E. cisplatensis and E. vollonoides can be undertaken using this marker. The nuclear gene haplotypes, by comparison, are inappropriate for discriminating between the species post-release because some are shared by both species in laboratory cultures (Fig. 2). Typically, this pattern of mito-nuclear discordance would preclude the use of mitochondrial genes as diagnostic markers. However, several observations suggest that nuclear genetic admixture is not the result of recent hybridisation. Few nuclear gene haplotypes are shared by both species. Of the CAD haplotypes that were assigned to a species that is atypical for the associated haplotype group, three out of five of these atypical haplotypes were unique to the atypical species (CAD-Hap13, CAD-Hap16 and CAD-Hap17 in Fig. S1). We would expect more haplotypes to be shared between the Eueupithecia species, compared with what was observed, if cross-mating were common and had occurred recently. Further, if the atypical CAD haplotypes originated from recent hybridisation, they should be present in the typical species as well, and probably at a higher frequency than in the atypical species.

Nevertheless, the cause of the mito-nuclear discordance requires further investigation. We caution that the molecular data presented here were not intended to address this question and that additional population genetics studies using individuals collected from a larger area in the native range of these Eueupithecia species are required to properly investigate this pattern and its biological relevance. Incomplete lineage sorting, or species-biased mating of any hybrid individuals, might also be influencing the observed patterns. The cause of mito-nuclear discordance can be difficult to resolve, and nuclear introgression without mitochondrial introgression is rarer than the inverse (Toews & Brelsford 2012), but it has been recorded before in Lepidoptera (Mullen et al. 2008). The geographic distribution of E. cisplatensis and E. vollonoides in their native range in Argentina is largely allopatric (Hausmann et al. 2016), but there may be a zone of overlap near central Santa Fe and Corrientes provinces around 29° south latitude where mating may occur and where such comparisons can be made in sympathy.

Based on the host specificity test results, approval was granted by the Commonwealth of Australia to release E. cisplatensis and E. vollonoides in Australia in 2012 and 2014, respectively. Extensive releases are currently underway, with early signs indicating establishment of both species on P. aculeata infestations across northern Australia. These Eueupithecia species may also be potentially useful as biological control agents in countries of tropical Africa and the Pacific islands where P. aculeata infestations are recorded.

**RESEARCH PERMITS**

The exportation of the biological control agents from Argentina were made under permits from the Dirección de Fauna Silvestre and Dirección Nacional de Ordenamiento Ambiental y Conservación de la Biodiversidad de Argentina (Permit No. 33952/12; 03982/13) and Servicio Nacional de Sanidad y Calidad Agroalimentaria (DNPV Permit No. 599/12; 129/13). Importation and release of the biological control agents in Australia were made under Commonwealth of Australia permits (IP12015739; IP12015735; IP09095710; IP13002539) and
Biosecurity WA Permits (00767; 00768; 00770; 001537; 002098; 002100).

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**Figure S1.** Haplotype networks of the (a) COI gene region, (b) CAD gene region, and (c) 28S gene regions for *Eueupithecia cisplatensis* and *E. vollonoides* individuals. Each individual is coloured according to their origin (cultured in lab, collected in native range and field collected in the introduced range). The size of each circle represents the number of sequences that were found with each haplotype. In the legend, AR indicates Argentina, and AUS indicates Australia. A duplication of Figure 2, but with haplotypes numbered separately for each network (Table S3).

**Table S1.** The plant species and number of replicates undertaken when testing the host specificity of *Eueupithecia cisplatensis* and *E. vollonoides* in laboratory conditions in Argentina and Australia.

**Table S2.** The number of *Eueupithecia cisplatensis* and *E. vollonoides* individuals that sequences were successfully obtained for is listed for each of the gene regions that were included in this study. AR indicates samples from Argentina, and AUS indicates samples from Australia.

**Table S3.** Gene regions and primers used in mitochondrial and nuclear gene barcoding of *Eueupithecia vollonoides* and *E. cisplatensis* samples.

**Table S4.** Details of the *Eueupithecia vollonoides* and *E. cisplatensis* samples used in mitochondrial and nuclear gene barcoding. The haplotype code for the COI, CAD and 28S genes are also listed for individuals where sequence data was obtained. 28S and CAD have two haplotypes each because they are phased (i.e., the haplotypes for these individuals have been estimated from a single ambiguous sequence).