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Assessment of genetic structuring in the *Lygodium* fern moths *Austromusotima camptozonale* and *Neomusotima conspurcatalis* in their native range: implications for biological control

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ABSTRACT

Assessing the genetic diversity and structuring of potential biological control agents in their native range can be a vital step in developing efficient biological control programs. These assessments, however, are not routinely conducted prior to release of agents. Assessments of these variables after releases have been made may also provide valuable insights into why some agents fail to establish in the introduced range. We therefore examined, within their native distribution, the phylogeographic structuring of two biological control agents previously released for the control of *Lygodium microphyllum* in Florida, USA: *Neomusotima conspurcatalis* (which has successfully established) and *Austromusotima camptozonale* (which has failed to establish). Strong regional genetic structuring was detected within *N. conspurcatalis*, with three distinct geographical clades identified. The Australian populations were, surprisingly, not recovered as monophyletic. In *A. camptozonale*, by contrast, regional genetic structuring was far less clear. Seven distinct haplotypes were identified from Cape York Peninsula of northern Queensland, Australia (the putative geographic origin of the Florida *L. microphyllum*). The moths released in Florida were from southern Queensland, with material from this region having a unique haplotype not present in any of the Cape York Peninsula material. Further testing is required to confirm the species status of the distinct mitochondrial lineages in both *A. camptozonale* and *N. conspurcatalis*, and to assess whether any of these lineages are better adapted and more damaging to the Florida *L. microphyllum* genotype than the lineages previously released.

1. Introduction

Biological control is often considered the most economical and environmentally acceptable means of managing invasive weeds (Hoddle, 2004; Seastedt, 2015). One of the great practical challenges, though, is the lack of predictability in terms of establishment success and efficacy of the introduced agent (Greathead, 1986; Ehler, 1990; Harris, 1998). Indeed, a recent review indicated that only 27% of invasive plant biological control programs have contributed to significant control of the target weed (Van Driesche et al., 2010), and the extent of control contributed by individual species is usually only partial (McFadyen, 1998).

Incorporating modern genetic tools into biological control research has the potential to substantially improve the success rate of biological control programs (see Gaskin et al., 2011; Vorsino et al., 2012). Such research, however, often focuses solely on identifying the provenance of invasive weeds, so that natural enemies from this locality can be sourced and released in the invasive range. This approach is based on the belief that local adaptations lead insects to be more damaging to plants from populations with which they have co-evolved (Kniskern and Rausher, 2001). While this has been demonstrated in some systems (Goolsby et al., 2006) the generality of this hypothesis has not yet been tested, despite this issue having been identified over a decade ago (Hufbauer and Roderick, 2005).

We believe that molecular approaches can be used for more extensive purposes. For instance, assessing phylogeographic structuring within potential biological control agents can be very helpful in recognising host-plant specific cryptic species from among reputed host plant generalists (Rafter et al. 2013). Indeed, in recent years cryptic species have been identified in candidate biological control agents

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(Rauth et al., 2011; Toševski et al., 2013) as well as in biological control agents that have already been released (Roehrdanz et al., 2011; Paterson et al., 2016).

A high level of genetic diversity within the populations of agents released into the field is often considered to be important in successful establishment, as it is considered to increase the probability of local adaptation after establishment in the field (Force, 1967; Remington, 1968; Messenger et al., 1976; Reed et al., 2003; Phillips et al., 2008). Knowledge of the genetic structuring of arthropod taxa in their native range may also help determine their dispersal capacities (Gaskin et al., 2011). For instance, genetic homogeneity of an arthropod species over a broad spatial scale-indicating a high level of gene-flow-may suggest the potential for efficient spread in their new environment. Strong genetic structuring in the native range may indicate reduced dispersal ability, which may suggest that the biological control agent may not spread quickly and effectively in the new environment. Such information should be helpful in designing release strategies of potential biological control agents (Jonsen et al., 2007; Rauth et al., 2011).

An assessment of native range population structure of potential biological control agents prior to their release is optimal, as it could help direct the selection of agents for biological control and the geographic design of release strategies. Such assessments, after releases have been made, may also provide valuable insights into why some agents fail to establish in the introduced range, by determining whether the most genetically appropriate plant-herbivore match was made.

Lygodium microphyllum (Pteridophyta: Lygodiaceae) is a rapidly spreading invasive weed that already covers a broad area of southern Florida in the United States (Pemberton and Ferriter, 1998). *Lygodium microphyllum* is a fast growing vine-like fern that climbs over trees and shrubs, smothering and shading native understorey vegetation (Nauman and Austin, 1978; Pemberton and Ferriter, 1998). Native to Africa, Asia and Australia, it was first found naturalized on the south-eastern coast of Florida in 1968, and was considered well established by 1978 (Beckner, 1968; Nauman and Austin, 1978). Management of *L. microphyllum* in Florida has proved extremely difficult. Herbicides provide the primary management option in many areas (Hutchinson et al., 2007), but are expensive (Pemberton et al., 2002) and often have substantial non-target effects on native vegetation (Hutchinson, 2010). Mechanical removal and burning are not effective long term control methods as the fern readily reshoots from rhizomes (Goolsby et al., 2003; Stocker et al., 2008). Biological control is therefore considered the best prospect for long-term management of *L. microphyllum* (Pemberton and Ferriter, 1998).

Goolsby et al. (2006) identified the putative origin of the invasive Florida lineage of *L. microphyllum* as the Iron Range area of Cape York Peninsula in north eastern Australia, with subsequent molecular analysis suggesting this lineage is found throughout the Cape York Peninsula (McCulloch et al., unpublished). It was demonstrated that leaf-galling mites (*Floracarus perrepae*) from this region were more damaging to *L. microphyllum* than mites collected from other areas, many of which could not survive on Florida *L. microphyllum* (Goolsby et al. 2006). *Floracarus perrepae*, sourced from Iron Range National Park, was released in Florida from 2008 to 2010, but its establishment in Florida was unexpectedly low, with populations initially failing to establish in most release plots (Boughton and Pemberton, 2011). Populations have, however, begun to establish in isolated patches in Martin County and the Everglades National Park (Lake et al., 2014).

In addition, two defoliating moths (Pyralidae: Musotiminae) have also been released in Florida to control *L. microphyllum*: *Austromusotima camptozonale* (Hampson) and *Neomusotima conspurcatalis* (Warren). These closely related moths have overlapping native range across northern Australia and southern Asia, and laboratory host-range testing demonstrated that their larvae were able to develop fully only on members of the genus *Lygodium* (Goolsby et al., 2003). Both species have similar ecological requirements, and were highly damaging to *L. microphyllum* in laboratory host range tests (Goolsby et al., 2003; Boughton et al., 2009), but their establishment and spread in Florida has been very different. *Austromusotima camptozonale* was first released in Florida in 2004, but despite numerous release efforts, persistent populations have not yet established in the field (Boughton and Pemberton, 2008, 2012). *Neomusotima conspurcatalis* was released at a number of sites in 2008 and 2009 and large, persistent field populations established readily at several sites (Boughton and Pemberton, 2009). This species has also spread beyond the points of initial release, but still only exists in isolated patches in central Florida (Smith et al., 2014).

In this paper we examine the genetic diversity and phylogeographic structuring of these two pyralid moth species across their native range using mitochondrial (COI) and nuclear (28S rRNA) sequences. We then assess whether the contrasting establishment success of these species in Florida may be correlated to the particular mitochondrial lineages released, and highlight potential directions for further exploration and evaluation for impact and host-range involving these species. The results, and the approach taken, also have implications for the wider and more effective use of molecular techniques in biological control.

2. Materials and methods

2.1. Collection of samples

Extensive surveys of *L. microphyllum*, *Lygodium japonicum*, *Lygodium flexuosum* and *Lygodium reticulatum* were conducted in sub-tropical and tropical Australia from northern New South Wales to Cape York and west through the tropical regions of the Northern Territory and Western Australia as well as in Indonesia, Singapore, Hong Kong, Malaysia Thailand, and China (Fig. S1). Adults and larvae of *N. conspurcatalis* and *A. camptozonale* were primarily collected from *L. microphyllum* stands, however several specimens were collected from *Lygodium flexuosum*. Collections were made using a combination of searches above and below ground, beating trays, sweep nets, and light traps, as described by Goolsby et al. (2003). Specimens were identified morphologically, and either dried on pins or stored in 95% ethanol prior to DNA extraction.

2.2. DNA extraction, amplification and sequencing

Between one and ten specimens were sequenced per collection site to examine intraspecific genetic structuring within *A. camptozonale* and *N. conspurcatalis*. In addition specimens of other *Lygodium* Pyralidae species (specifically, *Lygomusotima stria*, *Siamusotima aranea*, and an undescribed *Ambia* species) were sequenced to provide outgroups for the molecular analyses. Genomic DNA was extracted using either CTAB (Doyle, 1991) or using spin columns (following the protocols of Ridley et al., 2016). A 732-bp portion of the mitochondrial cytochrome oxidase I (COI) locus was amplified from ethanol preserved insects and more recent (< 10 year old) pinned samples using the primers TYJ1460 (Simon et al., 1994) and HCO2198 (Folmer et al., 1994). In cases where this region could not be amplified (mainly older pinned specimens) a smaller (450-bp) fragment was amplified using the primers C1J1718 (Simon et al., 1994) and HCO2198. A 1000-bp fragment of 28S rRNA was amplified using primers D2-3665F (Belshaw and Quicke, 1997) and D5-4749R (Danforth et al., 2006). PCRs were carried out as 12 µl reactions containing 0.03 units of My Taq™ (Bioline, London, UK), 1 × buffer, and 0.2 µm of forward and reverse primers, and 2 µl of template DNA. Amplification involved an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 50 °C for 30 s, and 72 °C for 45 s, with a final extension for 7 min at 72 °C. PCR products were cleaned using one unit each of Exonuclease I and Antarctic Phosphatase (New England Biolabs, Massachusetts, USA), with sequencing reactions performed using an ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at Macrogen (Korea).