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Assessing the scope for genetic rescue of an endangered butterfly: the case of the Eltham copper

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Abstract

1. Genetic rescue is an under-utilised conservation strategy used to boost the genetic diversity and/or fitness of small, isolated populations through the introduction of novel genes. Effective implementation of genetic rescue requires an understanding of the evolutionary history and genetic distinctiveness of populations.
2. The Eltham copper butterfly (ECB) is an endangered subspecies that exists as small, isolated populations across four disjunct regions in Victoria, Australia.
3. Mitochondrial and nuclear sequence data were used to assess the genetic distinctiveness of ECB from its nearest relative: the fiery dull copper butterfly (FDC), explore the evolutionary history and population structure of ECB across its contemporary distribution, and assess the scope for genetic rescue of the most-threatened ECB population in Eltham-Greensborough.
4. Findings support the current status of ECB and FDC as distinct evolutionary units, which should continue to be managed separately given current knowledge.
5. Modest population genetic structure across the range of ECB was detected. However, low levels of historical gene flow and shared haplotypes between Eltham-Greensborough and the northern regions suggest relatively recent divergence.
6. Given relatively recent divergence, low population numbers and low genetic diversity, the population located in the Eltham-Greensborough region is a strong candidate for genetic rescue.
7. Using established criteria, explicit recommendations are made for the implementation of genetic rescue via translocations. Genetic rescue, together with ongoing habitat management, should improve genetic diversity, fitness and persistence of the ECB in the region most threatened by urban disturbance.
8. Any risks associated with genetic rescue in conservation should be weighed against extinction risk as a result of inaction.

Keywords

Genetic diversity, conservation recommendations, population structure, Lepidoptera, *Paralucia pyrodiscus lucida*, *Paralucia pyrodiscus pyrodiscus*, Lycaenidae

Introduction

Human-induced destruction of natural habitats and associated habitat fragmentation are major drivers of global biodiversity loss (Foley et al., 2005, Rands et al., 2010). Many Lepidoptera (butterfly and moth) species have experienced population decline as a result of human-induced habitat loss and fragmentation (Habel et al., 2010, Thomas et al., 2004, Wenzel et al., 2006, Wilcove et al., 1998). Butterflies in particular have proven to be charismatic flagship species, eliciting public sympathy and attracting a substantial amount of resources relative to other invertebrate taxa. Butterflies are also commonly used as indicator taxa for environmental conditions and umbrella taxa for a broader guild or habitat-type (Caro and O'Doherty, 1999, Finnell and Lehn, 2007, Guiney and Oberhauser, 2009, New, 1997).

In fragmented landscapes, populations typically exist as small, isolated subunits that are at elevated risk of extinction due to the combined influences of environmental, demographic and genetic stochasticity (Caughley, 1994, Spielman et al., 2004). Strong effects of genetic drift in small populations lead to loss of genetic diversity, associated loss of fitness and inbreeding depression, and reduced capacity to evolve in response to environmental change (Crnokrak and Roff, 1999, Frankham, 1996).

Dispersal and associated gene flow are fundamental population processes that can counteract the effects of genetic drift in small populations, boosting genetic diversity and, in turn, increasing a population's capacity to adapt to environmental changes (Wardell-Johnson et al., 2011). Gene flow is often restricted between populations due to geographic isolation. Thus, gene flow in the form of 'genetic rescue', where novel alleles are introduced to increase genetic diversity and/or fitness in small populations, can be a powerful conservation tool (Hufbauer et al., 2015, Hoffmann et al., 2015, Whiteley et al., 2015, Weeks et al., 2011). Many taxa have been shown to respond positively to genetic rescue (Frankham, 2015), but concerns about outbreeding depression (the production of low-fitness progeny as a result of outcrossing) and genetic swamping (loss of locally-adapted or phenotypically distinctive genotypes) hamper the utilisation of genetic rescue as a conservation strategy (Hoffmann et al., 2015, Wootton and Pfister, 2015).

With sufficient knowledge about the evolutionary history of taxa, scenarios in which outbreeding depression is likely to be problematic are typically quite readily identifiable, and the potential harm to populations tends to be overstated (Frankham et al., 2011). Conversely, inbreeding and low genetic variation are strongly linked to increased extinction probability (Frankham, 2010, Frankham et al., 2011). This is exemplified by the classic case of the

Glanville fritillary butterfly, in which genetic variation at only a few neutral genetic loci was the major predictor of extinction-proneness of patch populations in the wild, even compared to important ecological variables (Saccheri et al., 1998). Thus, for endangered taxa, any risk of outbreeding depression and swamping as a result of genetic rescue should be weighed against the risk of extinction due to inaction (Frankham et al., 2011, Weeks et al., 2011).

In this study we explored the evolutionary history and scope for genetic rescue of a threatened invertebrate, the Eltham copper butterfly *Paralucia pyrodiscus lucida* (henceforth 'ECB'). The ECB has become an important flagship taxon for insect conservation and was one of the first Australian invertebrates to be listed as threatened and endangered under State, and later national, legislation (*Victorian Flora and Fauna Guarantee Act 1988*, *Environment Protection and Biodiversity Conservation Act 1999*, respectively). Despite its conservation status, to date, no studies have explored the evolutionary distinctiveness of the ECB.

The ECB is currently regarded as a subspecies of *P. pyrodiscus* (Braby, 1990, Canzano et al., 2007). The ECB is morphologically distinguished from the more widespread *P. pyrodiscus* subspecies, the fiery dull copper butterfly *P. p. pyrodiscus* (henceforth 'FDC'), primarily by the extent of copper scale brightness on the wings (Braby, 1990, Braby et al., 1999). The current ranges of the subspecies do not overlap; the FDC has a patchy distribution from north Queensland to east Victoria and the ECB is confined to central and west Victoria (Braby et al., 1999). Both subspecies are restricted to open eucalypt woodland habitat and have complex lifecycles that include obligate ecological relationships with plants belonging to the Pittosporaceae family and ants of the genus *Notoncus* (Braby et al., 1999). In contrast to the FDC larvae, which are known to feed on at least three different plant species, the ECB is strictly associated with the plant sweet bursaria *Bursaria spinosa*; the ECB lays its eggs on the stems or foliage of sweet bursaria and larvae feed on the leaves of the plant (Braby, 1990, Braby et al., 1999, Canzano et al., 2007). *Notoncus* ants protect the butterfly larvae during nocturnal feeding in return for sugary secretions (Braby, 1990). There are no records of ECB occurring in the absence of *Notoncus* ants (Vaughan, 1988).

The ECB was first discovered in the Eltham-Greensborough area of outer north-eastern Melbourne in 1938, and was thought to have gone extinct by the 1950s, until its rediscovery there in 1987 (New, 2011, Yen et al., 1989) (Figure 1). Within the Eltham-Greensborough region the ECB is known to occur in 10 isolated locations. Since 1987, additional small, disjunct populations of ECB have been identified in the Horsham (3 locations), Bendigo (6

locations) and Castlemaine (2 locations) regions of northern Victoria up to ~335 km away from Eltham-Greensborough (Figure 1) (personal communication, New, T. R.). The natural range of the ECB prior to human influences is unknown, but extinctions at Tallarook, Murtoa, Dimboola, Keilor and Broadmeadows over the course of the 20th century support a historically broader and more continuous range in Victoria (Braby et al., 1999) (Figure 1). Given the apparent low mobility of ECB, genetic exchange among regions, or among colonies within regions isolated by an urban or agricultural matrix, is considered unlikely (Braby et al., 1999, Webster, 1993).

The decline in range and abundance of the ECB is largely attributed to loss of suitable habitat through large-scale agricultural expansion and urbanisation (Canzano et al., 2007) (Figure 1). ECB numbers fluctuate each year but remain generally low (no more than a few hundred adults and larvae observed on any site, commonly far fewer), particularly in the Eltham-Greensborough region (New, 2011). Of the locations where ECB occurs, the Eltham-Greensborough region is the most urbanised and fragmented, with conservation-dependent sites subject to natural successional changes and a variety of anthropogenic threats including vandalism and dumping of rubbish (Canzano et al., 2007). The small isolated Eltham-Greensborough colonies are therefore of particular conservation concern.

The rediscovery of the ECB in 1987 prompted the formation of a volunteer working group in 1989, the “Friends of the Eltham Copper Butterfly”, which works with local councils, community and state government to monitor and manage the subspecies. To date, conservation efforts have focused on the protection, preservation and restoration of sweet bursaria habitat across its known range (Webster, 1993) and include the control of weeds and pests. However, there is scope for future implementation of genetic rescue via translocations among extant populations, perhaps especially by boosting the small periurban populations near Melbourne with individuals from larger, more rural populations. Understanding the extent of recent and past genetic exchange between colonies can provide useful insights into the evolutionary distinctiveness of populations and the suitability of different colonies as source- and recipient populations in any future translocations (Frankham et al., 2011, Weeks et al., 2011).

In this first genetic study of *P. pyrodiscus*, we used mitochondrial and nuclear sequence data to assess the genetic distinctiveness of ECB from FDC, and to explore the evolutionary history and population structure of ECB across its contemporary distribution. We sought to

determine the appropriate scale of management for ECB (regional or state-wide) and assessed the scope for genetic rescue of the most-threatened ECB population in Eltham-Greensborough.

Materials and methods

Study Region and Sampling

A total of 66 ECB individuals were sourced from all four geographic regions in Victoria where extant populations of ECB are known to occur: Eltham-Greensborough, Castlemaine, Bendigo and Horsham (Table 1; Figure 1). Each region was represented by two sampling locations pooled for analysis (Table 1). Samples were collected during the breeding season (October to December) and, where possible, early instar larvae were collected, to reduce the demographic and genetic impacts of sampling, given that this life stage has the highest rate of natural mortality (Table 1). Given the threatened status of the ECB, sample sizes were necessarily limited to 5-10 individuals per sampling location per year (Table 1). Larvae were either frozen fresh and stored at -80°C or placed in 95% ethanol, and stored at -20°C. Adult samples were pinned and mounted. Legs from an additional 20 ECB pinned adults were sourced from museum and private collections (Table 1). Five adult FDC samples were collected from a single location at Mt Noorinbee in Gippsland, Victoria (Figure 1), chosen to represent the FDC geographically closest to the contemporary range of the ECB and thus likely to have most gene flow with ECB.

Laboratory methods

DNA was extracted from larvae using a modified salting-out protocol (Sunnucks and Hales, 1996). The detailed extraction protocol is provided in Appendix A. DNA from adult specimens was extracted from 2-3 legs or ~2 mm³ muscle tissue, using the Qiagen DNeasy Tissue Kit following the standard protocol (Qiagen, Valencia, CA).

Individuals were screened for sequence variation at two mitochondrial regions (ND5 and CO1), chosen for their different but complementary evolutionary rates and expected ability to resolve different taxa (Hill, 2015, Norgate et al., 2009). A 617 base pair (bp) section of the CO1 mitochondrial gene was amplified using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994). New primers were designed to amplify a 389 bp section of the ND5 mitochondrial gene (details in Appendix B). Because DNA from the older (pre-1990) samples sourced from museum and private collections was highly degraded, sets of primers

were developed for amplifying and sequencing CO1 and ND5 in a series of shorter fragments (< 180 bp each) (Appendix B). We found no conclusive evidence of temporal patterning between historical (1987, 1989) and recent (2011, 2015) samples (Table 1).

Sequencing only mitochondrial regions can lead to biased inferences about the evolutionary history of species (Ballard and Whitlock, 2004). For this reason, individuals were also screened for variation at five anonymous single copy nuclear sequence loci: Z5V8 (302 bp), 63EA (286 bp), S5ZW (286 bp), A7D8 (264 bp), and 03W6 (246 bp). These nuclear loci were designed based on whole genome shotgun sequencing data. Shotgun sequencing was performed on the Roche 454 GS-FLX Titanium platform (Roche, 454 Life Sciences, Branford, CT, USA) at the Australian Genome Research Facility (AGRF; Brisbane, Australia) following the methodology of Gardner et al. (2011) and Bertozzi et al. (2012). The bioinformatics pipeline used to target sequence variable, single-copy, non-coding nuclear markers avoided mitochondrial DNA, known highly conserved regions, open reading frames and members of complex gene families. Details of nuclear primer design and testing are provided in Appendix C. Primer sequences are provided in Appendix B. Locus-specific PCR conditions are provided in Appendix D. PCR products were sequenced commercially at Macrogen, South Korea, using forward primers. Reverse primers were used for resolving ambiguous peaks. Sequence chromatograms were edited and aligned using Geneious Pro 7 (Kearse et al., 2012). The absence of insertions/deletions and stop codons in the CO1 and ND5 genes supported their mitochondrial origin (rather than being mitochondrial pseudogenes, Sunnucks and Hales, 1996). For the nuclear loci, the gametic phases of heterozygous individuals were resolved using the Phase 2.1 algorithm (Stephens and Donnelly, 2003) in DnaSP 5.10.1 (Librado and Rozas, 2009) using PHASE default settings (0.90) for the confidence probability thresholds (Garriick et al., 2010).

Longer-term, phylogeographic signatures of genetic divergence of P. pyrodiscus populations

Genetic differences between populations can accumulate on different time-scales via the processes of genetic drift, mutation and natural selection. Pairwise distances (proportion of nucleotide differences) between subspecies and regions were calculated in MEGA 5.0 (Tamura et al., 2011). To assess the genetic distinctiveness of *P. pyrodiscus* populations on phylogeographic (i.e. longer-term, accumulated mutations/sequence differences) timescales

we constructed median-joining haplotype networks for each locus and concatenated mitochondrial regions using PopART (<http://popart.otago.ac.nz>) (Bandelt et al., 1999). Input files for PopArt were edited so that deletions would be recognised as nucleotide differences. Because networks for nuclear loci do not account for within-locus recombination they may not represent true genealogical relationships, but are useful for visualising geographic patterns of haplotype distributions. Single-locus networks are not necessarily expected to represent species phylogeny because unlinked nuclear loci have different evolutionary histories due to the stochastic effects of drift (Page and Charleston, 1997).

To build species phylogeny, we constructed a multilocus species tree and estimated divergence time between the ECB and FDC using *BEAST implemented in BEAST 2.4.2 (Bouckaert et al., 2014, Heled and Drummond, 2010). PartitionFinder 1.1.1 (Lanfear et al., 2012) was used to determine appropriate substitution models for the mitochondrial loci. For all nuclear loci, bModelTest (Bouckaert and Drummond, 2017) was set as the site model, run with the default model-averaging setting. The Yule process model was used for the species tree prior to best resemble species-level phylogenetic processes (Drummond et al., 2007). Mitochondrial trees were linked. The species trees population function was assumed to be linear with constant root. Strict clocks were applied to all partitions. Clock rates for the CO1 and ND5 mitochondrial regions were assigned log normal distributions. For CO1 the clock rate prior distribution was defined by a mean = 0.0113, with 95% credibility intervals of 0.008-0.0146. For ND5, the clock rate prior distribution was defined by a mean = 0.0159, with 95% credibility intervals of 0.012-0.0198. Clock rates for mitochondrial regions were based on substitution rates estimated for beetles in the genus *Carabus* (Coleoptera, Carabidae) using geological and fossil calibrations (Andujar et al., 2012). These rates have previously been used to estimate divergence time across *Polyura* butterflies in the Solomon Islands (Toussaint et al., 2016). Clock rate priors for anonymous nuclear loci were assigned uniform distributions with uninformative credibility intervals (0.001-0.05). Because we sampled clock rates from a distribution rather than setting fixed rates, our analysis accounted for uncertainty in both mitochondrial and nuclear clock rates. The analysis was run for 1×10^7 generations and sampled every 1×10^4 steps. Three replicate runs were checked for convergence, combined and analysed in Tracer version 1.6 (Rambaut et al., 2014), after discarding the first 10% of samples in each run as burn-in. Runs were combined using LogCombiner 2.4.2. Trees were summarised using TreeAnnotator 2.4.2 and visually assessed using FigTree 1.4.2 and DensiTree 2.4.2.

Shorter-term signatures of divergence and population structure of P. pyrodiscus populations

Population structure and shorter-term signatures (i.e. allele frequency differences due to genetic drift) of genetic divergence among *P. pyrodiscus* populations were explored using three different genotype- and allele- frequency-based methods as outlined below.

First, we used STRUCTURE 2.3.4 (Pritchard et al., 2000) to infer patterns of population structure based on individuals' genotypes at nuclear loci. STRUCTURE uses a Bayesian clustering algorithm to identify the most likely number of genetic groups or clusters (K) across samples and to proportionally assign individuals to each of the clusters. STRUCTURE was run using the admixture model with correlated allele frequencies. Twenty replicates (of 3×10^6 Markov Chain Monte Carlo, after a burn-in period of 10^6 repetitions) were run for K from 1 to 10. Information on sampling location was not included in the analysis. Results were summarised using the web-server CLUMPAK (Kopelman et al., 2015). The most likely number of K was chosen according to the Evanno ΔK method (Evanno et al., 2005).

Second, we used allele-frequency based methods in Arlequin 3.5.2.2 (Excoffier and Lischer, 2010) to assess the extent of genetic differentiation across sampled *P. pyrodiscus* populations. Pairwise genetic differentiation between ECB and FDC and between the four ECB regions was measured using F_{ST} (based on haplotype frequencies alone) and Φ_{ST} (based on haplotype frequencies as well as level of sequence divergence).

Third, we used hierarchical analysis of molecular variance (AMOVA) to calculate the proportion of genetic variance in allele frequencies across ECB samples explained by different levels of geographic hierarchy. Genetic variance was partitioned into components corresponding to three different geographic scales: 1) among individuals within regions; 2) among regions; and 3) between Eltham-Greensborough and northern Victoria.

Assessing genetic diversity across the four ECB regions

Characterising levels of genetic diversity across the distribution of the ECB can help to identify populations that are at elevated risk of extinction (e.g. populations that could benefit from genetic rescue), as well as populations with higher levels of genetic diversity and/or different diversity (e.g. populations that could act as source populations for translocations).

To assess genetic diversity across the ECB range we used DnaSP 5.10.1 (Librado and Rozas, 2009) to calculate a suite of genetic diversity measures for each sampling region: the number

of variable sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and the average number of nucleotide differences (K). We used GenAlEx 6.503 (Peakall and Smouse, 2006, 2012) to measure heterozygosity among nuclear loci across populations.

Testing for historical gene flow between the four ECB regions

According to established criteria of Frankham et al. (2011), a history of gene flow within the last 500 years (i.e. a recent period encompassing most human-induced impacts on wildlife globally, including habitat clearance) would suggest a low risk of outbreeding depression under assisted migration across the ECB's contemporary distribution. We used Bayesian coalescent modelling in MIGRATE-N (Beerli and Palczewski, 2010) to test for and quantify historical ($4N_e$ generations into the past, where N_e = effective population size) gene flow between the four geographically disjunct ECB regions (Horsham, Castlemaine, Bendigo and Eltham-Greensborough). The MIGRATE-N model estimated the mutation-scaled effective population size (Θ) for each of the four regions, and the mutation-scaled migration rate (M) between each pair of regions. For nuclear loci, Θ is equal to $4 \times$ effective population size (N_e) $\times$ mutation rate per site per generation (μ) and M is the immigration rate per generation (m) divided by μ . Mutation-scaled migration rates were converted to number of migrants per generation (N_m) using the formula: $N_m = M\Theta/4$ (Beerli and Felsenstein, 1999). Nuclear loci and mitochondrial regions were assigned inheritance scalars of 1 and 0.25, respectively, so that Θ for all loci was expressed as $4N_e\mu$. MIGRATE-N was run using the sequence model and Bayesian inference. We set uniform priors on both Θ and M ($U[0-0.2]$ and $U[0-50000]$, respectively) (Appendix E, Table E1). Four heated chains were run using default (or recommended) temperatures (1.0, 1.5, 3.0 and 1×10^6), and the auto-tune option was implemented. For each locus we ran 20 replicate chains, recording samples every 100 steps for a total of 10000 samples per replicate chain. The first 5×10^6 steps of each replicate chain were discarded as burn-in. Convergence was assessed by examining the posterior distribution of parameters across loci, ESS values and acceptance ratios (Appendix E, Table E2).

Results

Support for evolutionary distinctiveness of ECB from FDC

Haplotype networks supported the evolutionary distinctiveness of the ECB from the geographically closest FDC population at all loci except Z5V8, where the FDC haplotype was shared with ECB individuals from the Horsham, Castlemaine and Eltham-

Greensborough regions (Figure 2). Mean sequence divergence between ECB and FDC was 4.5% for the COI locus and 5.9% for ND5 (Appendix F, Table F1). Because the sorting of nuclear genes is slower than of mitochondrial genes, unsurprisingly, nuclear sequence divergence between the ECB and the FDC was lower (mean = 1.2%, range: 0.5 – 2.3%; Appendix F, Table F1). Nevertheless, this difference between subspecies was greater than the difference between Eltham-Greensborough and the northern Victorian regions (Bendigo, Castlemaine and Horsham; mean = 0.5%, range: 0.0 – 2.0%; Appendix F, Table F1). Long-term divergence between the two subspecies was also reflected in very strong genetic differentiation (nuclear mean $\Phi_{ST} = 0.600$, $p < 0.05$, mitochondrial mean $\Phi_{ST} = 0.951$, $p < 0.05$; Appendix F, Table F2), which was greater than that within ECB (Appendix F, Table F2). FDC samples from the single location at Mt Noorinbee were represented by a single haplotype at all loci, unique among all samples reported here at all but one locus (Figure 3).

The *BEAST analysis converged and all parameters had effective sample size (ESS) values above 200, with the exception of the “hasEqualFreqs.s” statistic for the S5ZW and Z5V8 loci (ESS = 184 and 94, respectively). *BEAST estimated that ECB and FDC diverged c. 1.3 Mya (95% HPD: 0.67-2.01) (Appendix G).

Longer-term signatures of divergence between ECB regions

The geographic distribution of haplotypes across all loci supported low-to-moderate divergence of the Eltham-Greensborough region from the Horsham, Castlemaine and Bendigo regions on evolutionary timescales (i.e. that based on accumulated new sequence variation, rather than redistribution of existing variation) (Figure 2). There was little evidence of strong phylogeographic structure among the Bendigo, Castlemaine and Horsham regions at nuclear loci (Figure 2). However, among the northern Victorian regions there was some evidence of weak geographic structuring of mitochondrial haplotypes, particularly at the ND5 region (expected to be the most resolving sequence given a typically higher mutation rate than that of COI and rapid lineage sorting expected for mtDNA, Norgate et al., 2009). Concatenated ND5 and COI mitochondrial regions supported distinct haplotypes for each of the Eltham-Greensborough, Castlemaine and Bendigo regions, with one additional haplotype shared between Castlemaine and Horsham (Figure 2).

Population structure and shorter-term signatures of distinctiveness of P. pyrodiscus populations

Results of analyses of population structure based on genotype- and allele frequencies were consistent with the evolutionary distinctiveness of ECB from FDC, and with some divergence of the Eltham-Greensborough region from the northern Victorian regions on phylogeographic timescales (Figure 3). Under the delta K method, STRUCTURE analysis supported the presence of three main genetic clusters across *P. pyrodiscus* samples, corresponding to the Eltham-Greensborough region, the northern Victorian regions and the FDC samples from Gippsland (Figure 3; Appendix H, Figure H2). Genetic distinctiveness of the Eltham-Greensborough region from the three northern Victorian regions was supported by strong genetic differentiation (mean Φ_{ST} = 0.49-1.0 and 0.28-0.48, at mitochondrial and nuclear loci, respectively; Appendix F, Table F2). Lack of strong phylogeographic structure across northern Victoria was supported by lower pairwise genetic differentiation among Bendigo, Castlemaine and Horsham (mean Φ_{ST} = 0.34-0.5 and 0.10 – 0.23 for mitochondrial and nuclear loci, respectively) than for comparisons between Eltham-Greensborough and each of the three northern Victorian regions (Appendix F, Table F2). Although there was no support for strong genetic structure across northern Victoria, consideration of higher values of K ($K=4$) in the STRUCTURE analysis did indicate that there may be some additional weak substructuring present across the northern Victorian regions, in particularly associated with weak differentiation of Horsham (Appendix H, Figure H3).

Analyses of variation within and among ECB populations showed the strongest patterns of geographic structure being detected by the more-resolving mitochondrial regions (AMOVA in Table 2). Based on F_{ST} and Φ_{ST} statistics for mitochondrial DNA regions, 72.8%-74.6% of genetic variance was explained by differences among geographic regions within northern Victoria, with smaller percentages of variance explained by differences between northern Victoria and Eltham-Greensborough (10.9%-18.0%) and by variation among individuals within regions (9.2%-14.5%; Table 2). For nuclear loci, 55.3% -56.4% of genetic variance was among individuals within regions, with smaller proportions explained by differences among regions within northern Victoria (17.2%-27.9%) and by differences between northern Victoria and Eltham-Greensborough (24.0%-26.5%; Table 2).

Assessing levels of genetic diversity across the distribution of the ECB

Each locus was typically represented by a single common haplotype and a small number of rare haplotypes (Table 3). The Eltham-Greensborough region was represented by only a single haplotype for all but one locus, and was the least diverse region (Table 3). The Eltham-

Greensborough region was associated with some diversity (3 haplotypes across all markers) not present in individuals sampled from the other regions (Table 3). Across all loci, samples from Horsham, Bendigo and Castlemaine had 13 haplotypes (~62%) not represented in the Eltham-Greensborough region (Figure 2). The most diverse regions were Bendigo and Castlemaine (Table 3). Overall, observed heterozygosity for the ECB, averaged across the four regions, ranged from 0.000 – 0.181 across the nuclear loci (Table 3). MIGRATE-N analysis found that all four regions analysed (Eltham-Greensborough, Horsham, Bendigo, and Castlemaine) had very small mutation-scaled effective population sizes, with 95% confidence intervals of Θ estimates encompassing zero (Table 4).

Evidence of historical gene flow between ECB regions

Very low, but non-zero, levels of historical (approximately $4N_e$ generations in the past) gene flow were detected among the four regions analysed (Table 4). The highest historical level of genetic exchange was between the two geographically closest regions, Castlemaine and Bendigo (0.16 migrants per generation; Table 4). Historical levels of genetic exchange between the other more geographically distant pairs of regions were lower (0.053-0.085 migrants per generation; Table 4).

Discussion

Our results support the current subspecies status of ECB and FDC on criteria recommended by Braby et al. (2012). We detected some evidence of population genetic structure across the range of ECB. However, evidence of some historical gene flow and shared haplotypes between the Eltham-Greensborough region and the northern Victorian regions suggest relatively recent divergence. Based on this and additional criteria discussed below (see *Scope for genetic rescue of the Eltham-Greensborough ECB region*), we consider that assisted migration between ECB regions should carry a low risk of outbreeding depression (Frankham et al., 2011). Given very low population numbers and low levels of genetic diversity on small, isolated sites, the Eltham-Greensborough region is an especially strong candidate for genetic rescue. The ECB represents a potentially useful case study to examine the genetic and demographic outcomes of genetic rescue, due to short generation times and physical ease of translocations as compared to vertebrates (Brown, 1997, Knisley et al., 2005). Although not in the context of genetic rescue, translocations have been highly successful in re-establishing populations of another myrmecophilous (i.e. associates with ants) butterfly species in the United Kingdom (Andersen et al., 2014). We stress that any genetic rescue should be carried

out in conjunction with assured maintenance of the receptor sites and with genomic and population monitoring to assess effects on genetic diversity, fitness and abundance.

Genetic analyses support genetic distinctiveness of the ECB from the FDC

Long divergence time estimates (1.3 Mya) between ECB and FDC supports the genetic distinctiveness of the two subspecies. The extent of CO1 mitochondrial divergence between ECB and FDC (4.5%) was approaching levels typically reported for congeneric species of Lepidoptera (~6-7%) (Hebert et al., 2003). However, because the five adult FDC individuals analysed in this study were sourced from a single geographic location and thus did not represent the complete FDC distribution, the extent of divergence between the subspecies cannot be fully resolved here. Although the FDC sampling location was chosen to approximately represent the FDC population that is geographically closest to the distribution of ECB, and is therefore assumed to have diverged most recently from the ECB, more comprehensive sampling of the FDC from across its broader range would help to put the extent of divergence between the two subspecies in context. In the absence of information on biological functional differences between the subspecies (additional to existing information on differences in morphology and host-plant range) and wider geographic sampling of FDC, our findings support the continued management of the threatened ECB as an evolutionary unit distinct from the FDC.

Genetic distinctiveness of the Eltham-Greensborough region, but evidence of historical gene flow between extant ECB regions

Similar levels of genetic divergence between each of the three northern-Victorian regions and the Eltham-Greensborough region suggest that any of the northern regions could potentially act as source populations for translocations to Eltham-Greensborough. Based on the number of shared haplotypes across all loci and fixation indices for highly resolving mitochondrial regions, the most similar of the three northern Victorian locations to the Eltham-Greensborough region was Bendigo. However, different loci exhibited different patterns of population structuring and all pairs of regions shared haplotypes at some loci, consistent with relatively recent divergence.

Evidence of some historical gene flow among all pairs of geographically isolated regions of ECB (e.g. between Horsham and Castlemaine, between Castlemaine and Bendigo, etc.) was found. This is consistent with a more-continuous geographic distribution before substantial and widespread clearance of native forests and woodlands over the last ~200 years for timber,

mining, agriculture and urbanisation (Figure 1). Negative effects of fragmentation on gene flow and genetic diversity have been reported for other low-mobility butterfly species (Berwaerts et al., 1998, Habel et al., 2013, Hill et al., 2006).

Scope for genetic rescue of the Eltham-Greensborough ECB region

Given demographic and genetic data (low population numbers and very low MIGRATE-N estimates of mutation-scaled effective population size) are consistent with population size reductions associated with habitat loss and isolation, all ECB populations are likely to be experiencing the negative effects of drift and inbreeding and be characterised by low evolutionary potential (Frankham et al., 2010, Frankham et al., 2014, Harrisson et al., 2014). The genetic rescue effect is expected to be greater with larger divergence between target and source populations, up to the point where outbreeding depression arises (Frankham et al., 2011, Frankham, 2015). There are no simple metrics that can be used to determine what level of divergence is too much, however molecular data can be used to estimate whether proposed crosses are likely to be problematic (Frankham et al., 2011, Harrisson et al., 2016). In cases where there are no suitable populations for genetic rescue, crosses to other taxa are increasingly being considered (Smith et al., 2014, Love Stowell et al., 2017). However, given the presence of other suitable source populations and the reported levels of divergence between FDC and ECB approaching those of congeneric Lepidopteran species (Hebert et al., 2003), we would not recommend considering FDC as a source for genetic rescue of ECB populations until the taxonomy has been fully resolved (Frankham et al., 2011). Nevertheless, translocation of individuals from the northern Victorian regions to the most threatened Eltham-Greensborough region may constitute a promising management strategy for boosting genetic diversity and/or increasing fitness (Whiteley et al., 2015). Even a source population with low genetic diversity has been shown to improve allelic diversity and fitness traits in another inbred population following translocation (Heber et al., 2013). Individuals for translocation could be sourced from a single region, or from multiple source regions to maximise diversity and minimise the impact on the source population, such as described by the composite provenancing model (Prober et al., 2015). The number of migrants per generation (Table 4) suggests that historically the highest level of genetic exchange was between the two geographically closest regions, Castlemaine and Bendigo, indicating that both locations are similarly suitable as source populations for genetic rescue to the Eltham-Greensborough region. However, the largest populations near Bendigo may be the most diverse and resilient sources from which to transfer individuals.

Many studies of vertebrates and invertebrates have demonstrated positive effects of gene flow on genetic diversity, fitness and population persistence in both natural and laboratory environments (Frankham, 2015, Tallmon et al., 2004). In many instances, a single genetically-effective migrant per generation from a genetically divergent source is sufficient to increase average fitness and reduce extinction risk in small populations (Bryant et al., 1999, Ebert et al., 2002, Hufbauer et al., 2015, Spielman and Frankham, 1992, Waite et al., 2005), although more will usually be preferable. Despite strong empirical evidence demonstrating its potential benefits, there are only ~20 examples of genetic rescue being applied to conservation scenarios (Frankham et al., 2011, Frankham, 2015).

Genetic rescue may carry a risk of outbreeding depression, which must be carefully assessed (Edmands, 2007, Hoffmann et al., 2015, Wootton and Pfister, 2015). This risk is considered low for populations of the same species, with no fixed chromosomal differences, no substantial environmental differences, and with a history of gene flow within the last 500 years (Frankham et al., 2011). Given all ECB populations assessed here belong to the same subspecies and MIGRATE-N analysis supported historical gene flow among regions, fixed chromosomal differences between regions are unlikely. Climate does not vary substantially across the range of the ECB; the annual range of mean temperatures in the Eltham-Greensborough region (9.9-20.9 °C) is similar to Castlemaine (7.7-19.8 °C) and encompassed within the temperature ranges recorded for Bendigo (7.9-22.1 °C) and Horsham (7.2-22.2 °C) (Bureau of Meteorology). Occupied habitat is also broadly similar across the regions, although embedded within different matrix types. Some differences in the specific host ant species exist across the ECB's range. Eltham-Greensborough shares the ant species *N. capitatus* with the Castlemaine region, whilst Horsham hosts *N. ectatommoides* (Canzano et al., 2007). The Bendigo ant species is unknown, but is likely to be *N. capitatus* given Bendigo's proximity to Castlemaine. ECB in different regions may have adapted to their specific local *Notoncus* ant species, which could lead to incompatibilities following inter-region translocations. If the translocated butterflies failed to survive in one area, experimental host association work would be recommended. However, related myrmecophilous lycaenids typically associate with ecologically similar ants and some can switch to alternative host ants and food plants during extreme changes in climate (Andersen et al., 2014, Eastwood et al., 2006). Heritable endosymbiotic bacteria, such as *Wolbachia*, may threaten the reproductive compatibility between different arthropod populations (Nice et al., 2009). Although not currently known to exist in the ECB, this disease risk should be taken into account before

translocation. Given evidence of historical gene flow and a lack of strong environmental differences between the Eltham-Greensborough region and the northern Victorian regions, we consider the risk of outbreeding depression to be low, especially relative to the risk of extinction if no management action is taken (Frankham et al., 2011, Saccheri et al., 1998).

Management recommendations

Genetic rescue would ideally be trialled initially by translocating a small number (~10) of adult individuals from the Bendigo region into a single site of the Eltham-Greensborough region. The exact number of individuals to translocate should be determined by balancing evolutionary genetic considerations with practicalities such as the availability of butterflies. Ideally, several genetically effective migrants per generation are recommended to improve genetic health of inbred populations (e.g. Mills and Allendorf (1996) suggest between 1 and 10 migrants per generation as an appropriate guideline). How much gene flow will be required into the future will depend on many factors including the success in alleviating other threats, and population size. Due to relatively small differences in estimated migration rates and genetic diversity, all three northern Victorian populations are likely to be similarly suitable for use as the source population for translocations. However, Bendigo is the best candidate as a source population, based on having the largest known populations of ECB, which should make it most resilient to donating individuals.

In the first instance, translocations would ideally be undertaken under controlled release (field cage) conditions. Taking adults from the first flight period (late November- early December) would give the greatest seasonal opportunity to monitor and determine outcomes of establishment, and to help to build up numbers on site for release in the following year. Following translocations, the Eltham-Greensborough region should be monitored for population trends and any potential fitness outcomes (e.g. hatching success, maturation success and fecundity). Ideally, changes in genetic diversity during any genetic rescue should be monitored using cost-effective genome-wide assays that offer optimal assessment of evolutionary potential and using larval exuviae to avoid interference with adults (Harrisson et al., 2014). Because myrmecophiles can be challenging to conserve successfully (Settele and Kühn, 2009), the interaction between the introduced individuals and the host ants in the Eltham-Greensborough region would ideally also be monitored for signs of incompatibility and/or any potential co-evolution that may have other ecological consequences.

To date, most studies aimed at promoting population persistence in threatened invertebrates have typically focussed on habitat restoration and subsequent reintroduction of captive-bred individuals (Daniels and Emmel, 2004, Knisley et al., 2005). In contrast to captive-breeding and release programs of endangered species, which are associated with large economic and logistic limitations, genetic rescue may offer a more cost-effective strategy with the capacity to deliver much stronger population viability outcomes (Frankham, 2015, Snyder et al., 1996). Enhanced but not replaced by genetic rescue, habitat restoration and site management should continue in all ECB regions to counter the variety of threats flowing from urban environments (Fitzpatrick et al., 2016, Wood and Pullin, 2002). Implementation of trial genetic rescue from Bendigo to Eltham-Greensborough, in conjunction with ongoing habitat restoration and protection, should facilitate an overall improvement of genetic diversity, fitness and persistence of the ECB in the region most threatened by urban disturbance. If genetic rescue proves a successful management strategy for the Eltham-Greensborough region, i.e. improves demographic parameters and increases genome-wide diversity, assisted migration could be implemented between the northern Victorian regions to boost the viability of ECB populations across the subspecies' range. Translocations into currently unoccupied habitat might be possible in the future for range expansion if populations recover sufficiently. As for all conservation management actions, appropriate resourcing, planning, political will and support of the local community will be critical to the long-term success of any genetic rescue efforts.

Concluding remarks

Currently, genetic rescue is an under-utilised tool in conservation, at least partially due to exaggerated or unverified concerns about outbreeding depression (Frankham et al., 2011, Hedrick and Fredrickson, 2010, Hoffmann et al., 2015, Weeks et al., 2011, Whiteley et al., 2015). Concerns over accountability for management actions perceived to be high risk may be preventing genetic rescue from being used more often as a conservation tool (Frankham et al., 2011, Weeks et al., 2011). However, there is also a risk of extirpation or extinction associated with management inaction, and it needs to be recognized that failure to act is as much a decision as acting (Frankham et al., 2011, Hedrick and Fredrickson, 2010). In the face of ever-changing environments, promoting the capacity of species to adapt to new conditions is increasingly important (Hoffmann et al., 2015). Better incorporation of evolutionary processes into conservation management strategies should have positive outcomes for the

viability and evolutionary potential of threatened taxa and for biodiversity in general
(Hoffmann et al., 2015, Pierson et al., 2015).

Dedication

We dedicate this publication to our late co-author, Alan L. Yen, whose passion for
invertebrate conservation and the Eltham copper butterfly provided the vision and enthusiasm
to initiate this work.

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Conflict of Interest

Authors declare to have no conflict of interest.

Data Archiving

Genetic data will be publicly archived on Genbank and PANGAEA at the time of
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874 conservation: Three case studies in south-eastern Australia. In: CLARK, T. W. &
875 SEEBECK, J. H. (eds.) *Management and Conservation of Small Populations*. Illinois:
876 Chicago Zoological Society.

877 Table 1. Taxonomic distinction, life stage (A, adult; L, larva) of the specimen at time of
878 collection, year of collection, location and region of collection, and total number of samples
879 (N) from each site. ECB = Eltham copper butterfly, FDC = fiery dull copper butterfly.
880 Individual sample breakdown is listed in Appendix I.

Region	Location(s)	Subspecies	N	Specimen type	Year
Horsham	Wail State Forest	ECB	5	A	2011
	Kiata	ECB	5	A	2011
Bendigo	Big Hill Channel	ECB	15	L, A	2011, 2013
	Big Hill Primary School	ECB	5	L	2011
Castlemaine ¹	Kalimna Park	ECB	5	L	2011
	Castlemaine Botanic Gardens	ECB	5	L	2011
	Castlemaine Botanic Gardens	ECB	5	A	1988
Eltham-	Eltham	ECB	15	A	1987, 1989

¹ Historically (1988) and recently (2011) sourced individuals from the Castlemaine Botanical Gardens show little evidence of temporal patterning with one haplotype across all measured loci except two: S5ZW and CO1. For the polymorphic loci S5ZW and CO1, allele frequencies did not differ significantly between the two time periods (non-significant pairwise F_{ST} values).

Greensborough (EG) ²	Greensborough - Yandell's Reserve	ECB	6	A	2015
Gippsland	Mt. Noorinbee	FDC	5	A	2011

881

882 Table 2. Hierarchical Analysis of Molecular Variance (AMOVA) of Eltham copper butterfly
883 populations as defined by 4 regions/sites (Bendigo, Castlemaine, Horsham and Eltham-
884 Greensborough) grouped into two groups (Eltham-Greensborough and northern Victoria).
885 F_{ST} , a fixation index, measures the extent of genetic differentiation among subpopulations
886 where 0 means no differentiation (identical frequencies of sequences) and 1 is complete
887 differentiation (fixed subpopulations). F_{ST} accounts for allelic haplotype counts, Φ_{ST} also
888 incorporates information from nucleotide divergences among haplotypes. F_{ST}/Φ_{ST} is variance
889 among sites relative to total variance, F_{SC}/Φ_{SC} is variance among sites within groups, and
890 F_{CT}/Φ_{CT} is variance among groups relative to total variance. ns = not significant, all other
891 values have $p < 0.05$.

Locus		within sites %	within groups %	among groups %	F_{ST}/Φ_{ST}	F_{SC}/Φ_{SC}	F_{CT}/Φ_{CT}
CO1	F_{ST}	3.79	-0.02	96.23	0.96	ns	ns
	Φ_{ST}	0.96	-0.01	99.05	0.99	ns	ns
ND5	F_{ST}	14.53	145.61	-60.15	0.854	0.909	ns
	Φ_{ST}	28.01	149.2	-77.21	0.72	0.842	ns
Mean (mtDNA)	F_{ST}	9.16	72.80	18.04	0.91	0.909	
	Φ_{ST}	14.49	74.60	10.92	0.86	0.842	
SD	F_{ST}	7.59	102.98	110.58	0.08		
	Φ_{ST}	19.13	105.51	124.64	0.19		
63EA	F_{ST}	77.98	26.74	-4.72	0.22	0.26	ns
	Φ_{ST}	73.97	73.97	-12.45	0.26	0.34	ns
03W6	F_{ST}	90.71	16.04	-6.75	0.093	0.15	ns
	Φ_{ST}	88.65	19.84	-8.49	0.113	0.183	ns
S5ZW	F_{ST}	96.17	4.6	-0.77	0.038	ns	ns
	Φ_{ST}	96.78	7.14	-3.91	0.032	ns	ns

² Historically (1987, 1989) and recently (2015) sourced individuals share a singular EG haplotype at all loci but 63EA, where a single rare unique individual was sourced from 1987.

A7D8	F_{ST}	0	0	100	1	ns	ns
	Φ_{ST}	0	0	100	1	ns	ns
Z5V8	F_{ST}	16.91	38.5	44.59	0.831	0.695	ns
	Φ_{ST}	16.91	38.5	44.59	0.831	0.695	ns
Mean	F_{ST}	56.35	17.18	26.47	0.44	0.37	
(nuclear)	Φ_{ST}	55.26	27.89	23.95	0.45	0.41	
SD	F_{ST}	44.62	15.82	46.24	0.45	0.29	
	Φ_{ST}	43.91	29.62	48.38	0.44	0.26	

892

893 Table 3. Basic diversity statistics for two mitochondrial regions and five nuclear loci,
894 calculated in DnaSP and GenAlEx, where N = number of samples, L = Length of sequence
895 analysed, S = number of segregating (variable) sites, h = number of haplotypes, Hd =
896 haplotype diversity, π = nucleotide diversity, K = average number of nucleotide differences,
897 He = expected heterozygosity, and Ho = observed heterozygosity.

Locus	Group/Region	N	L	S	h	Hd	π	K	He	Ho
CO1	All	57	553	27	4	0.526	0.0025	4.815	-	-
	FDC	4	553	0	1	0	0	0	-	-
	ECB	53	553	4	3	0.456	0.0031	1.733	-	-
	Bendigo	16	553	1	2	0.125	0.0002	0.125	-	-
	Castlemaine	11	553	0	1	0	0	0	-	-
	Horsham	10	553	0	1	0	0	0	-	-
	Eltham/Greensborough	16	553	0	1	0	0	0	-	-
ND5	All	66	290	18	4	0.611	0.0102	2.945	-	-
	FDC	5	290	0	1	0	0	0	-	-
	ECB	61	290	2	3	0.55	0.0003	0.622	-	-
	Bendigo	19	290	0	1	0	0	0	-	-
	Castlemaine	14	290	2	2	0.363	0.0025	0.725	-	-
	Horsham	9	290	0	1	0	0	0	-	-
	Eltham/Greensborough	19	290	0	1	0	0	0	-	-
03W6	All	66	182	3	4	0.248	0.0186	0.409	0.121	0.046

	FDC	5	182	0	1	0	0	0	0.000	0.000
	ECB	61	182	2	3	0.125	0.0071	0.156	0.151	0.058
	Bendigo	19	182	0	1	0	0	0	0.000	0.000
	Castlemaine	13	182	2	3	0.484	0.886	0.64	0.423	0.231
	Horsham	10	182	1	2	0.174	0.379	0.32	0.180	0.000
	Eltham/Greensborough	19	182	0	1	0	0	0	0.000	0.000
63EA	All	51	226	6	5	0.327	0.0033	0.739	0.179	0.145
	FDC	2	226	0	1	0	0	0	0.000	0.000
	ECB	49	226	6	4	0.272	0.0031	0.705	0.224	0.181
	Bendigo	13	226	3	2	0.492	0.0065	1.477	0.473	0.462
	Castlemaine	8	226	3	2	0.125	0.0017	0.375	0.095	0.100
	Horsham	8	226	1	2	0.325	0.0014	0.325	0.278	0.111
	Eltham/Greensborough	20	226	2	2	0.05	0.0004	0.1	0.049	0.050
A7D8	All	60	196	1	3	0.125	0.0007	0.125	0.000	0.000
	FDC	4	196	0	1	0	0	0	0.000	0.000
	ECB	56	196	0	1	0	0	0	0.000	0.000
	Bendigo	16	196	0	1	0	0	0	0.000	0.000
	Castlemaine	13	196	0	1	0	0	0	0.000	0.000
	Horsham	9	196	0	1	0	0	0	0.000	0.000
	Eltham/Greensborough	18	196	0	1	0	0	0	0.000	0.000
S5ZW	All	66	215	7	5	0.223	0.0037	0.793	0.070	0.076
	FDC	5	215	0	1	0	0	0	0.000	0.000
	ECB	61	215	3	4	0.096	0.0005	0.097	0.087	0.095
	Bendigo	20	215	1	2	0.185	0.0009	0.185	0.180	0.200
	Castlemaine	11	215	2	3	0.186	0.0009	0.19	0.169	0.182
	Horsham	10	215	0	1	0	0	0	0.000	0.000
	Eltham/Greensborough	20	215	0	1	0	0	0	0.000	0.000
Z5V8	All	67	224	6	2	0.503	0.0135	3.017	0.127	0.091
	FDC	5	224	0	1	0	0	0	0.000	0.000

ECB	62	224	6	2	0.504	0.0135	3.021	0.159	0.113
Bendigo	19	224	0	1	0	0	0	0.000	0.000
Castlemaine	14	224	6	2	0.349	0.0094	2.095	0.260	0.154
Horsham	10	224	6	2	0.337	0.009	2.021	0.375	0.300
Eltham/Greensborough	19	224	0	1	0	0	0	0.000	0.000

Table 4. MIGRATE-N model of mutation-scaled effective population size (Θ) for each of the four ECB regions and the mutation-scaled migration rate (M) between each pair of regions, where C is Castlemaine, B is Bendigo, H is Horsham and E is Eltham-Greensborough.

Parameter	2.5%	Mode	97.5% ³	No. Migrants per gen ($\Theta M/4$)
Θ_C	0.00000	0.00007	0.00120	-
Θ_B	0.00000	0.00007	0.00120	-
Θ_H	0.00000	0.00007	0.00133	-
Θ_E	0.00000	0.00007	0.00120	-
$M_{E \leftrightarrow C}$	833.3	3050.0	5466.7	0.053
$M_{E \leftrightarrow B}$	2066.7	4316.7	6400.0	0.076
$M_{E \leftrightarrow H}$	1733.3	3683.3	8166.7	0.064
$M_{C \leftrightarrow H}$	2433.3	4850.0	9366.7	0.085
$M_{B \leftrightarrow H}$	1533.3	3316.7	6133.3	0.058
$M_{C \leftrightarrow B}$	1333.3	3683.3	9066.7	0.16

³ 95% confidence intervals are represented between the lowest 2.5% of the distribution or the highest 2.5% of the distribution

Figure 1. Sampling locations in Victoria, south-eastern Australia, of Eltham copper butterfly (ECB) (black circles) and fiery dull copper butterfly (FDC) (black triangle) individuals used in analyses. All regions where extant populations of ECB are known to occur were sampled: Horsham (pink), Bendigo (yellow), Castlemaine (blue) and Eltham-Greensborough (green). FDC samples were sourced from Mt Noorinbee in Gippsland (orange). Extinct colonies of ECB are shown (open circles). Numbers correspond to current and extinct ECB population locations: 1) Kiata, 2) Dimboola, 3) Wail State Forest, 4) Murtoa, 5) Big Hill Primary School, 6) Big Hill Channel, 7) Castlemaine Botanic Gardens, 8) Kalimna Park, 9) Keilor, 10) Broadmedows, 11) Tallarook, 12) Greensborough, 13) Eltham, and 14) Mt Noorinbee. Shading represents modelled historical (pre-1750; light green) and contemporary (2005; dark green) extent of forest and woodland ecological vegetation classes. Spatial vegetation data (NV1750_EVC and NV2005_EVCBCS) were sourced from <https://www.data.vic.gov.au>. Range of the species *P. pyrodiscus* is shown in the inset (red circles represent all records from the Atlas of Living Australia).

Figure 2. Median-joining haplotype networks for five nuclear loci, two mitochondrial regions, and a concatenation of the two mitochondrial loci, where each circle represents a unique genetic sequence (haplotype). The size of the circle is proportional to the number of

individuals with the corresponding haplotype. Colours correspond to subspecies and/or
 geographic region (green = Eltham-Greensborough (ECB), pink = Horsham (ECB), blue =
 Castlemaine (ECB), yellow = Bendigo (ECB), orange = FDC (Figure 1). The number next to
 each line quantifies nucleotide differences between connected haplotypes, no number
 signifies a nucleotide difference of 1. The 11 bp deletion in locus A7D8 is marked with (del).
 Figure 3. Summary of STRUCTURE results (K=3) run based on genotypes for nuclear loci
 for all *P. pyrodiscus* individuals (N=70). Plots indicate proportional assignment of
 individuals to the ‘dark grey’, ‘light grey’ or ‘black’ genetic cluster. Samples are arranged
 according to sampling location. Vertical black lines separate sampling locations.

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