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RESEARCH

Population genetic structure and range limits of *Prostanthera cineolifera* (Lamiaceae), a vulnerable shrub with a patchy distribution

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Abstract

Integrating molecular data is essential for clarifying the distributions and genetic structures of species that have histories of misidentification and misapplication of names. There has been confusion about the species limits of the Vulnerable *Prostanthera cineolifera* with respect to morphologically similar specimens in the Hunter Valley, New South Wales, Australia and morphologically dissimilar specimens in the Lower Hawkesbury Valley, New South Wales, and from north-eastern New South Wales. To test the species limits of *P. cineolifera,* and related taxa, specimens were collected from across the range and augmented with herbarium specimens. We used morphometric analysis of 18 morphological characters across 51 samples. Using the DArTseq reduced representation sequencing platform, 4010 single-nucleotide polymorphisms (SNPs) across 110 individuals were recovered for molecular analysis. Both morphological and molecular analyses produced three concordant clusters (A) *P. cineolifera*, (B) a group sharing similarities with *P*. sp. Hawkesbury (B.J.Conn 2591), and (C) a group allied with *P. lanceolata* and *P. ovalifolia*. These results indicate that the specimens form north-eastern New South Wales are more likely to be *P. lanceolata*, not *P. cineolifera*, and that specimens from the Lower Hawkesbury are of an undescribed species with the phrase name *P*. sp. Hawkesbury (B.J.Conn 2591). Within *P. cineolifera* there was pronounced genetic differentiation among populations. Little evidence of inbreeding was observed, but the newly recognised, more isolated populations had the lowest genetic diversity. This study provides new information about the range of the species and its genetic structure that informs the conservation priorities for this species.

Keywords DArTseq · Molecular analysis · Morphometric analysis · Population genetics · *Prostanthera* · SNP

Introduction

The goal of conserving the evolutionary potential of species (Frankel 1974; Bowen 1999; Milot et al. 2020) can be hampered by a lack of information on the distribution and genetic diversity of species (e.g. Gitzendanner et al. 2012; Neel and Ellstrand 2003; Coates et al. 2018). Taxonomic uncertainty can lead to undiscovered biodiversity, which undermines conservation planning and decision-making (Dubois 2003). Misidentification of populations can also underestimate or overestimate the geographic distribution of species, and can risk misallocating conservation efforts (Mason et al. 2016; Conn et al. 2021; Collins et al. 2022).

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For many plant species, some of the most pressing needs for conservation are clarification of taxonomy and improved identification tools to accurately characterise distributions. Once species limits are well understood, population genetics can be used to characterize genetic diversity and population structure for the purpose of prioritizing conservation actions (Ottewell et al. 2016).

Disjunct species distributions raise questions about the biology, taxonomy and conservation of species (Llorens et al. 2015; Ottewell et al. 2016; Millar and Byrne 2020). Disjunct populations may have simply been misidentified, possibly as a result of unsuccessful identification keys or a lack of robust taxonomic work on the group (examples in Williams et al. 2006; Sassone et al. 2021; Wilde and Barrett 2021). Disjunctions of distributions, particularly across areas of endemism, call into question the status of species. However, if taxonomically sound, the small, range-edge populations may be at risk of losing genetic diversity by genetic drift and inbreeding and therefore have an increased

extinction risk (Frankham et al. 2012). Edge-of-range or disjunct populations could also be of high conservation value, as they often contain unique genetic diversity (Lesica and Allendorf 1995; Channell 2004; Razgour et al. 2013) that may play an important role in adaptation of the species to environmental change such as climate change (Kyrkjeeide et al. 2020). However, we risk over-investing or under-investing in such populations if species limits are poorly understood.

Developments in sequencing technologies have accelerated population genetics in the context of species delimitation and conservation (Hunter et al. 2018). Reduced representation sequencing approaches can generate robust single nucleotide polymorphism (SNP) datasets without the need for a time-consuming development stage (Andrews et al. 2016). Analysis of reduced representation sequence data does not rely on the availability of a reference genome. This makes the data uniquely suitable for studying the population structure, genetic diversity and species boundaries of non-model organisms (da Fonseca et al. 2016; Rossetto et al. 2021), as well as testing the identity of questionable populations or unknown samples (Renner et al. 2022).

Prostanthera Labill., an Australian endemic genus, includes 114 described species and 18 accepted phrase names (Australian Plant Census IBIS database 1970 onwards; Wilson et al. 2012, 2019; Conn et al. 2021; O'Donnell et al. 2021), and are mostly shrubs, some of which are so-called species complexes with morphological variability and wide distributions such as the *P. lasianthos* Labill. species complex (Conn 1993) and *P. ovalifolia* R.Br. *s.l.* (Althofer 1978). An integrative taxonomic approach has been successful in partially resolving the *P. lasianthos* species complex (Conn et al. 2021), but confusion remains within the *P. ovalifolia* complex. For example, *P. cineolifera* R.T.Baker & H.G.Sm. has been frequently confused with *P. ovalifolia* and *P. lanceolata* Domin. Although *P. cineolifera* is listed as a "vulnerable" species under both Commonwealth (Environment Protection and Biodiversity Conservation Act 1999) and New South Wales (Biodiversity Conservation Act 2016) legislation, the genetics and species limits of *P. cineolifera* have not yet been examined.

Prostanthera cineolifera was described from Broke in the Hunter Valley (Baker and Smith 1912) and has a patchy distribution on shallow or skeletal soils in this region of New South Wales. While development and agricultural intensification have occurred in the regions it inhabits, it is unclear how much this has contributed to the patchiness of populations. Populations of *Prostanthera* vary from scattered individuals (Palsson 2020) to multi-aged stands or large stands of seemingly a single age cohort that are possibly the result of mass germination after a disturbance such as fire or drought (Althofer 1978 and pers. obs.). We thus expect patterns of genetic diversity to vary among populations, and some isolated populations may be at risk from inbreeding or the loss of genetic diversity. According to Office of Environment & Heritage (2019), *P. cineolifera* is found at Scone, Cessnock (both in the Lower Hunter Valley) and St Albans. The latter population is in the Hawkesbury River catchment and specimens are morphologically different from *P. cineolifera*. An obvious difference is that they have persistent, hairy bracteoles (floral prophylls), whereas those of *P. cineolifera* are shed early in bud development (Fig. 2). These specimens from St Albans are morphologically similar to *P.* sp. Hawkesbury (B.J.Conn 2591) NSW Herbarium. Another taxonomic problem is that specimens in the Upper Hunter Valley similar to *P. cineolifera* have been determined as *P. ovalifolia*. Finally, two populations from north-eastern New South Wales (Tabbimoble Creek and Pillar Valley), approximately 450 km from the Hunter Valley, have also been identified as *P. cineolifera* (AVH 2017) although they occur within the range of *P. lanceolata* (Althofer 1978). If these populations are indeed *P. cineolifera*, they may warrant extra protection because of the likelihood that they possess unique genetic variation or experience distinct threatening processes (e.g. hybridization). These taxonomic questions suggest the distribution described in Office of Environment & Heritage (2019) is misleading, and the need for taxonomic revision has been noted in the Department of the Environment (2008). Reduced representation sequencing is well suited to investigating the taxonomic questions and the fragmented population structure of *P. cineolifera*.

Using field observations, herbarium searches, morphological study and next-generation sequencing-based genetic analysis, we examined variation in *P. cineolifera* and relatives to address three principal questions. (1) Are the disjunct populations in north-eastern New South Wales (Tabbimoble Creek and Pillar Valley) and in the Lower Hawkesbury River region isolated, ecologically distinct populations of *P. cineolifera*, or do they belong to different, perhaps undescribed, species? This question has bearing on conservation priorities. (2) To what extent are populations subdivided within *P. cineolifera*? (3) Is there cause for concern about the genetic or demographic health of any *P. cineolifera* populations, or the species as a whole? We use genetic diversity and inbreeding coefficients, as well as site observations of age structure, to identify any genetically depauperate populations. While more work is needed, we also lay the groundwork for further taxonomic assessment of related species by clarifying their limits relative to *P. cineolifera*.

Materials and methods

Sampling

We used AVH (2017) data for *P. cineolifera*, *P. lanceolata* and *P. ovalifolia* and specimens held at N.C.W. Beadle

Herbarium to identify sampling locations to target the New South Wales and south-eastern Queensland populations of the study group (Fig. 1; Table 1). Populations that we determined to be *P. cineolifera* based on morphology, including those that had been determined as *P. ovalifolia* in the past, were sampled from 11 sites in four broad locations in the Hunter Valley: Wingen Maid Nature Reserve (one), Wallaby Rocks near Sandy Hollow (one), Cousins Creek in Wollemi National Park (one) and near the type locality in the Broke-Pokolbin area near Singleton (seven). We sampled at Tabbimoble Creek and Pillar Valley, the site of two putative outlying collections of *P. cineolifera* from the north coast of New South Wales (AVH 2017). We aimed to test whether these populations were *P. cineolifera* or not—perhaps *P. lanceolata* or *P. ovalifolia*. We collected a representative sample of populations that had been determined as *P. lanceolata* and *P. ovalifolia* in south-eastern Queensland and north-eastern New South Wales, including the type location of *P. lanceolata* (Tamborine Mountain, Queensland). Samples from north-eastern New South Wales were all morphologically consistent with *P. lanceolata*. The type locality for *P. ovalifolia* of Mount Westall, inside the Shoalwater Bay Training Area, was inaccessible. For *P. ovalifolia*, we restricted our sampling to populations close to the type locality of Mount Westall, i.e. Mount Stanley (225 km SSE of Mount Westall) and Mount Maria (270 km SSE of Mount Westall). Locations in the Hunter and Lower Hawkesbury Valleys where populations of *Prostanthera* had been determined as *P. ovalifolia* or *P. cineolifera* were also sampled and allocated field names based on morphology (Fig. 1; Table 1 and see Table S1 for voucher details). A population of *P*. sp. Oxley Wild Rivers National Park (J.B. Williams NE 91044) was included because of similarity to *P.* sp. Hawkesbury.

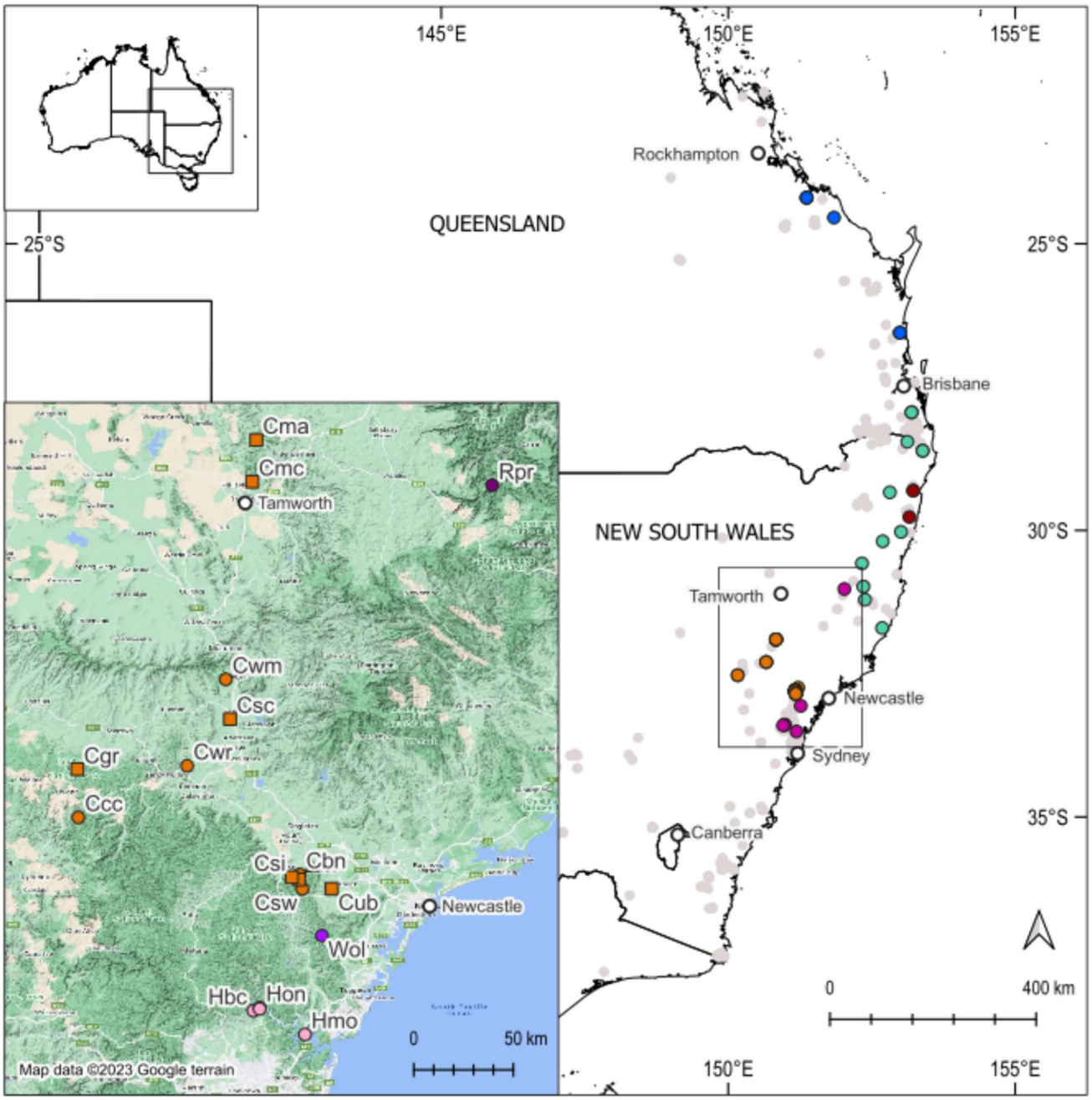
We refer to the morphologically similar specimens in the Lower Hawkesbury catchment that have been determined to be *P. cineolifera*, or *P. lanceolata* or *P. ovalifolia* as *P.* sp. Hawkesbury, as they are morphologically similar to *P*. sp. Hawkesbury (B.J.Conn 2591) NSW Herbarium. The specimens from Olney State Forest, which had been determined to be *P. ovalifolia*, were given the working name *P.* sp. Olney State Forest as they could be separated from *P.* sp. Hawkesbury on the basis of leaf shape, leaf texture and scent. The specimens from north-eastern New South Wales are referred to by their locations as their species identities were unknown. The final study group included *P. cineolifera, P. lanceolata, P. ovalifolia, P.* sp. Hawkesbury, *P*. sp. Olney State Forest, *P*. sp. Oxley Wild Rivers and the populations from Tabbimoble Creek (TB) and Pillar Valley (PV) previously determined as *P. cineolifera.* To increase the geographic coverage of our sampling of *P. lanceolata*, we included three samples from herbarium specimens (Table 1). We also included two samples from herbarium specimens for *P*. sp. Oxley Wild Rivers. One of each was later filtered out due to low call rates (Table S5).

Morphologically, *P.* sp. Hawkesbury, *P*. sp. Olney State Forest and *P.* sp. Oxley Wild Rivers National Park share relatively long, persistent hairy prophylls and recurved calyx lobes and are referred to hereinafter as the *P.* sp. Hawkesbury group (Fig. 2). The differences between *P. cineolifera* and *P. lanceolata* and *P. ovalifolia* are more subtle. In *P. cineolifera* the calyx lobes are adpressed to the corolla in the developing bud and in the fully opened corolla, unlike in *P. lanceolata* and *P. ovalifolia* where calyx lobes are reflexed away from the corolla in the developing bud and fully open flower (Fig. 2). The disposition of the calyx lobes is not always obvious in pressed material.

Fieldwork involved collection of herbarium vouchers and metadata including soil descriptions, digital images, leaf material for phytochemical and morphological analyses, material to be freeze dried, material stored in silica gel (Si Gel), material for cuttings, and occasionally seedling transplants. Fieldwork was conducted under New South Wales Office of Environment and Heritage Scientific Licence SL100305. All voucher specimens were lodged at N.C.W. Beadle Herbarium, with duplicates allocated to other Australian herbaria as appropriate. Population size estimates and the presence of juvenile and adult plants were also recorded. Further field work was undertaken in 2019 and 2022 to search for previously unknown populations of *P. cineolifera.* With the exception of the populations at Tabbimoble Creek and Pillar Valley, field collections were assigned to species based on diagnostic morphological features (Baker and Smith 1912; Domin 1928; Conn 1993), such as stem indumentum, bud and calyx shape. We treated the populations at Tabbimoble Creek and Pillar Valley as belonging to *P. cineolifera* to enable us to test the validity of protecting them as such, although initial examination in the field caused us to question this assignment.

Morphological analysis of species boundaries in *Prostanthera cineolifera*

Morphological analysis included representative specimens from our field collections and from the following herbaria: Queensland Herbarium (BRI), Australian National Herbarium (CANB), N.C.W. Beadle Herbarium (NE), and National Herbarium of New South Wales (NSW). Herbarium codes follow the Index Herbariorum (see http://sweetgum.nybg.org/ih/, accessed 17 July 2022). Herbarium searches revealed specimens morphologically consistent with *P. cineolifera*—all determined as *P. ovalifolia*—from Goulburn River National Park, Manilla, Tamborine Mountain, Queensland (BRI AQ0336457) (Fig. S1) and Upper Moore Creek (Tamworth) These specimens had the diagnostic features of



Legend - inset

- *P. cineolifera* morphology only
- *P. cineolifera* genetics only
- *P. cineolifera* morphology and genetics
- *P.* sp.Oxley Wild Rivers NP morphology and genetics
- *P.* sp. Olney SF morphology and genetics
- *P.* sp. Hawkesbury morphology and genetics

Legend - main map

- *P. ovalifolia* AVH records at 2017-07-13
- *P. ovalifolia* collection sites
- *P. lanceolata* collection sites
- *P. cineolifera* collection sites
- *P.* sp. Hawkesbury group collection sites
- Tabbimoble Creek and Pillar Valley collection sites

◂**Fig. 1** Sampling locations. *Main map* Distribution in eastern Australia of *P. ovalifolia* according to the Australasian Virtual Herbarium (AVH; 13 July 2017) with our collection localities for *P. lanceolata, P. ovalifolia, P. cineolifera* and the *P*. sp. Hawkesbury group. *Coloured inset* Sampling sites in the Peel, Hunter and Hawkesbury River valleys. Labels are species and location codes. C = *P. cineolifera,* W = *P*. sp. Olney State Forest and H = *P*. sp. Hawkesbury. For location codes see Table 1. *Note* AVH data were cleaned of cultivated specimens and specimens with geocoding errors that placed them offshore

P. cineolifera (features of stem indumentum, bud, and calyx shape) and were included in the morphological analysis.

Characters used were guided by character lists in Williams et al. (2006) and Conn et al. (2013), augmented by characters chosen following our examination of specimens. Characters included quantitative (10), unordered multistate (6) and binary characters (2). Quantitative characters were scored as a mean of three measurements. Measurements were made either by steel rule or eyepiece graticule at 5X magnification, calibrated against a steel rule. Initially, information for 18 morphological characters (see Table S2) across 50 samples were recorded into DELTA (Dallwitz 1980), then exported via Excel to PATN v4 (Belbin 2004–2013) for morphological analysis (Fig. S2, Table S3). Samples with more than two missing data points were removed, leaving 48 samples (Omp1 and Omw1-T, which had 5 and 7 missing data points, respectively).

Morphometric analysis used the Gower metric for association. The flexible unweighted pair-group method with arithmetic mean (UPGMA) with the default $\beta = -0.1$ (Belbin 2013) was used for clustering, and semi-strong hybrid multidimensional scaling (SSH-MDS) was used for ordination with the maximum number of iterations set to 100 with other parameters at their default settings. The Kruskal–Wallis (KW) statistic was used to evaluate the ability of variables to discriminate between a set of groups in the cluster; higher KW values have more discriminating power (Belbin 2004–2018). Principal-component correlation (PCC) values were used to evaluate the contributions of specific variables to the ordination. These values, expressed as r^2, are an indication of the amount of variance accounted for by that character (Belbin 2004–2018).

Genetic analysis of species boundaries and population structure

Genotyping and filtering

For DNA extraction, leaf samples were generally freeze-dried or dried on Silica gel. Genotyping-by-sequencing using DArTseq platform (Wenzl et al. 2004; Kilian et al. 2012; Li et al. 2015) was conducted by Diversity Arrays Technology P/L (DArT) (Canberra, Australia), producing SNP data. High quality DNA was extracted by DArT from dried leaf samples and digested using at least one methylation-sensitive restriction enzyme (*Pst*I) before ligation of sequencing adapters and Illumina 75 bp single-end sequencing. One hundred and thirty three samples from the study group plus three samples of *P. rotundifolia* R.Br. *sens. strict*. for use as an outgroup and additional *Prostanthera* samples from an unrelated study were randomized across two plates prior to sequencing (Meirmans 2015). Thirteen of the samples had a technical duplicate in the second plate. Following sequencing, the resulting 75 bp reads were filtered, trimmed and assembled de novo, then variants were scored by DArT using their proprietary pipeline.

All filtering and downstream analysis of SNPs was conducted in R 4.0.2 or earlier (R Core Team 2023). The data returned from DArT were filtered using *dartR* v2.9.7 (Mijangos et al. 2022). Loci were filtered on repeatability (min. 95%), then on call rate (min. 95%). Individuals with call rates less than 90% were removed to filter out poor quality samples, and loci with $H_O > 0.6$ were removed, as they likely represented paralogous sequence regions. Loci with < 5% minor allele frequency and those that deviated significantly from Hardy–Weinberg equilibrium ($p < 0.01$) in two or more populations with at least 5 samples were removed. Secondary SNPs within sequence tags were removed, and loci with similar trimmed sequence tags were removed if their Hamming distance was less than 0.2. Finally, loci with linkage disequilibrium greater than 0.2 were pruned so that only one was retained (Table S4). A subset of the filtered data consisting only of *P. cineolifera* samples, as determined by genetic and morphological data, was created for downstream analyses. This subset was refiltered on call rate with the same thresholds, and monomorphic loci were removed.

Preliminary ordination of this dataset via principal component analysis (PCA) (Gower 1971) was conducted using the 'gl.pcoa' function in *dartR* (Mijangos et al. 2022). Scrutiny of the resultant plot indicated three anomalous specimens that plotted halfway between two groups. For each of these specimens, other samples from the same population behaved as expected. The possibility of hybrids was rejected because this group of *Prostanthera* are pollinated by beetles and flies (Wilson et al. 2017) and the putative parents are 180–800 km apart. These anomalous specimens were possibly caused by errors in plate setup and were removed. A fourth anomalous specimen, supposed to be a sample of *P*. sp. Hawkesbury, grouped with the *P. ovalifolia*. These two species are very distinct morphologically (Fig. 2). This fourth specimen was possibly mislabeled and was removed.

Table 1 Summary of populations of the study group sampled for morphological and genetic analyses, after filtering

Species and location code[a]	Taxon	Location[b]	No of samples for morphological analysis	No of samples for genetic analysis
Oms	*P. ovalifolia*	Mt Stanley, Central Qld	4	8
Omm	*P. ovalifolia*	Mt Maria, Central Qld	3	7
Omn	*P. ovalifolia*	Mt Ninderry, south-eastern Qld	4	6
Ltm	*P. lanceolata*	Tamborine Mtn, south-eastern Qld	2	2
Lbm	*P. lanceolata*	Bar Mtn, north-eastern NSW	2	2
Lmf	*P. lanceolata*	Minyon Falls, north-eastern NSW	2	2
Lma	*P. lanceolata*	Mt Marsh, north-eastern NSW[c]	1	1
Lsw	*P. lanceolata*	Sherwood NR, north-eastern NSW	1	2
Lny	*P. lanceolata*	Nymboida, north-eastern NSW[c]	1	1
Lne	*P. lanceolata*	New England NP, north-eastern NSW		2
Lmb	*P. lanceolata*	Middle Brother Mtn, north-eastern NSW		2
Lcr	*P. lanceolata*	The Castle NR, north-eastern NSW	1	
Lbs	*P. lanceolata*	Mt Boss SF, north-eastern NSW	1	
Cma	*P. cineolifera*	Manilla, Peel Valley, NSW	1	
Cmc	*P. cineolifera*	Upper Moore Creek, Peel Valley, NSW	1	
Cwm	*P. cineolifera*	Wingen Maid, Hunter Valley, NSW	2	6
Cwr	*P. cineolifera*	Wallaby Rocks, Hunter Valley, NSW	1	2
Cgr	*P. cineolifera*	Goulburn River NP, Hunter Valley, NSW	1	
Ccc	*P. cineolifera*	Cousins Creek, Hunter Valley, NSW	1	8
Csc	*P. cineolifera*	Scone, Hunter Valley, NSW	1	
Cbk	*P. cineolifera*	Broke Cessnock Rd, Hunter Valley, NSW		1
Csi	*P. cineolifera*	Siberia, Broke, Hunter Valley, NSW	1	
Cbn	*P. cineolifera*	Bees Nest Ridge, Hunter Valley, NSW	1	21[d]
Cbb	*P. cineolifera*	Broken Back Trail, Hunter Valley, NSW	1	6
Csw	*P. cineolifera*	Sawpit Rd, Hunter Valley, NSW	1	6
Cub	*P. cineolifera*	Upper Bellbird, Hunter Valley, NSW	1	
Rpr	*P*. sp. Oxley Wild Rivers National Park	Paradise Rocks, Oxley Wild Rivers NP, NSW[c]	1	1
Wol	*P*. sp. Olney State Forest	Olney SF, Central Coast, NSW	3	6
Hmg	*P*. sp. Hawkesbury	Mangrove Creek, Hawkesbury River catchment, NSW	1	
Hwf	*P*. sp. Hawkesbury	Wisemans Ferry, Hawkesbury River catchment, NSW	1	
Hon	*P*. sp. Hawkesbury	Old Northern Rd, Wisemans Ferry, Hawkesbury River catchment, NSW	3	6
Hbc	*P*. sp. Hawkesbury	Bicentenary Rd, Webbs Creek, Hawkesbury River catchment, NSW	2	6
Hmo	*P*. sp. Hawkesbury	Bar Point, Hawkesbury River catchment, NSW	1	1
TB	TB	Tabbimoble Creek, NSW	1	2
PV	PV	Pillar Valley, NSW		3
	Totals		48	110

[a]The leading letter of the species and location code is generally the leading letter of the specific epithet of the *Prostanthera* species expected at that site (C = *P. cineolifera*, H = *P*. sp. Hawkesbury, L = *P. lanceolata*, O = *P. ovalifolia*, R = *P*. sp. Oxley Wild Rivers National Park, W = *P*. sp. Olney State Forest). The second and third letters are codes for the collection site. The populations of putative *P. cineolifera* from Tabbimoble Creek and Pillar Valley are labelled by their location codes only; *PV* Pillar Valley, *TB* Tabbimoble Creek

[b]*NP* National Park, *NR* Nature Reserve, *NSW* New South Wales, *Qld* Queensland, *Rd* Road, *SF* State Forest

[c]Herbarium specimens sampled for genetic analysis. For details of vouchers, see Table S1

[d]Four separate subpopulations of 5, 4, 5 and 7 samples

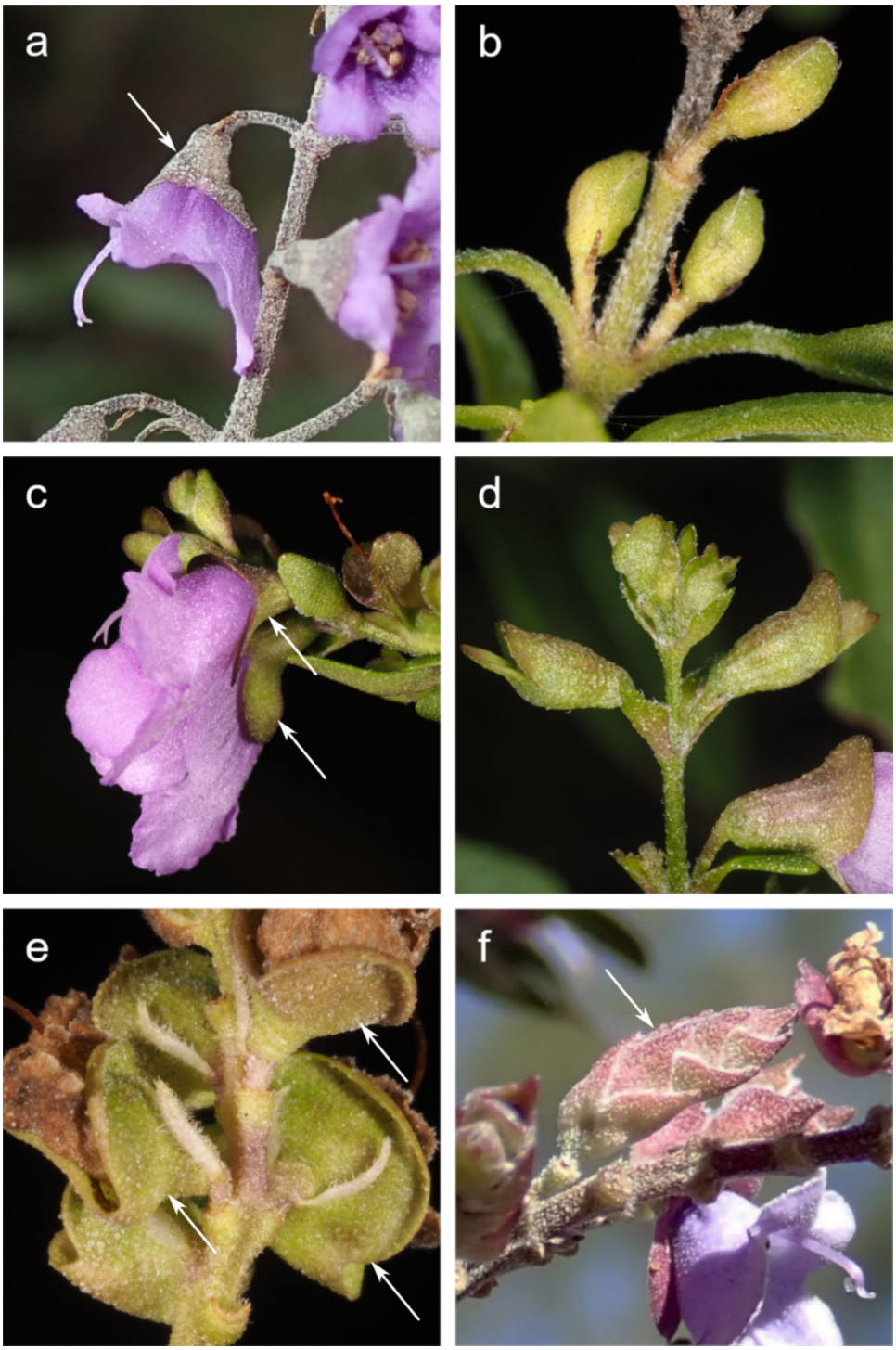


Fig. 2 Comparison of some morphological features in the study group. Left hand side: calyx disposition (arrows), right hand side: buds. **a** and **b** *P. cineolifera*, **c** and **d** *P. ovalifolia,* **e** *P*. sp. Hawkesbury group with recurved calyx lobes and persistent hairy bracteoles; **f** *P*. sp. Hawkesbury group with floral bracts (arrow) enclosing buds. Photographs: **a**, **f** R.L.Palsson, **b–e** J.J.Bruhl

Species delimitation and identification of TB and PV populations

To examine the relationships of the populations in the study group, a neighbour-joining tree was constructed among populations using 'gl.tree.nj' in *dartR* (Mijangos et al. 2022) with a matrix of Euclidean distances among poulations, and rooted with specimens of *P. rotundifolia* from Launceston and Genoa Falls as the outgroup. *P. rotundifolia* samples were then removed.

To examine underlying data structure and visualize the genetic differences, PCA was conducted using the function 'gl.pcoa' in *dartR* (Mijangos et al. 2022). PCA was conducted on the full dataset and on each of the major clusters. We included all sampled populations within the study group (*P. cineolifera, P. lanceolata, P. ovalifolia,*

P. sp. Hawkesbury and *P.* sp. Olney State Forest, *P.* sp. Oxley Wild Rivers National Park and the populations at Tabbimoble Creek and Pillar Valley that had been determined as *P. cineolifera*).

Non-hierarchical clustering was conducted using sparse nonnegative matrix factorization (sNMF) in *LEA* v3.12.2 (Frichot and François 2015). The number of genetic clusters (K) best supported by the data was determined from the decay of mean cross-entropy values (Frichot and François 2015). The 'snmf' command was run with K = 1–15 with 10 replicates for each value of K, and the run with the lowest entropy selected for graphing the estimated individual ancestry coefficients.

As isolation by distance (IBD) may drive population structure, it was important to distinguish genetic discontinuities between species from apparent clustering due to patchy sampling across a continuum (Frantz et al. 2009). We used conStruct v1.0.4 (Bradburd et al. 2018) for non-hierarchical clustering of populations within a spatially explicit population-level model that estimates ancestry proportions while accounting for IBD. It characterizes the contributing ancestral populations as 'layers' in which allele frequencies may vary according to an estimated degree of spatial autocorrelation. Evaluation of model fit via cross-entropy allows the spatial model to be compared with one in which allele frequencies do not vary geographically, as well as comparing different values of *K*, the number of ancestral populations. The software takes allele frequencies as input and produces estimates of population-level ancestry proportions, as well as parameters describing the rate of decay of covariance with spatial distance in each layer. With both the spatial and non-spatial models, five independent MCMC chains were run for each value of *K* from 1 to 10, each with 100,000 iterations and an 'adapt_delta' parameter of 0.99. In addition to cross-validation, contributions of layers to overall population covariance were used to inform the model selection.

Basic population summary statistics

Basic genetic summary statistics were calculated for the putative species in the study region (Table S6) and for populations within *P. cineolifera*, excluding those with $N < 5$ for within-species analyses. Mean and standard deviations of observed heterozygosity and unbiased expected heterozygosity (Nei 1978) across loci were estimated using the 'gl.report.heterozygosity' command in *dartR* (Gruber et al. 2017). Within *P. cineolifera*, 1000 bootstraps across loci within the package *heirfstat* version 0.5–11 (Goudet 2005) were used to estimate 95% confidence limits for the inbreeding coefficient, *F*, and alleles private to each population were counted using *poppr* version 2.9.4 (Kamvar et al. 2014). Inbreeding coefficients were not calculated for the species-level analysis, as small population sample sizes in some species meant that hierarchical analyses could not be used to account for population subdivision within species. Differentiation among putative species and among populations within *P. cineolifera* (excluding those with $N < 5$) was compared using pairwise F_{ST}, calculated with the 'stamppFst' command in the *StAMPP* package with 1000 permutations (Pembleton et al. 2013).

Genetic structure and isolation-by-distance in *P. cineolifera*

Population clustering was explored using sNMF as described above. IBD was tested using the 'gl.ibd' function in *dartR* with 1000 permutations. This function was used to run a Mantel test on the proportion of shared alleles among pairs of individuals and the pairwise linear geographic distance between sampling locations. Population-level analyses with varying thresholds of sample size were also conducted.

Results

Field observations

Only populations within the Hunter Valley region were consistent with the morphology of *P. cineolifera*. The morphological boundary between *P. lanceolata* and *P. ovalifolia* was indistinct in the field; therefore, we refer to them hereinafter as 'the *P. lanceolata* group'. However, our tentative field determinations were supported by observations of flowering time. In this group, there is a gradient of flowering time with a discontinuity at the Brisbane River, so we use *P. ovalifolia* and *P. lanceolata* to refer to specimens collected north and south of the Brisbane River, Queensland, respectively.

Following additional field work in 2019 and 2022, a further visit to the National Herbarium of New South Wales and communication with SAJ Bell and KH Stokes in 2021, a total of 22 populations of *P. cineolifera* are known. In unexplored parts of Broken Back Range, Goulburn River National Park and Wollemi National Park, there is considerable suitable habitat of slopes, ridge crests and gullies for more potential populations of *P. cineolifera*.

The number of individuals in populations of *P. cineolifera* ranged from three (Broke Road) to c. 1,000 individuals at Bees Nest Ridge 4 and Yellow Rock Rd 4 (Table 2). The minimum number of individuals for the species is estimated at 5,200, as of October 2022 (Table 2). We found no immature plants of *P. cineolifera* on Wallaby Rocks or Broke Road. All other known populations of *P. cineolifera* were multi-aged including immature plants. Some populations (Bees Nest Ridge 1 and 2, Yellow Rock Rd 2 and 3) were composed of all relatively young individuals, with all

Table 2 Estimated population size and age structure of *Prostanthera cineolifera* populations known to the authors

Locality	Collection site	Population estimate	Age structure
Broken Back Range	Bees Nest Ridge 1	30	most shrubs less than 2 m high
	Bees Nest Ridge 2	80	most shrubs less than 2 m high
	Bees Nest Ridge 3	320	multi-aged
	Bees Nest Ridge 4	1,000	multi-aged
	Broken Back Trail	100	multi-aged, but lacking very old shrubs
	Sawpit Rd	300	multi-aged, but lacking very old shrubs
	Broken Back Rd[a]	40	multi-aged
	Yellow Rock Rd 1	500	multi-aged
	Yellow Rock Rd 2	45	most shrubs less than 2 m high
	Yellow Rock Rd 3	130	most shrubs less than 2 m high
	Yellow Rock Rd 4	1000	multi-aged
	Yellow Rock Rd 5	400	multi-aged
Broke Cessnock area	Broke Rd[b]	3	mature plants only
	Singleton Military Area (two sites) [c]	100	multi-aged
Goulburn River NP	Morrisons Flat Trail	500	multi-aged
Scone	Owens Gap[d]	140	unknown
Sandy Hollow	Wallaby Rocks	10	mature plants only
Upper Bellbird	Limestone Creek (two sites) [e]	40	multi-aged
Wingen Maid NR	Wingen Maid	200	multi-aged
Wollemi NP	Cousins Creek	300	multi-aged
Estimated minimum total population		**5,200**	

Multi aged populations generally included a range of ages from young shrubs less than 1 m high to mature shrubs up to 6–7 m; some mature populations lacked the very old shrubs. Shaded populations were not known to the authors at the time of sampling. NP = National Park, NR = Nature Reserve, Rd = Road

[a]KH Stokes, pers. comm. 2021

[b]These plants did not survive the severe drought that ended early 2020

[c]SAJ Bell, pers. comm. 2021

[d]NSW 785391

[e]LMC Foster, pers. comm. 2018

plants less than 2 m, in contrast to the maximum height for the species of 6–7 m.

Morphological analysis of species boundaries

Morphological clustering via UPGMA and ordination by semi-strong hybrid multidimensional scaling separated the 48 samples scored for morphological characters into three distinct groups: (A) *P. cineolifera*; (B) a mixed group of *P.* sp. Hawkesbury *P*. sp. Olney State Forest and *P.* sp. Oxley Wild Rivers National Park and (C) a mixed group of *P. lanceolata* and *P. ovalifolia*. The top five Kruskal–Wallis characters useful for distinguishing these groups included calyx and pedicel characters (Table 3). Morphometric ordination analysis produced a low stress value (0.0839) indicating an excellent representation of the data in reduced dimensions. The characters contributing most to the discrimination of these groups (Table 3) were four of the five most important in the UPGMA.

The putative population of *P. cineolifera* at Tabbimoble Creek (TB1) in north-eastern New South Wales clustered with the *P. lanceolata* and *P. ovalifolia* group in both the morphometric phenogram (C in Fig. 3a) and the morphometric ordination (arrowed in Fig. 3b). The immature Pillar Valley samples were not included. In Group B, the three samples from the one population in Olney State Forest formed a separate branch as did the eight samples of *P*. sp. Hawkesbury with the one sample of *P.* sp. Oxley Wild

Table 3 Characters contributing strongly to the distinction of three groups in the morphometric ordination and in the morphometric classification (Fig. 3)

Character (units)	r^2	KW	A	B	C
Calyx lobes, shape in bud	**0.902**	**37.4**	Lobes appressed	Indeterminate	Lobes reflexed
Prophylls, persistent or caducous	**0.859**	25.1	Persistent	Caducous	Persistent
Hairs on calyx rim, density	**0.849**	**35.7**	Usually none—sparse	Usually dense	Ranges from none to dense
Leaf length (mm)	**0.843**	19.4	16–30	18.5–36	10.5–28
a_1 axis length[a] (mm)	0.792	**34.2**	1.6–3.1	0–1.7	0.5–2.0
a_1 axis + anthopodium length[a] (mm)	0.784	**33.7**	1.9–4.4	0.5–1.9	1.4–2.8
Hairs on the calyx rim length (mm)	0.658	**34.1**	0–0.06	0.04–0.14	0–0.06

For the morphometric ordination, r^2 (principal-component correlation) values ≥ 0.8 are shown in bold. For the morphometric classification, the top five Kruskal–Wallis (KW) values are in bold. A = *Prostanthera cineolifera*; B = *P.* sp. Hawkesbury + *P.* sp. Olney State Forest + *P.* sp. Oxley Wild Rivers National Park; C = *P. lanceolata* + *P. ovalifolia*. The a_1 axis of the pedicel describes the portion between the bracteoles and the base of the pedicel

[a]Pedicel characters

Rivers National Park sister to *P.* sp. Hawkesbury (Fig. 3a). In Group C, two of the three main branches included samples of both *P. lanceolata* and *P. ovalifolia* (Fig. 3a).

Genetic analysis of species boundaries

The unfiltered SNP data consisted of 185,761 SNPs scored for 157 samples, including 13 technical duplicates. Following filtering, the technical duplicates differed by 2.3% on average (standard deviation 1.2%). After thinning of technical duplicates and removal of anomalous samples (Table S4), the filtered data consisted of 3,915 SNPs scored for 110 individuals, with an average of 0.92% missing data and a mean read depth of 13.93.

The neighbour-joining tree (Fig. 4a), showed distinct clusters of populations in concordance with the three clusters of the morphometric classification and morphometric ordination (Fig. 3). All samples of *P. cineolifera* clustered together in Clade A. The populations assigned a priori to *P.* sp. Hawkesbury, *P.* sp. Olney State Forest and *P.* sp. Oxley Wild Rivers National Park, represented here as 'Paradise Rocks', formed Clade B. Clade C included all individuals assigned a priori to *P. lanceolata* or *P. ovalifolia* and the populations from Tabbimoble Creek and Pillar Valley that had been previously determined as *P. cineolifera*. Within Clade C, Clades D, E and F corresponded to phenology and partly to geographic distribution. Clade E, assigned a priori to *P. ovalifolia* was sister to Clade F, in which all samples were collected south of the Brisbane River and assigned a priori to *P. lanceolata*. Clade D, also assigned a priori to *P. ovalifolia*, was sister to Clades E and F.

The first two axes of the principal component analysis, which explained 40.6% of the total variation (Fig. 5a), identified the same three major clusters as the morphological data. Importantly, all samples that had been determined to be *P. cineolifera* based on morphology clustered together, as did the *P.* sp. Hawkesbury group and the *P. lanceolata* group, which included the populations in north-eastern New South Wales that had been previously identified as *P. cineolifera* and are recorded here by their location codes (TB and PV). However, further resolution of genetic structure within groups was also possible. PCA of genetic data for the *P. cineolifera* group identified four clusters corresponding to the geographic location of collection sites, with all collections in the Pokolbin area forming a tight cluster (Fig. 5b). In the *P.* sp. Hawkesbury group, PCA identified four clusters, mainly corresponding to the geographic location (Fig. 5c). Principal component analysis of genetic data for the *P. lanceolata* group identified four clusters. Populations flowering in November (Mount Stanley, Mount Maria and Mount Ninderry) formed three separate clusters while populations flowering in September formed one cluster (Fig. 5d).

In conStruct analysis, the spatial models were best supported up to K = 5 (Figs. S3, S4, S5), and the posterior distributions of the parameters describing the decay of genetic similarity with distance excluded zero for most layers examined (results not shown). The most stable results with high predictive accuracy were obtained at K = 3 (Figs. S3, S4, S5), and additional layers contributed little to the covariance among populations (Figs. S3, S4, S5). Strong differentiation was observed between *P. cineolifera* and the *P. lanceolata* group (Fig. 4b), but no differentiation was observed within the latter for values of K up to 5 (Fig. S5). The genetic composition of the *P.* sp. Hawkesbury group differed substantially from that of *P. cineolifera* and the *P. lanceolata* group. These results support discontinuities between the major groups, but not within the *P. lanceolata* group. The sampling was not suitable for testing differentiation within the *P.* sp. Hawkesbury group, as it was represented by only four populations.

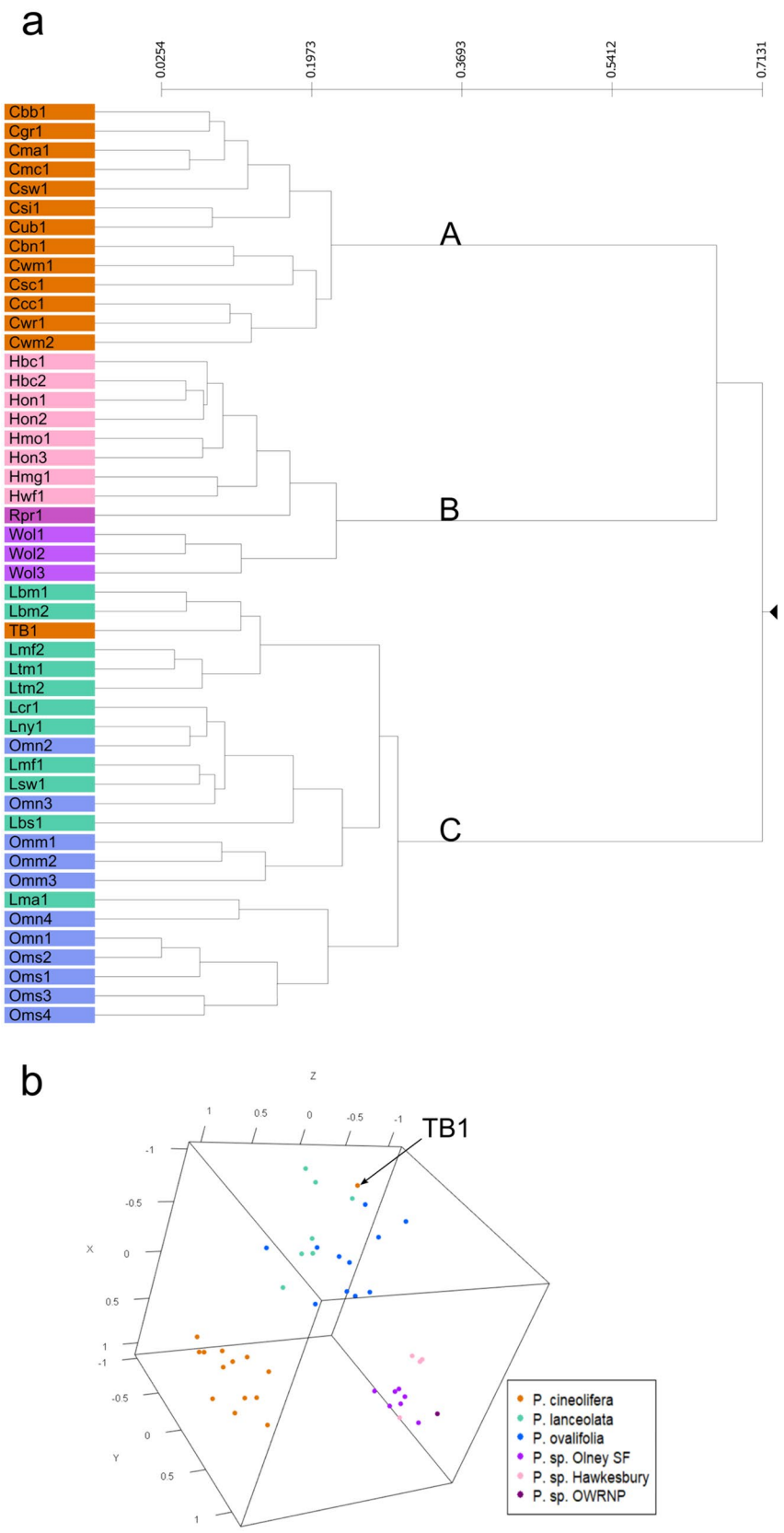


Fig. 3 **a** Phenogram from morphometric classification of 48 samples showing three groups. *A Prostanthera cineolifera; B* a group consisting of *P.* sp. Hawkesbury, *P.* sp. Olney State Forest and *P.* sp. Oxley Wild Rivers National Park; and *C* a mixed group of *P. lanceolata* and *P. ovalifolia* including the specimen from Tabbimoble Creek (TB1) previously determined as *P. cineolifera*. See Table 1 for sample codes. **b** Morphometric ordination plot of semi-strong hybrid multidimensional scaling results. The Tabbimoble Creek specimen (TB1) is indicated by the arrow

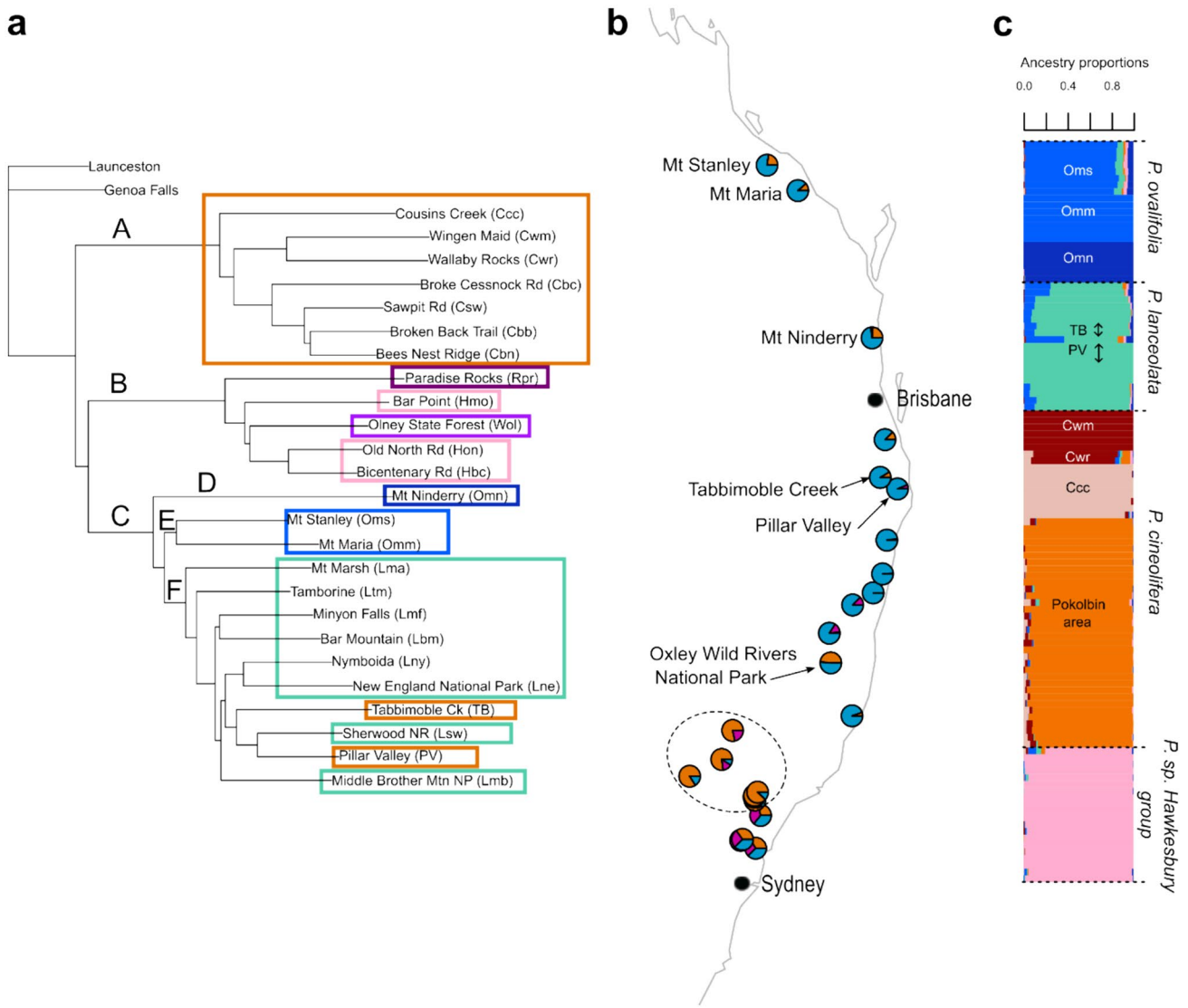


Fig. 4 **a** Neighbour-joining tree of genetic data. Branch names are sampling locations with location code. Distances are based on 4,010 loci across 113 individuals. Clade A = *Prostanthera cineolifera*, Clade B = *P.* sp. Hawkesbury, *P.* sp. Olney State Forest and *P.* sp. Oxley Wild Rivers National Park and Clade C = *P. lanceolata*, *P. ovalifolia*, and the specimens from Tabbimoble Creek and Pillar Valley that had been previously determined as *P. cineolifera*, Clades D, E = *P. ovalifolia,* Clade F = P. *lanceolata*. **b** Non-hierarchical clustering with spatial model in conStruct (Bradburd et al. 2018) of *P. cineolifera, P. lanceolata, P. ovalifolia, P.* sp. Hawkesbury, *P.* sp. Olney State Forest, *P.* sp. Oxley Wild Rivers National Park and the populations from Tabbimoble Creek and Pillar Valley previously determined as *P. cineolifera*. Pies represent estimated proportions of ancestry derived from three layers (see also Figs S3, S4, S5). Dashed ellipse surrounds sampling locations for *P. cineolifera.* **c** Individual ancestry proportions from model-based clustering with sNMF of all individuals, assuming there were 7 genetic clusters/ancestral populations. Location codes (Table 1) included for some samples. (See Fig. S6 for K = 3, 6, 7, 8)

In addition to the species-level discontinuities demonstrated by conStruct analysis, model-based clustering with sNMF also detected strong population structure within *P. cineolifera* and *P. ovalifolia.* At K = 3, the three genetic clusters were concordant with the three clusters in the morphometric analysis (Fig. 3) and conStruct (Fig. S4). Cross-entropy values had a minimum at K = 7 indicating seven genetic clusters/ancestral populations are represented in the data (Fig. S6a). At K = 7, *P. ovalifolia* had two clusters/ancestral populations, corresponding to the geographically separate collection localities, and *P. cineolifera* had three genetic clusters/ancestral populations: Wallaby Rocks and Wingen Maid combined as one cluster, with the Pokolbin area and Cousins Creek as two separate clusters corresponding to the collection locations (Fig. 4c). This is concordant with the model-based clustering with sNMF of the *P. cineolifera* individuals where the cross-entropy values had a

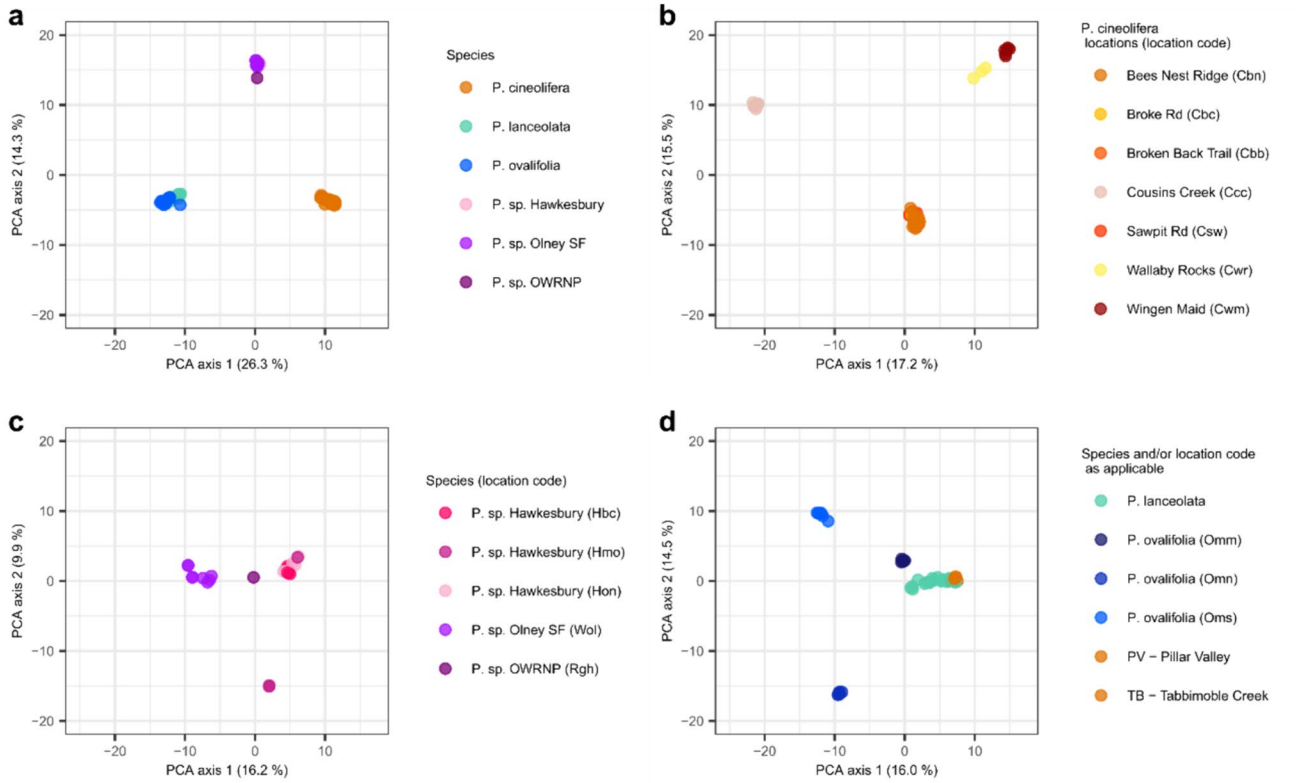


Fig. 5 Genetic structure in *Prostanthera cineolifera* and related taxa identified by principal component analysis (PCA) of SNP data. **a** The filtered dataset for the study group (110 individuals). **b** The *P. cineolifera* taxa of 53 individuals. **c** The *P.* sp. Hawkesbury (Hbc, Hmo and Hon) group, which also includes *P.* sp. Olney State Forest (Wol) and *P.* sp. Oxley Wild Rivers National Park (Rgh). **d** The *P. lanceolata* group, which also includes *P. ovalifolia* (Omm, Omn and Oms) and the populations at Tabbimoble Creek (TB) and Pillar Valley (PV) that had been previously determined as *P. cineolifera.* Percentage of genetic variation explained by each of the PCA axes is provided in parentheses

minimum at K = 3 indicating the same three ancestral populations (Fig. 6c).

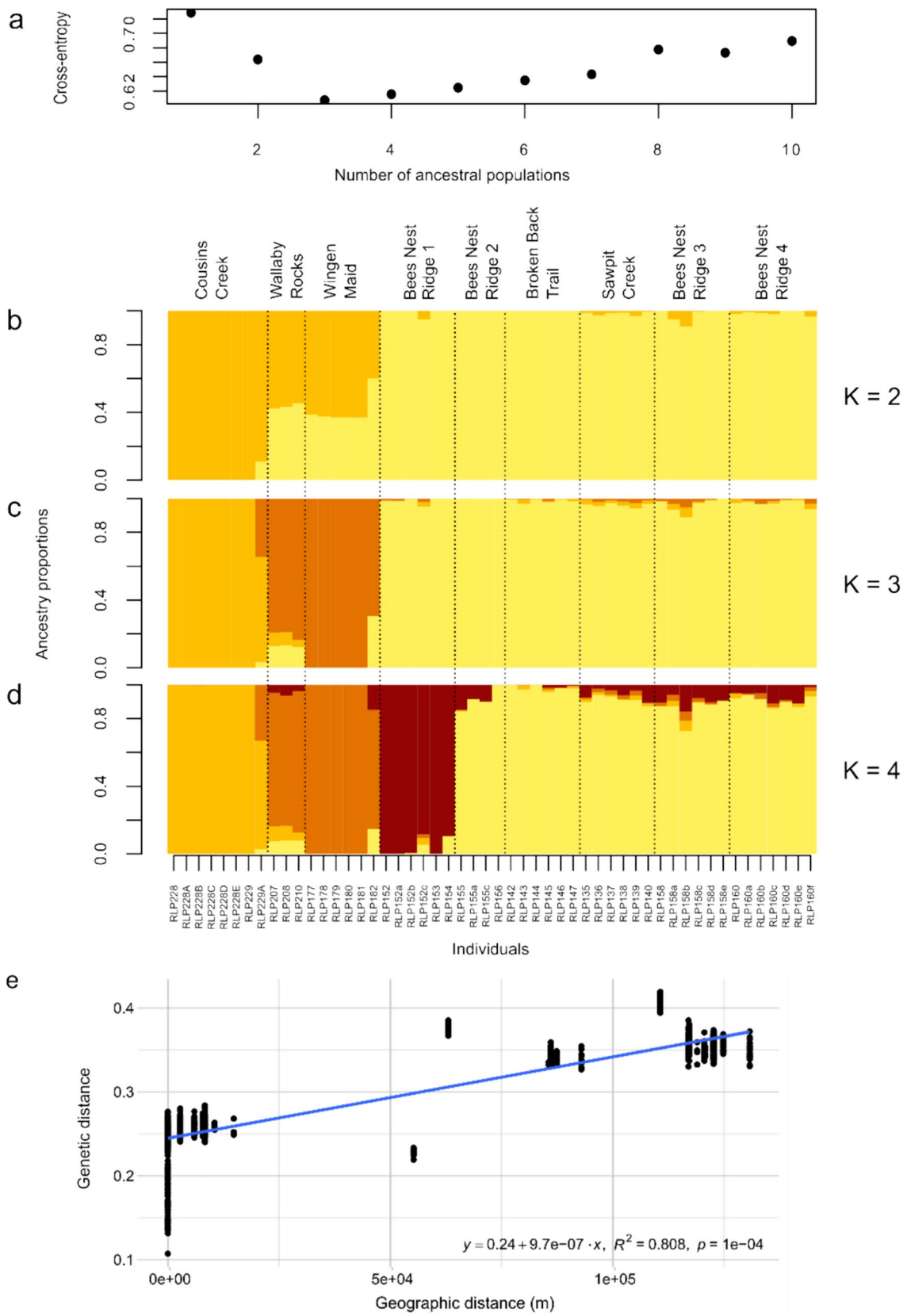
The pairwise F_{ST} values between *P. cineolifera* and *P. lanceolata*, *P. ovalifolia*, *P.* sp. Hawkesbury and *P.* sp. Olney State Forest were all greater than 0.5, indicating pronounced genetic differentiation of *P. cineolifera* from the other species (Table 4). Concordant with the genetic neighbour joining tree (Fig. 4a), there was significant genetic differentiation between *P. lanceolata* and *P. ovalifolia* ($F_{ST} = 0.354$, $p < 0.001$) and between *P.* sp. Hawkesbury and *P.* sp. Olney State Forest ($F_{ST} = 0.205$, $p < 0.001$; Table 4).

Population structure within *Prostanthera cineolifera*

Within *P. cineolifera*, the refiltered dataset consisted of 1,623 SNPs scored for 50 individuals, with an average of 0.46% missing data and a mean read depth of 14.17%. The resultant PCA indicated clusters according to the geographic location of collection site, with all collections in the Broken Back Range/Pokolbin area forming a tight cluster (Fig. 5b). Inbreeding was low or absent in most populations, with a maximum at Bees Nest Ridge 3, which had an inbreeding coefficient of 0.083 (95%CI: 0.064–0.123; Table 5). Gene diversity varied from 0.177 (s.e. 0.005) at Wingen Maid to 0.303 (s.e. 0.005) at Bees Nest Ridge 3.

The global F_{ST} was 0.274, indicating high genetic differentiation among populations within *P. cineolifera*. Pairwise F_{ST} ranged from 0.028 (little genetic differentiation), between the Bees Nest Ridge populations 3 and 4, to 0.544 (pronounced genetic differentiation) between the Cousins Creek and Wingen Maid populations (Table 6). The geographically close Broken Back Range populations of Bees Nest Ridge 1, Bees Nest Ridge 3, Bees Nest Ridge 4, Broken Back Trail and Sawpit Rd occur within an area of c. 8 km^2. For these populations, F_{ST} was less than 0.13 between all pairs. Greater divergence ($F_{ST} > 0.34$) occurred between the Broken Back Range populations and the geographically distant populations of Cousins Creek and Wingen Maid.

Many of the pairwise F_{ST} values between populations of *P. cineolifera* (Table 6) were approaching the between-species values (Table 4), for example between *P. cineolifera* and *P. lanceolata*, $F_{ST} = 0.576$, and higher than the


a
Cross-entropy
0.62
0.70
2
4
6
8
10
Number of ancestral populations
Cousins Creek
Wallaby Rocks
Wingen Maid
Bees Nest Ridge 1
Bees Nest Ridge 2
Broken Back Trail
Sawpit Creek
Bees Nest Ridge 3
Bees Nest Ridge 4
b
c
d
Ancestry proportions
0.0
0.4
0.8
K = 2
K = 3
K = 4
Individuals
e
Genetic distance
0.1
0.2
0.3
0.4
0e+00
5e+04
1e+05
Geographic distance (m)
y = 0.24 + 9.7e−07 · x, R² = 0.808, p = 1e−04

◂**Fig. 6** Model-based clustering and isolation by distance in *Prostanthera cineolifera*. Individual ancestry proportions estimated by sNMF, assuming there are 2–4 genetic clusters/ancestral populations. **a** Cross entropy values. The minimum at $K=3$ indicates that three genetic clusters/ancestral populations are represented in the data. For K = 2 and K = 3, all 20 replicate runs indicated clusters as in **b** and **c** respectively. For K = 4, 26 of 30 replicates indicated clusters as shown in **d**. **e** IBD plot comparing geographic and genetic distances (population based F_{ST}), shown with linear regression

between-species divergence of *P. lanceolata* and *P. ovalifolia* ($F_{ST}=0.354$) and for *P.* sp. Hawkesbury and *P.* sp. Olney State Forest ($F_{ST}=0.205$).

Further model-based clustering of *P. cineolifera* with sNMF provided informative distinctions between geographic sampling locations, indicating the presence of population substructure at relatively fine scales (Figs. S7, S8). For K = 4, the south-eastern-most population on Bees Nest Ridge, Bees Nest Ridge 1, formed a fourth cluster, distinct from the cluster comprised of Sawpit Rd, Broken Back Trail and the other three populations on Bees Nest Ridge (Fig. S7c). Concordant with their very low pairwise F_{ST} values (Table 6), Sawpit Rd, Bees Nest Ridge 3 and Bees Nest Ridge 4 were consistently clustered together for $K<8$ (Fig. S7, S8). The three lowest pairwise F_{ST} values were observed between three populations (between Bees Nest Ridge 3 and Bees Nest Ridge 4: $F_{ST}=0.028$, between Bees Nest Ridge 3 and Sawpit Rd: $F_{ST}=0.042$ and between Bees Nest Ridge 4 and Sawpit Rd: $F_{ST}=0.049$).

Isolation-by-distance was supported by Mantel tests of the relationship between geographic distance and either the proportion of alleles shared by individuals (Mantel's $r=0.899$, $p=0.001$) or Nei's genetic distance between populations (Mantel's $r=0.856$, $p=0.002$); (Fig. 6e).

Discussion

Our study aimed to establish the species boundaries of *P. cineolifera* with respect to related species in the region of its distribution, and to examine its population genetics. Analyses of both the morphological and genetic data returned the same three distinct groups (Figs. 3, 4): (A) *P. cineolifera*; (B) the *P.* sp. Hawkesbury group, a mixed group of *P.* sp. Hawkesbury, *P.* sp. Olney State Forest and *P.* sp. Oxley Wild Rivers National Park; and (C) the *P. lanceolata* group, a mixed group of *P. lanceolata*, *P. ovalifolia* and populations at Pillar Valley and Tabbimoble Creek in north-eastern New South Wales that had previously been determined as *P. cineolifera* of interest.

Geographic extent of *Prostanthera cineolifera*

Analyses of both the morphological and genetic data supported the conclusion that there are no outlying populations of *P. cineolifera* in north-eastern New South Wales, but the distribution does include populations in the Upper Hunter previously determined to be either *P. discolor* or *P. ovalifolia.* The taxonomic concept of *P. cineolifera* as a morphologically, genetically, geographically, chemically (Sadgrove et al. 2020) and ecologically distinct species is supported. We used multiple methods to assess species boundaries in the study group. Patchy sampling may result in apparently discrete population structure, purely due to the presence of isolation-by-distance (IBD) among continuous or connected subpopulations, rather than species boundaries (Pritchard et al. 2000; Tapper et al. 2014). The IBD pattern in *P. cineolifera*, and more broadly within the study group, suggested that it should be accounted for when testing for discrete population structure, and conStruct models incorporating spatial autocorrelation consistently performed better in cross-validation than did nonspatial models (Fig. S3). Clear distinctions exist between *P. cineolifera*, and the other two major groups, but the overall differentiation between *P. lanceolata* and *P. ovalifolia* (Table 4) could be explained by the underlying spatial autocorrelation due to IBD. This result was consistent with morphometric analysis, which did not separate *P. lanceolata* and *P. ovalifolia*.

The north-eastern New South Wales specimens from Tabbimoble Creek and Pillar Valley (Fig. 1), which have sometimes been determined as *P. cineolifera*, correspond to descriptions of *P. lanceolata,* and cluster with other specimens determined by us to be *P. lanceolata* in the morphometric and genetic analyses. Our results indicate that these populations belong to *P. lanceolata* and are not *P. cineolifera*. This confusion may be avoided in the future by updating a couplet in the PlantNET key (Conn 1993) to use calyx characters rather than the currently used indumentum characters. The southern limit of *P. cineolifera* was also clarified, as all populations south of the Hunter Valley were clearly distinct from *P. cineolifera* in all morphological and genetic analyses (Figs. 2, 3, 4, and 5).

Prostanthera cineolifera, distinguished by stem indumentum, calyx features, bud shape and a loose, open inflorescence is distributed in the Sydney Basin and Nandewar Bioregions (IBRA v7.0) (Department of Climate Change Energy the Environment and Water 2020). The two populations from Manilla and Upper Moore Ck (Tamworth) in the Nandewar Bioregion appear to belong to *P. cineolifera* on the basis of morphology, but they have not been recorded since historical herbarium collections were made in 1913 and 1933 respectively. One area of uncertainty remains. Populations of *Prostanthera* in the West Wyalong to Wagga Wagga districts of New South Wales that were originally

Table 4 Pairwise F_{ST} among the *Prostanthera* study species. For all pairs, $p < 0.001$ based on 1000 random permutations

	P. lanceolata	*P. ovalifolia*	*P.* sp. Hawkesbury	*P.* sp. Olney SF
P. ovalifolia	0.354			
P. sp. Hawkesbury	0.667	0.660		
P. sp. Olney SF	0.700	0.693	0.205	
P. cineolifera	0.576	0.572	0.589	0.587

For ease of interpretation, cells are shaded. The darker the shading, the higher the F_{ST} value

Table 5 Genetic summary statistics for populations of *Prostanthera cineolifera*, averaged over 1623 SNP loci

Population	*N*	Polymorphic loci (%)	H_O (s.e)	H_E (s.e.)	F_{IS} (95% CI)	*pa* (%)
Bees Nest Ridge_1	5	69.2	0.306 (0.007)	0.272 (0.005)	-0.128 (-0.174, -0.117)	0.9
Bees Nest Ridge_3	5	74.6	0.277 (0.006)	0.303 (0.005)	0.083 (0.064, 0.122)	0.0
Bees Nest Ridge_4	7	80.5	0.295 (0.006)	0.299 (0.005)	0.012 (-0.011, 0.039)	0.0
Sawpit Rd	6	78.0	0.291 (0.006)	0.296 (0.005)	0.017 (-0.007, 0.045)	0.0
Broken Back Trail	6	73.4	0.284 (0.006)	0.285 (0.005)	0.003 (-0.023, 0.029)	0.1
Cousins Creek	8	55.9	0.201 (0.006)	0.209 (0.005)	0.041 (0.014, 0.075)	58.5
Wingen Maid	6	46.1	0.174 (0.006)	0.177 (0.005)	0.016 (-0.017, 0.054)	25.4

Standard errors and 95% confidence intervals were based on resampling of loci, not individuals. N = number of individuals, H_O = observed heterozygosity, H_E = unbiased expected heterozygosity, F_{IS} = inbreeding coefficient, *pa* = private alleles

Table 6 Pairwise F_{ST} among the populations of *P. cineolifera*, where N > 5

	Bees Nest Ridge_1	Bees Nest Ridge_3	Bees Nest Ridge_4	Sawpit Rd	Broken Back Trail	Cousins Creek
Bees Nest Ridge_3	0.093					
Bees Nest Ridge_4	0.099	0.028				
Sawpit Rd	0.103	0.042	0.049			
Broken Back Trail	0.126	0.061	0.062	0.071		
Cousins Creek	0.402	0.356	0.344	0.344	0.369	
Wingen Maid	0.432	0.377	0.365	0.373	0.397	0.544

For all pairs, $p < 0.001$ based on 1000 random permutations. For ease of interpretation, cells are shaded. The darker the shading, the higher the F_{ST} value

described by Bentham (1834) as *P. atriplicifolia* A.Cunn. ex Benth appear somewhat morphologically similar to *P. cineolifera.* More work is underway to ascertain if they are a separate entity or *P. cineolifera.*

Genetic relationships and population structure of *Prostanthera cineolifera*

Population structure within *P. cineolifera* was expected, as our sampling localities for *P. cineolifera* were up to 100 km apart. We detected substantial structure at multiple scales, using diverse analytical techniques. The three main groups (A–C; Figs. 3a, 4a), correspond to geographic areas (Fig. 3b). At that scale, F_{ST} was similar to or greater than that between species in this group. For example, $F_{ST} = 0.537$ between Cousins Creek and Wingen Maid (Table 6), compared with $F_{ST} = 0.439$ between *P. cineolifera* and *P. lanceolata* (Table 4). In an outcrossing perennial plant, fragmentation is likely to take many generations to produce such pronounced population divergence. The patchy distribution of the species appears to result primarily from a combination of historical biogeographical processes and specialized soil requirements. However, fragmentation due to agricultural intensification or other human impacts in the Hunter Valley may have contributed, as *P. cineolifera* was apparently more

common in the Millfield, Broke and Singleton areas in the early 1900s (Baker and Smith 1912).

Differentiation among individual sampling sites was also seen at finer scales, including between neighbouring populations on Bees Nest Ridge that were only c. 300 m apart. The strong genetic structure within *P. cineolifera* indicates a lack of migration between populations and the most isolated known site (Wingen Maid and by association Wallaby Rocks) has the lowest genetic diversity ($H_E = 0.15$). The genetic structure may be partly explained by geographic separation, as it displays a significant pattern of IBD ($P < 0.0001$). *Prostanthera cineolifera* is probably pollinated by beetles and flies (Wilson et al. 2017), and although some beetle pollinators fly up to 70 m between plants (Englund 1993), insect pollination and gravity-dispersed seeds do not favour long distance gene flow. This limited gene flow would contribute to the very high F_{ST} estimates between samples such as Cousins Creek and Wingen Maid ($F_{ST} = 0.537$), and to the moderate F_{ST} estimate between Broken Back Trail and Bees Nest Ridge 1 ($F_{ST} = 0.124$). Model-based clustering with sNMF also detected genetic structure at a range of scales (Figs. 6, S7, S8).

While there is good evidence to support at least three distinct management units within *P. cineolifera*, it is not known exactly how many populations remain unsampled in remote patches of suitable habitat between localities. We now know of several more recently-discovered populations in the Pokolbin area that were not sampled for genetic analysis: within the Singleton Military Area (SMA), five populations on Yellow Rock Road, another population on Broken Back Trail and several populations in the Cedar Creek biodiversity offset, now Cedar Creek Nature Reserve (Bell 2017). A large population was recently discovered in Goulburn River National Park. Nevertheless, it is clear from our fieldwork that the distribution of *P. cineolifera* is highly patchy, rather than continuous. While our population sampling was exhaustive based on our knowledge at the time, judging from satellite imagery (for example https://maps.six.nsw.gov.au/), there are other areas that likely contain other populations of *P. cineolifera*.

Conservation status of *Prostanthera cineolifera*

Restricted gene flow can have negative consequences for populations, including inbreeding and decreases in genetic diversity, and hence a decrease in adaptive potential of the population (Ellstrand and Elam 1993; Reed and Frankham 2003; Garant et al. 2007; Markert et al. 2010). In this study, inbreeding coefficients did not seem to be related to population size, but rather to demography. Bees Nest Ridge 3 (population approximately 320) had a relatively high inbreeding coefficient of ($F = 0.135$) unlike Bees Nest Ridge 1 (population approximately 30) which had an inbreeding coefficient of -0.128. The 95% confidence intervals estimated for F_{IS} at Bees Nest Ridge 1 should be interpreted with caution, because they are based on resampling of loci, rather than individuals. As clonality was absent in the data, the most likely explanation for the negative value was the sampling of individuals ($N = 5$). Nevertheless, these populations differ in age structure, with Bees Nest Ridge 1 being a relatively young population lacking large old shrubs, whereas Bees Nest Ridge 3 had a more even distribution of growth stages. As all plants in the young population are adjacent to the road, we suspect that recruitment of the relatively young populations has been triggered by road building or maintenance, either through introduction of seeds by the grader or by stimulation of the soil seed bank by disturbance. Although no obvious signs of low fitness were observed at Bees Nest Ridge 3, further monitoring may be warranted.

Genetic diversity is needed for populations to adapt to changes in the environment, and gene flow is an important source of genetic variation, particularly when other populations possess potentially adaptive variants (Sexton et al. 2011). The predicted global warming of 2–7 °C by 2100 (Stott and Kettleborough 2002; O'Neill et al. 2016) will place smaller, isolated populations with lower genetic diversity, such as the populations of *P. cineolifera* at Wingen Maid and Wallaby Rocks, at greater risk of extinction (Aitken et al. 2008). While these isolated populations may have evolved low rates of inbreeding depression or maintain sufficiently large populations to avoid it, adaptive capacity may be limited without historical or recent connectivity. The Wingen Maid population is of concern, as it has low genetic diversity (as measured by expected heterozygosity: $H_E = 0.177$, s.e. 0.0.005) in comparison with other populations of the species (Table 5) despite a relatively healthy population size and structure. Further study of the Wingen Maid population, as well as several small populations, may be needed to determine whether the fitness of the population is vulnerable to changes in precipitation and temperature regimes. The very small population (c. 10) at Wallaby Rocks is of particular concern. This population is genetically similar to the Wingen Maid population, but it is much smaller and is threatened by overgrazing, possibly by both feral goats and macropods. The landholder should be encouraged to control the feral goat population, and further monitoring is recommended.

The total known census size of *P. cineolifera* is at least 5200 plants (Table 2), with the majority in the Pokolbin area. Except for the small populations on Broke Road (no longer extant as of July 2022) and at Wallaby Rocks, all populations are multi-aged, although several sites on Bees Nest Ridge and Yellow Rock Rd lack large, old shrubs. Many populations of *P. cineolifera* are likely unknown in the less explored areas of the region. *Prostanthera cineolifera* is currently listed as Vulnerable under both Commonwealth and New South Wales legislation. However, we have presented

evidence that supports delisting of *P. cineolifera*. While the demographic information, genetic data and estimated population sizes of *P. cineolifera* suggest that the species is not in immediate danger of extinction, the apparent lack of genetic connectivity and signs of reduced genetic diversity or inbreeding do raise concern about conservation of certain populations as mentioned above. Although the species is morphologically consistent across the range that we have determined here, it seems likely that unique genetic diversity would be lost if any populations became extinct. Indeed, the apparent disappearance of populations in the Nandewar Bioregion may have already compromised the evolutionary potential of the species.

Other entities

Prostanthera sp. Olney State Forest is currently known from one population, and efforts to find further populations have not been successful. The population suffered a major population decline with the severe drought that ended in early 2020, with little recruitment following good rains. This taxon needs conservation and has informal protection. The known population is in a section of state forest marked as "forest management zone 2- a permanent exclusion to harvesting" (Mark Drury, Forestry Corporation of NSW, pers. comm. 2022). Further work is needed to understand the ecology, particularly the fire ecology, of *P.* sp. Olney State Forest.

Group B (Figs. 3, 4) includes the only available sample of *P.* sp. Oxley Wild Rivers National Park. It is genetically most similar to *P.* sp. Hawkesbury but is morphologically distinct. We therefore consider it to be a separate entity that will be the subject of a future paper.

Further work is needed to test and clarify species boundaries in the *P. lanceolata* group with more extensive sampling, particularly for *P. ovalifolia*. There is some evidence of genetic clustering in ordinations based on genetic data, and populations of this group differ in phenology: specimens from north of the Brisbane River flower in November, while the populations from south of the Brisbane River flower in September. However, the morphological boundary is not clear, and ConStruct analysis did not support the boundary when accounting for spatial autocorrelation. This suggests that the morphological and phenological variation may reflect isolation-by-distance in a more-or-less continuously distributed species, rather than two reproductively isolated species. Indeed, Althofer (1978, p. 47) observed that "as the [*P. ovalifolia*] moves southward", down the New South Wales coast, "an almost imperceptible change takes place. The leaves become progressively larger, and small indentations become perceptible….". Additional sampling is needed to characterise the morphological and genetic variation in the group, which may reflect adaptation to climatic, latitudinal and elevational gradients.

Conclusions

By clarifying the species boundaries of *P. cineolifera*, this study has shown that populations outside of the Peel and Hunter Valleys belong to other species and are not of conservation concern as unusual isolates of *P. cineolifera*. The northern populations are part of the *P. lanceolata* and *P. ovalifolia* group, while the populations in the Lower Hawkesbury are distinct from *P. cineolifera* and will be the subject of a separate taxonomic paper. The study has also documented deep genetic divergence and IBD among populations of *P. cineolifera*. Finer-scale subdivision was also observed, likely due to isolation-by-distance, consistent with insect pollination and passive seed dispersal. While *P. cineolifera* does not appear at immediate risk of extinction, certain populations exhibited lower genetic diversity or elevated inbreeding coefficients, although neither was related to population size. This work highlights the genetic consequences of restricted dispersal and isolated populations and identifies a need for further research on the adaptive capacity of such populations, especially in the face of changing climate.

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Author contributions Jeremy Bruhl, Rose Andrew and Ian Telford contributed to the study concept and design. Sample collection was performed by Ruth Palsson, Jeremy Bruhl, Rose Andrew and Ian Telford. Analyses were performed by Ruth Palsson with advice from Rose Andrew, Jeremy Bruhl and Ian Telford. The first draft of the manuscript was written by Ruth Palsson and all authors commented on versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Morphological data are available in the accompanying Supplementary_file2. The SNP datasets generated during and/or analysed during the current study are available in the RUNE repository at https://hdl.handle.net/1959.11/58744 (https://doi.org/10.25952/rgw2-sc64).

Declarations

Competing interest The authors declare no competing interests.

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