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# Molecular phylogenetic analysis of the *Prostanthera phylicifolia* (Lamiaceae) assemblage resolves relationships of the 'Critically Endangered' *P. gilesii* and other putative new species

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## Abstract

*Prostanthera gilesii* Althofer ex. B.J.Conn & T.C.Wilson is a critically endangered species from the Central Tablelands of New South Wales. A conservation management strategy is currently underway for this species, whose phylogenetic affinities are not known. Morphologically, *P. gilesii* resembles *P. phylicifolia* F.Muell. and a population of uncertain identity from Evans Crown Nature Reserve in the Central Tablelands of New South Wales known as *P.* sp. Evans Crown (G.M.Taseski NSW1055966). The taxonomy of *P. phylicifolia,* however, is unclear. *Prostanthera phylicifolia* was described from populations in the Victorian Alps and Monaro; however, populations spanning from Victoria to southern Queensland have been variously identified as either *P. phylicifolia* or *P. scutellarioides* (R.Br.) Briq. despite substantial geographic disjunctions and morphological dissimilarity. To examine the relationship between populations identified as *P. phylicifolia, P. gilesii* and *P.* sp. Evans Crown, nuclear (external transcribed spacer, ETS) and chloroplast (*trn*H-*psb*A intergenic spacer) regions were sequenced and combined with an existing *Prostanthera* dataset and analysed with maximum-likelihood and Bayesian-inference methods. *Prostanthera gilesii* and *P.* sp. Evans Crown were recovered as sister taxa within a clade consisting of populations morphologically similar to the type of *P. phylicifolia* from the Victorian Alps and Snowy Mountains, Monaro and Southern Tablelands of New South Wales. Populations from northern New South Wales and southern Queensland identified as *P. phylicifolia* or *P. scutellarioides* were recovered as an assemblage of unrelated clades. The molecular phylogeny supports *P. gilesii* and *P. phylicifolia* as closely related as hypothesised based on morphology and supports *P.* sp. Evans Crown as a population which requires additional study to assess its taxonomic status.

## Introduction

Globally, isolated mountain masses and rock outcrops (e.g. shield volcanoes, inselbergs, monadnocks, tors) harbour a diverse range of narrowly endemic, highly specialised and threatened taxa (Porembski and Barthlott 2000; Michael *et al.* 2010). As these rocky outcrops often function as relictual ecosystems or refugia, they are particularly vulnerable to environmental threats such as climate change, land degradation, and altered fire regimes (Fitzsimons and Michael 2017; IUCN 2019; Selwood and Zimmer 2020). As an overwhelming body of evidence suggests that human-induced species loss is accelerating (Barnosky *et al.* 2011; Vos *et al.* 2014; Ceballos *et al.* 2015), there is an imperative to prioritise the understanding, documentation, and conservation of such fragmented biota.

Australian mint-bush, *Prostanthera* Labill., is the largest group of Australian Lamiaceae and many species have restricted ranges that are associated with isolated rock outcrops (Conn 1984, 1988; Conn and Wilson 2015). With striking floral displays and strongly aromatic leaves, various *Prostanthera* have horticultural, agricultural, and pharmaceutical value (Althofer 1978; Collins *et al.* 2014; Tang *et al.* 2017; Sadgrove *et al.* 2020). The Australian Plant Census (APC) currently recognises 100 species, 8 subspecies, and 18 standardised phrase-named species (APC 2021) in *Prostanthera*. Of the 100 accepted species, 19 are listed as "threatened" at a Commonwealth level (Department of Agriculture 1999, 2021) and 13 are listed as "threatened" in New South Wales (Government 2016; OEH 2021) representing almost a fifth of these species. One such species is the critically endangered *P. gilesii* Althofer ex. B.J.Conn & T.C.Wilson which is known from two small subpopulations on the shield volcano of Mount Canobolas State Conservation Area, south-west of Orange on the Central Tablelands of New South Wales (Figure 1A, Figure 2) (Conn and Wilson 2015). The species is the subject of targeted conservation research and management projects (OEH 2019; Scott and Auld 2020; Andrew *et al.* unpublished data) which includes population monitoring, genomic analysis, *ex situ* conservation and weed and pest management.

The phylogenetic relationship between *P. gilesii* and other *Prostanthera* is not known, which has impeded possible genomic conservation efforts. Althofer (1978) first recognised this species as *P.* sp. aff. *phylicifolia* (1978) and Conn and Wilson (2015) also identified a strong morphological similarity between *P. gilesii* and *P. phylicifolia* F.Muell. However, these similarities are difficult to reconcile as the taxonomic identity of *P. phylicifolia* has become unclear following changes implemented in Bentham's (1870) revision of the genus.

*Prostanthera phylicifolia s. str.* (Figure 1B, Figure 2) was described by Mueller (1858) from material collected from Mt Macfarlane (now Macfarlane's Lookout) near Omeo in northern Victoria and the "Maneroo" (present-day Monaro) region of New South Wales. Bentham's (1870) subsequent treatment of *P. phylicifolia* broadened its circumscription through citing specimens from the New England region, New South Wales, and the Glass House Mountains, Queensland, in addition to the Victorian type locality. Bentham's broad circumscription has caused taxonomic confusion, and populations from northern New South Wales and southern Queensland (Figure 2) have been variously assigned as either *P. phylicifolia* or *P. scutellarioides* (R.Br.) Briq. (Figure 1D), depending on herbaria curatorship. As an example, gatherings from these populations identified as *P. phylicifolia* (Figures 1E–F) sent as duplicates from the Queensland Herbarium (BRI) to the National Herbarium of New South Wales (NSW) have been reidentified at NSW as *P. scutellarioides*. While these populations share glabrous, narrow ovate leaves, floral morphology between northern and southern populations is largely variable (Figure 1). For example, southern populations share anthers with a distinctly elongated outgrowth of the connective tissue found between both pollen sacks (i.e. an anther appendage), while this appendage is reduced to absent in northern populations. Prior studies of floral types in *Prostanthera* (Wilson *et al.* 2017) suggest that such pronounced differences in floral type indicate that these groups are not closely related. Thus, further investigation is warranted across these populations.

Previous research into the systematics of *Prostanthera* included a sample from a southern population of *P. phylicifolia*, located at Deua National Park (Population 8, Figure 2), on the Southern Tablelands of New South Wales (Wilson *et al.* 2012; Conn *et al.* 2016). In these studies, this accession, and an accession of *P. scutellarioides* from Castlereagh Nature Reserve in the Sydney Basin were recovered as sister taxa, within a clade referred to as "Clade C". In Bentham's (1870) series classification, these two species were circumscribed within series *Convexae* Benth. While Wilson *et al.* (2012) revealed inconsistencies with Bentham's series and sectional classifications, they reported that most members of the *Convexae* were recovered within a well-supported "Clade C". Bentham's (1870) series *Convexae* encompasses species with small, shortly petiolate leaves with a convex–revolute margin and flowers that appear axillary. This may account for some confusion surrounding the identity of northern populations variously identified as either *P. phylicifolia* or *P. scutellarioides*. As none of these disjunct populations have been included in prior molecular studies of *Prostanthera*, their relationship with *P. phylicifolia* and *P. scutellarioides* is still unknown.

**Fig. 1.** Photographs of species and populations of *Prostanthera* investigated in this study. A = *P. gilesii*; B = *P. phylicifolia s. str.* (Tinderry); C = *P.* sp. Evans Crown; D = *P. scutellarioides s. str.* (Castlereagh Nature Reserve); E = *P. phylicifolia s. lat.* (Gibraltar Range National Park); F = *P.* sp. Minyon Falls. Images: T. C. Wilson (A); D. Indiarto (B); R. P. O'Donnell (C); G. M. Taseski (D); J. J. Bruhl (E); M. Colpus (F).

Another entity of uncertain identity that is morphologically similar to *P. phylicifolia s. str.* and *P. gilesii* is known from Evans Crown Nature Reserve on the Central Tablelands of New South Wales (Figure 1C, Figure 2). This entity (hereafter referred to as *P.* sp. Evans Crown (G.M.Taseski NSW1055966)) was first identified from herbarium material held at NSW while curating collections of *P. phylicifolia.* Collections from Evans Crown superficially resembled both *P. phylicifolia* and *P. gilesii* but appeared morphologically distinct on the basis of a dense indumentum of appressed retrorse hairs on all organs (vs ± glabrous to sparsely hairy, hairs antrorse for *P. phylicifolia* and *P. gilesii*), large elliptic prophylls (vs linear-terete for *P. phylicifolia* and narrow-elliptic for *P. gilesii*), and mericarps that are rugose and papillose with occasional long pilose hairs (vs reticulate, not distinctly papillose and glabrous for *P. phylicifolia*, mature mericarps not observed for *P. gilesii*). The phylogenetic affinities of *P.* sp. Evans Crown are also unknown. *Prostanthera gilesii* and *P.* sp. Evans Crown are most morphologically similar to *P. phylicifolia s. str.* and on this basis, we can posit that they are also members of "Clade C".

In this study, we used molecular evidence to determine the phylogenetic affinities of *P. gilesii* and *P.* sp. Evans Crown and investigate their relationship with populations variously determined as *P. phylicifolia* or *P. scutellarioides*. Molecular phylogenetic studies of *Prostanthera* using Sanger sequencing have allowed for the resolution of taxonomic issues between closely related taxa (Conn *et al.* 2013) and the separation of new species from morphologically similar accessions (Conn *et al.* 2016), but have also revealed the presence of species complexes (e.g. *P. howelliae* Blakely) that still require attention (Wilson *et al.* 2012). Here, we aim to extend our understanding of *P. phylicifolia* across its broader distribution, reconstruct the phylogenetic relationships of *P. gilesii, P. phylicifolia* and *P.* sp. Evans Crown, and test the monophyly of these taxa.

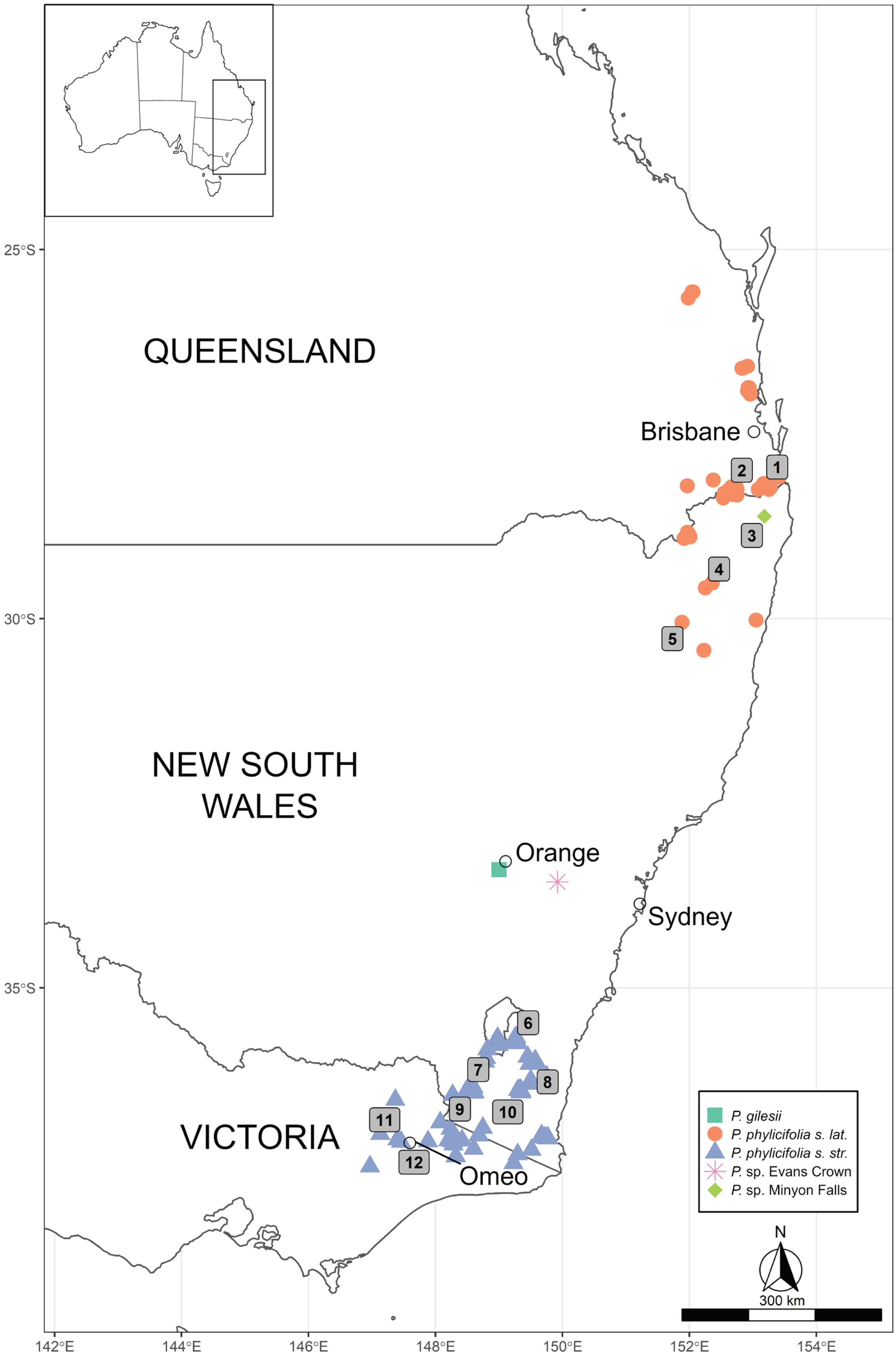

**Fig. 2.** Distribution map and sampled populations of *Prostanthera gilesii, P. phylicifolia s. lat., P. phylicifolia s. str., P.* sp. Evans Crown and *P.* sp. Minyon Falls based on AVH occurrence records (AVH 2021). 1 = Lamington NP; 2 = Mt. Barney NP; 3 = Minyon Falls; 4 = Gibraltar Range NP; 5 = Warra NP; 6 = Tinderry Ranges; 7 = Adaminaby; 8 = Deua NP; 9 = Kosciuszko NP; 10 = Dangelong NR; 11, 12 = Cobrunga.

## Materials and Methods

### *Specimens*

Leaf material for genetic analysis was sampled from material dried using silica gel or from herbarium material (Table 1). Samples of *P. gilesii* from the two known subpopulations at Towac Creek and The Walls, and two samples of *P.* sp. Evans Crown were used for analysis. Accessions representing 12 populations variously identified as *P. phylicifolia* or *P. scutellarioides* were included in our analyses. Populations sampled included five populations variously identified as *P. phylicifolia* or *P. scutellarioides* from northern New South Wales to southern Queensland (Figure 2, Populations 1–5), and seven populations considered to represent *P. phylicifolia s. str.* spanning from Victoria to the Southern Tablelands of New South Wales (Figure 2, Populations 6–12). As the northern accessions differ in their assignation between herbaria, they will be treated here as *P. phylicifolia s. lat.* following Bentham (1870). This includes one accession more recently assigned as *P.* sp. Minyon Falls (J.B.Williams *s.n.*, NE61356) I.Telford, which is maintained within the *P. phylicifolia* assemblage for simplicity (Figure 2, Population 3). An additional accession of *P. scutellarioides* from the Hunter Valley was sampled to test the northern limits of this species. Several specimens from Victoria identified as *P. phylicifolia* were observed as either glabrous or with a dense indumentum on all organs of entirely antrorse hairs, or occasionally retrorse hairs, which is consistent with Mueller's (1858, p.19) description of individuals of *P. phylicifolia* from the type locality as ranging from glabrous to velutinous ("glabra v. velutina"). Individual plants that are either entirely glabrous or densely hairy have previously been observed occurring within the same population (N.G. Walsh pers. comm. 2020). Leaf material from both glabrous and velutinous forms from the Cobungra region were collected for analysis from cultivated material held at the Royal Botanic Gardens Melbourne (MEL) and are here referred to as *P. phylicifolia* '*glabra*' and *P. phylicifolia* '*velutina*' *sensu* Mueller (1858) respectively.

**Table 1.** Accession details for specimens of *Prostanthera* and *Westringia* sampled for Sanger sequencing of nuclear (external transcribed spacer, ETS) and chloroplast (*trn*H-psbA intergenic spacer) markers in this study. Several accessions from previous studies were found to have been published with erroneous collection details that did not match those found on their respective herbarium voucher sheet or AVH database records. These samples are noted below and have been corrected to reflect their respective voucher sheets. GenBank accessions are given for each marker. P = *Prostanthera*; W = *Westringia*; CP = Conservation Park; NP = National Park; NR = Nature Reserve; SCA = State Conservation Area; SF = State Forest; N.S.W. = New South Wales; Qld = Queensland; S.A. = South Australia; Tas. = Tasmania; Vic. = Victoria; W.A. = Western Australia. New accessions for this study are indicated by bold text, ^ = previously published accessions with amended collection details.

| Taxon | Locality | Voucher | Herbarium and accession # | ETS | *trn*H-*psb*A |
|---|---|---|---|---|---|
| *P. albohirta* | Mt Emerald, Qld | Ford 6174 | CNS 010353.1 | KU532856 | KU563336 |
| *P. althoferi* | Yalgoo, W.A. | Wilson 271 | NSW 901182 | KU532857 | KU563337 |
| *P. althoferi* | Kambalda East, W.A. | Wilson 360 | NSW 902815 | KU532858 | KU563338 |
| *P. ammophila* | Pinkawillinie CP, S.A. | Te 854 | AD 232799 | KU532859 | KU563339 |
| *P. athertoniana* | Kahlpahlim Rock, Qld | Ford 1835 | NSW 1001033 | KU532860 | KU563340 |
| *P. baxteri* | Ravensthorpe, W.A. | Wilson 380 | NSW 845708 | KU532861 | KU563341 |
| *P. campbellii* | East Pilbara, W.A. | Perkins WA101 | PERTH 8461236 | KU532862 | KU563342 |
| *P. canaliculata* | Toodyay, W.A. | Hislop *et al.* 178–7 | PERTH 7433239 | KU532863 | KU563343 |
| *P. centralis*^ | Central Ranges, W.A. | Wilson 417 | NSW 972502 | KU532864 | KU563344 |
| *P. clotteniana* | Ravenshoe SF, Qld | Ford 5982 | NSW 933508 | KU532865 | KU563345 |
| *P. densa* | Port Stephens, N.S.W. | Wilson 37 | NSW 789038 | JX047677 | KF145100 |
| *P. eckersleyana* | Mt Marshall, W.A. | Horn 2508 | PERTH 5686180 | KU532866 | KU563346 |
| *P. eungella* | Eungella, Qld | McDonald 5131 | NSW 452207 | KU532867 | KU563347 |
| *P. galbraithiae* | Holey Plains, Vic. | Conn 5222 | NSW 808463 | JX047667 | KU563348 |
| ***P. gilesii*** **(Towac)** | **Towac Creek, Mt. Canobolas SCA, N.S.W.** | **Bruhl 3602** | **NE 108967** | **OK524300** | **OK562349** |
| ***P. gilesii*** **(Walls)** | **The Walls, Mt. Canobolas SCA, N.S.W.** | **Medd 7111** | - | **OK524301** | **OK562350** |
| *P. granitica*^ | Warrumbungles NP, N.S.W. | Tuft 162 | NSW 844192 | JX047639 | KU563349 |
| *P. howelliae*^ | Sackville, N.S.W. | Wilson 216 | NSW 799701 | JX047644 | KU563350 |
| *P. laricoides* | Yilgarn, W.A. | Edinger 6582 | PERTH 7772394 | KU532868 | KU563351 |
| *P. lasianthos* | Grampians NP, Vic. | Conn 5311 | NSW 799716 | KU532869 | KU563352 |

| Taxon | Locality | Voucher | Herbarium and accession # | ETS | *trn*H-*psb*A |
|---|---|---|---|---|---|
| *P. lasianthos* | Mt Field, Tas. | Henwood 864 | NSW 836127 | KU532871 | KU563353 |
| *P. lasianthos* | Mt Wilson, N.S.W. | Proft 3 | NSW 105895 | KU532870 | KU563355 |
| *P. lasianthos* | Blue Mountains NP, N.S.W. | Wilson 44 | NSW 799707 | JX047663 | KU563354 |
| *P. marifolia* | Sydney, N.S.W. | Conn 4380A | NSW 743858 | JX047657 | KF145108 |
| *P. mulliganensis* | Mt Mulligan, Qld | Clarkson 5241 | NSW 797165 | KU532872 | KU563356 |
| *P. nivea* var. *induta*^ | Warrumbungle Mountains, N.S.W. | Wilson 203 | NSW 803949 | JX047651 | KU563357 |
| *P. nudula* | Sentinel Hill, S.A. | Latz 24527 | AD 240053 | KU532873 | KU563358 |
| *P. ovalifolia s. l.* | Lamington NP, Qld | Forster PIF40534 | AQ 836012 | - | KU564718 |
| *P. ovalifolia s. l.* | Whian Whian SCA, N.S.W. | Anon. AA 873108 | NSW 4200941 | JX047633 | - |
| *P. ovalifolia s. l.* | Sherwood NR, N.S.W. | Wilson 139 | NSW 799705 | JX047622 | - |
| *P. patens* | Blue Hills Range, W.A. | Markey & Dillon 3715 | PERTH 7461267 | KU532874 | KU563359 |
| **_P. phylicifolia s. l._ (1)** | **Lamington NP, Qld** | **Beesley 1005** | **NSW 276830** | **OK524294** | **OK562342** |
| **_P. phylicifolia s. l._ (2)** | **Mt. Barney NP, Qld** | **Mathieson 485** | **NSW 815397** | **OK524293** | **OK562343** |
| **_P. phylicifolia s. l._ (4)** | **Gibraltar Range NP, N.S.W.** | **Taseski 961** | **NSW 1057452** | **OK524297** | **OK562346** |
| **_P. phylicifolia s. l._ (5)** | **Warra NP, N.S.W.** | **Telford 13500** | **NE 101293** | **OK524292** | **OK562341** |
| **_P. phylicifolia s. s._ (6)** | **Tinderry Ranges, N.S.W.** | **O'Donnell 61** | **NSW 1100407** | **OK524307** | **OK562356** |
| **_P. phylicifolia s. s._ (7)** | **Adaminaby, N.S.W.** | **Medd 217100a1** | **NE 109825** | **OK524306** | **OK562355** |
| *P. phylicifolia s. s.* (8) | Deua NP, N.S.W. | Liney 2039 | NSW 887109 | JX047643 | - |
| **_P. phylicifolia s. s._ (9)** | **Kosciuszko NP, N.S.W.** | **Medd 217096a** | **NE 109821** | **OK524305** | **OK562354** |
| **_P. phylicifolia s. s._ (10)** | **Dangelong NR, N.S.W.** | **Medd 217099b** | **NE 109826** | **OK524304** | **OK562353** |
| **_P. phylicifolia s. s. 'velutina'_ (11)** | **Cobrunga, Vic.** | **Walsh 8979** | **MEL 2470086** | **OK524302** | **OK562352** |
| **_P. phylicifolia s. s. 'glabra'_ (12)** | **Cobrunga, Vic.** | **Walsh 9013** | **MEL 2470120** | **OK524302** | **OK562351** |
| *P. prostantheroides* | Geeraning Rock, W.A. | Wilson 333 | NSW 902203 | KU532875 | KU563360 |
| *P. ringens* | Dubbo, N.S.W. | Wilson 153 | NSW 844195 | JX047605 | KU563361 |
| *P. scutate* | Northampton, W.A. | Porter 320 | PERTH 7452268 | KU532876 | KU563362 |
| *P. scutellarioides*^ | Castlereagh NR, N.S.W. | Wilson 215 | NSW 799702 | JX047642 | - |
| **_P. scutellarioides_** | **Myall River, N.S.W.** | **Bell s.n.** | **NSW 847837** | **OK524296** | **OK562345** |
| *P. serpyllifolia* subsp. *microphylla* | Breakaway Ridge NR, W.A. | Byrne 3929 | PERTH 8274665 | KU532877 | KU563363 |
| *P. serpyllifolia* subsp. *serpyllifolia* | Wickepin, W.A. | Pigott s.n. | PERTH 5098955 | KU532878 | KU563364 |
| **_P._ sp. Evans Crown** | **Evans Crown NR, N.S.W.** | **O'Donnell 28** | **NSW 1100357** | **OK524298** | **OK562347** |
| **_P._ sp. Evans Crown** | **Evans Crown NR, N.S.W.** | **O'Donnell 29** | **NSW 1100369** | **OK524299** | **OK562348** |
| **_P._ sp. Minyon Falls (3)** | **Nightcap NP, N.S.W.** | **Palsson 115** | **NE 105981** | **OK524295** | **OK562344** |
| *P. tallowa* | Kangaroo Valley, N.S.W. | Conn 5313 | NSW 749547 | JX047664 | KF692284 |
| *P. tallowa* | Morton NP, N.S.W. | Wilson 236 | NSW 978753 | KU532879 | KU563365 |
| *P. verticillaris* | Albany, W.A. | Wilson 388 | NSW 903073 | KU532880 | KU563366 |
| *P. violacea* | Blue Mountains NP, N.S.W. | Wilson 401 | NSW 904871 | KU532881 | KU563367 |
| *W. longifolia* | Goonoowigal SCA, N.S.W. | Wilson 458 | NSW 977896 | KU532882 | KU563368 |
| *W. lucida* | Kosciuszko NP, N.S.W. | Orme 600 | NSW 619652 | JX047601 | - |
| *W. senifolia* | Mount Buffalo NP, Vic. | Wilson 159 | NSW 799706 | JX047600 | - |

### *Selection of molecular markers*

Previous phylogenetic studies of *Prostanthera* have used the external transcribed spacer (ETS) region of nuclear ribosomal DNA, and the *ndh*F-*rpl*32, *trn*T-F, and *trn*H-*psb*A chloroplast regions (Wilson *et al.* 2012; Conn *et al.* 2013; Conn *et al.* 2016). Wilson *et al.* (2012) found that nuclear ETS data provided species-level resolution within *Prostanthera*, while the *ndh*F-*rpl*32 and *trn*T-F chloroplast regions provided poorer species-level resolution, as they have in other genera within the Westringieae (Guerin 2008). Ensuant molecular phylogenetic studies within *Prostanthera* have continued to incorporate nuclear ETS data but have used the *trn*H-*psb*A chloroplast region in place of the *ndh*F-*rpl*32 and *trn*T-F chloroplast regions (Conn *et al.* 2013; Conn *et al.* 2016). Analysis of the *trn*H-*psb*A region by Conn *et al.* (2013) provided resolution at an interspecific level, but did not resolve relationships at the population scale. Conn *et al.* (2016) provided a phylogeny of the genus that exhibited moderate-to-strong support values; however, only the results of a concatenated (ETS + *trn*H-*psb*A) analysis were reported. It is unclear from the results of Conn *et al.* (2016) whether the *trn*H-*psb*A region itself provides robust results across *Prostanthera*, or whether the reasonable support values are more strongly influenced by the ETS dataset. This study presented an opportunity to assess the relative utility of the *trn*H-*psb*A region. To augment existing genetic studies within *Prostanthera* and to supplement our analyses using existing datasets, the nuclear ETS region and *trn*H-*psb*A chloroplast region were selected for use in this study.

### *Specimens sampled for Sanger sequencing*

Sixteen samples of *Prostanthera* were newly sequenced for this study. Three samples of *Westringia* were used as an outgroup as they are also members of the Westringieae (Wilson *et al.* 2012; Conn *et al.* 2016). GenBank (Clark *et al.* 2016) accessions of the ETS and *trn*H-*psb*A regions used in previous studies of *Prostanthera* (Wilson *et al.* 2012; Conn *et al.* 2013; Conn *et al.* 2016) were combined with newly sequences from this study to provide a total nuclear dataset of 58 samples and a total chloroplast dataset of 53 samples (Table 1).

### *DNA extraction, amplification, and sequencing*

DNA extractions, polymerase chain reaction (PCR) to amplify the selected regions, and sequencing were carried out by the Australian Genome Research Facility (AGRF) following methods outlined in Wilson *et al.* (2012), Conn *et al.* (2013) and Conn *et al.* (2016). Primers are listed in Table 2.

**Table 2.** Primers used for amplification of markers

| Fragment | Primer Name | Direction | Sequence | Author(s) |
|---|---|---|---|---|
| ETS | ETS-PROS2 | 5′ to 3′ | GCAGCGACGACATCCCAACC | (Baldwin and Markos 1998) |
| | 18S-E | 3′ to 5′ | GCAGGATCAACCAGGTAGCA | (Wilson *et al.* 2012) |
| *trn*H-*psb*A | *psb*A | 5′ to 3′ | GTTATGCATGAACGTAATGCTC | (Tate and Simpson 2003) |
| | *trn*H$^{\mathrm{GUG}}$ | 3′ to 5′ | CGCGCATGCTGGTGGATTCACAATCC | (Sang *et al.* 1997) |

### *Sequence assembly and phylogenetic analysis of Sanger data*

Contigs were assembled using UGENE (Okonechnikov *et al.* 2012) and the "Map Sanger Reads to Reference" function. Reads were mapped using the default quality settings, with a trimming quality threshold of 30 and a minimum mapping similarity of 80%. For mapping the ETS region, an existing sequence of *P. phylicifolia* (JX047643.1) was used as a reference. For mapping the *trn*H-*psb*A chloroplast region, a sequence of *P. lasianthos* Labill. (KU563352.1) was used as a reference as there were no prior existing *trn*H-*psb*A sequences of *P. phylicifolia.* Chromatograms of forward and reverse reads were combined and then inspected visually to assess base call accuracy. Bases that were ambiguous due to overlapping base peaks were coded as polymorphic using IUPAC ambiguity codes.

Sequences were aligned in MEGA X (Kumar *et al.* 2018) using Clustal W and edited manually following visual inspection of the completed alignment. In addition to separate ETS and *trn*H-*psb*A datasets, a partitioned concatenated dataset was created from the two by using the "Concatenate Sequence Alignments" function in MEGA X. Substitution models were tested and implemented for each dataset (Table 3) using ModelFinder (Kalyaanamoorthy *et al.* 2017) as implemented in IQ-TREE (Nguyen *et al.* 2015).

Maximum-likelihood (ML) analyses were performed using IQ-TREE (Nguyen *et al.* 2015). Substitution models for each partition were unlinked (Chernomor *et al.* 2016). To estimate branch length and support, 1000 bootstrap replicates were calculated using ultrafast bootstrap approximation (UFBoot) (Hoang *et al.* 2018). For bootstrap replicates in partitioned analyses, a resampling strategy as outlined by Gadagkar *et al.* (2005) and Seo *et al.* (2005) was implemented to reduce the likelihood of false positives and incorrect branch support assignation. This method first resamples partitions and then sites within resampled partitions (Minh *et al.* 2021). Bootstrap values were considered strong if they provided ≥95% support, moderate from 80–94% and weak from 50–79%.

Bayesian analysis was conducted using MrBayes 3.2.7 (Ronquist *et al.* 2012). Substitution models for each partition were unlinked. For each analysis, two independent Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses were run. Four chains were used (one heated, three cold), running for 5 million generations, and sampled every 1000th generation. A relative burn-in duration of 25% was used. Posterior probability (PP) values of 1 (Larget and Simon 1999) were considered as strong support, 0.9–0.99 as moderate support, and 0.8–0.89 as weak support.

The final best-scoring trees output from ML and Bayesian analyses were combined and visualised using TreeGraph 2 (Stöver and Müller 2010).

## Results

Statistical data for each sequence alignment and their respective analyses are outlined in Table 3. Analysis of the concatenated ETS and *trn*H-*psb*A dataset recovered a better resolved tree with higher branch support than analysis of either region individually (Figure 3, Table 3).

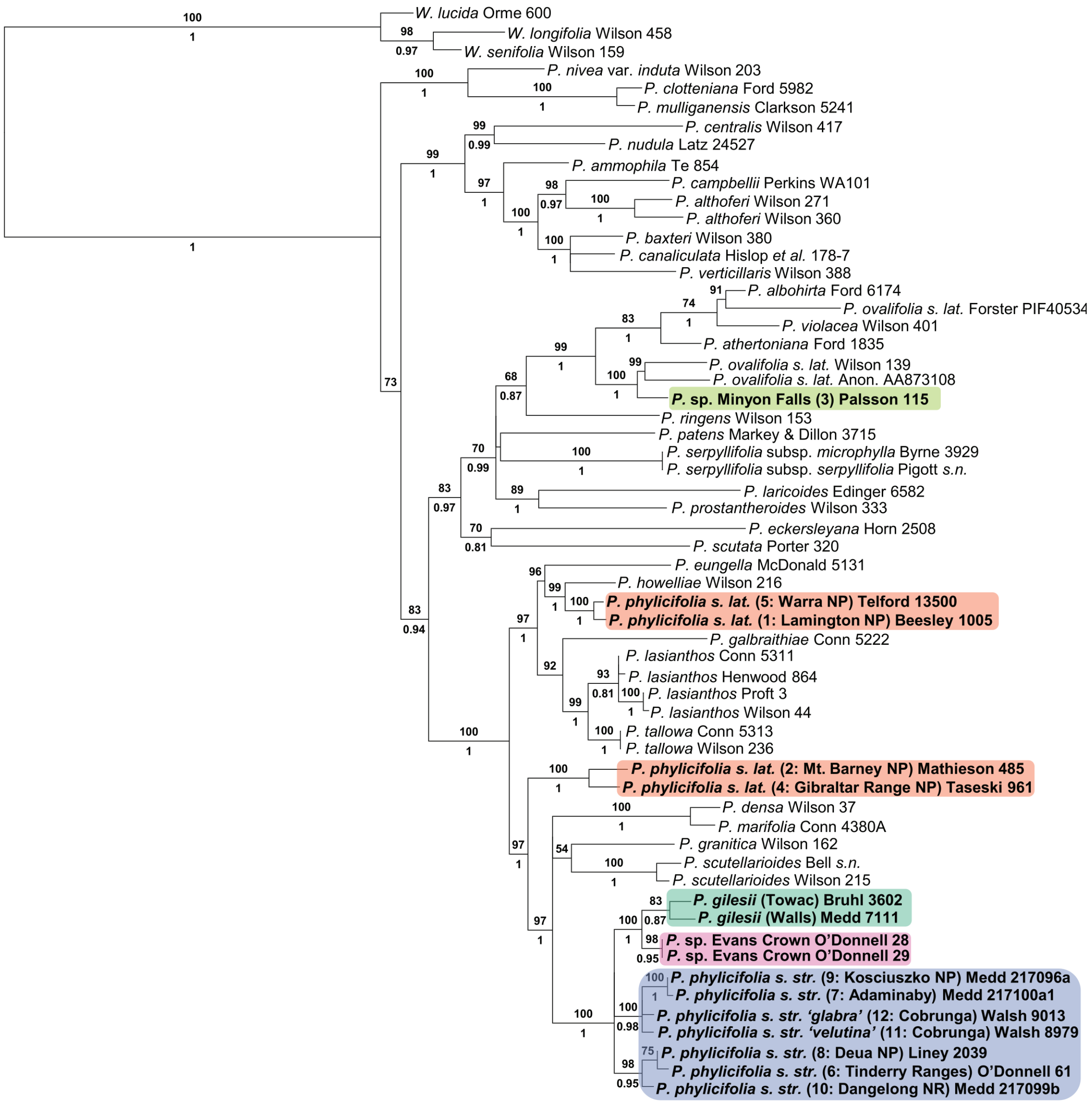

**Fig. 3.** Best scoring ML phylogram produced by IQ-TREE from analysis of the concatenated ETS and *trn*H-*psb*A sequence data for *Prostanthera* including three species of *Westringia* as outgroup representatives. Bayesian analysis posterior probabilities ≥0.8 from MrBayes and ML bootstrap values ≥50% are reported. *Prostanthera gilesii*, *P. phylicifolia s. str.*, *P. phylicifolia s. lat.*, *P.* sp. Minyon Falls and *P.* sp. Evans Crown are highlighted. Labels are species/phrase names, followed by primary collector and collection number. Labels for accessions of *P. phylicifolia* and *P. sp.* Minyon Falls include localities and population codes as outlined in Figure 2.

**Table 3.** Taxon information and statistics from Maximum Likelihood (ML) and Bayesian-inference analyses of nuclear (ETS) and chloroplast (*trn*H-*psb*A) markers**.**

| **Parameter** | **ETS** | ***trn*H-*psb*A** | **Concatenated** |
|---|---|---|---|
| Total number of taxa | 58 | 53 | 59 |
| Total number of ingroup taxa | 55 | 52 | 55 |
| Total number of outgroup taxa | 3 | 1 | 3 |
| Aligned length | 430 | 495 | 925 |
| Parsimony-informative sites | 188 | 44 | Partitioned |
| Invariant sites | 158 | 403 | Partitioned |
| ModelFinder model | SYM + $\Gamma$ | F81+I+$\Gamma$ | Partitioned |
| Number of nodes resolved (IQ-TREE) | 55 | 50 | 56 |
| Number of nodes resolved (MrBayes) | 46 | 20 | 49 |
| Number of nodes resolved with BS ≥ 95% | 34 | 7 | 36 |
| Number of nodes resolved with PP = 1 | 20 | 3 | 29 |
| % of nodes resolved with BS ≥ 95% | 62 | 14 | 64 |
| % of nodes resolved with PP = 1 | 43 | 15 | 59 |

Analysis of the concatenated dataset (Figure 3) recovered all samples of *P. phylicifolia* from southern New South Wales and Victoria (Populations 6–12, Figure 2) with *P. gilesii* and *P.* sp. Evans Crown in a well-supported clade (BS = 100%; PP = 1). This clade was recovered within a larger, strongly supported clade (BS = 97; PP = 1), sister to clades comprised of *P. granitica* Maiden & Betche*, P. scutellarioides, P. densa* A.A.Ham. and *P. marifolia* R.Br. *Prostanthera gilesii* and *P.* sp. Evans Crown were recovered together as sister taxa with strong support (BS = 100%; PP = 1). Both accessions of *P.* sp. Evans Crown were recovered as a distinct clade with moderate-to-strong support (BS = 98%; PP = 0.95), separate from *P. gilesii*. Both accessions of *P. gilesii* were recovered as a clade with weak-to-moderate support (BS = 83%; PP = 0.87). Specimens of *P. phylicifolia* formed two moderately-to-strongly supported clades. Accessions of *P. phylicifolia* from Cobrunga (including both glabrous and velutinous forms), Adaminaby, and Kosciuszko National Park formed a clade with moderate-to-strong support (BS = 100; PP = 0.98), and accessions from Tinderry, Deua National Park and Dangelong Nature Reserve were recovered as a clade with moderate-to-strong support (BS = 98%; PP = 0.95).

Accessions from populations from northern New South Wales and southern Queensland variously identified as *P. phylicifolia* or *P. scutellarioides* (Figure 2, Populations 1–5) were recovered in clades separated from the clade of accessions from southern New South Wales and Victoria. Specimens of *P. scutellarioides* from the Castlereagh type locality and from the Hunter Valley were recovered as a strongly supported clade (BS = 100%; PP = 1). Samples from Gibraltar Range National Park and Mt. Barney National Park were recovered together as a distinct, strongly supported clade (BS = 100%; PP = 1), sister to the clade comprised of *P. granitica, P. scutellarioides, P. densa, P. marifolia,* and the *P. gilesii-P. phylicifolia-P.* sp. Evans Crown clade. Samples from Warra National Park and Lamington National Park were recovered together in a strongly supported clade (BS = 100%; PP = 1) sister to *P. howelliae* within a strongly-supported clade (BS = 100%; PP = 1). This clade was recovered as a sister clade to *P. eungella* B.J.Conn & K.M.Proft. The accession of *P.* sp. Minyon Falls was recovered in a strongly supported clade (BS = 100%; PP = 1) with *P. ovalifolia* R.Br., sister to a clade with moderate-to-strong support (BS = 83%; PP = 1) containing *P. athertoniana* B.J.Conn & T.C.Wilson, *P. violacea* R.Br., and *P. albohirta* C.T.White.

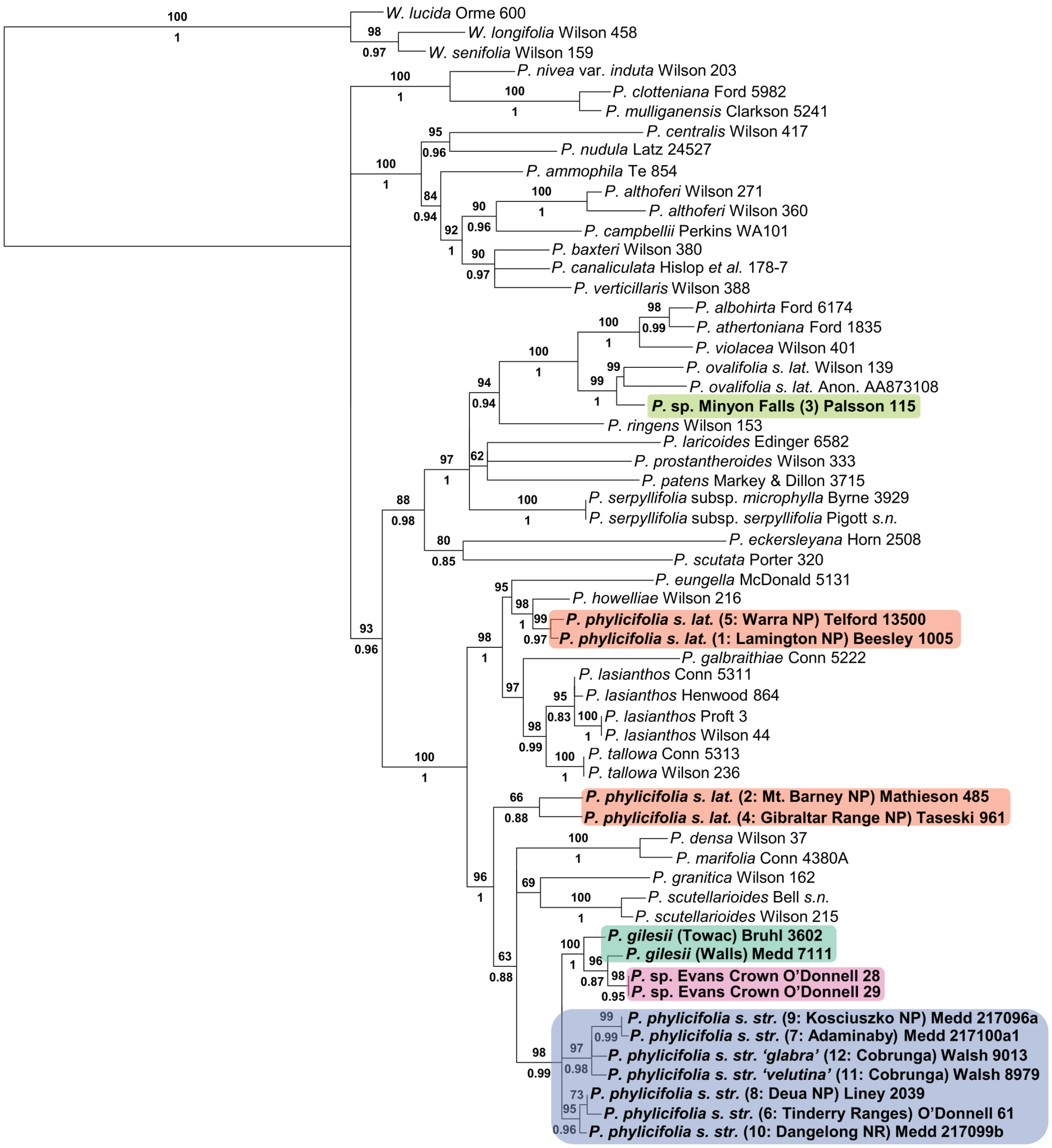

**Fig. 4.** Best scoring ML phylogram produced by IQ-TREE from analysis of nuclear ETS sequence data for *Prostanthera* including three species of *Westringia* as outgroup representatives. Bayesian analysis posterior probabilities ≥0.8 from MrBayes and ML bootstrap values ≥50% are reported. *Prostanthera gilesii*, *P. phylicifolia s. str.*, *P. phylicifolia s. lat.*, *P.* sp. Minyon Falls and *P.* sp. Evans Crown are highlighted. Labels are species/phrase names, followed by primary collector and collection number. Labels for accessions of *P. phylicifolia* and *P. sp.* Minyon Falls include localities and population codes as outlined in Figure 2.

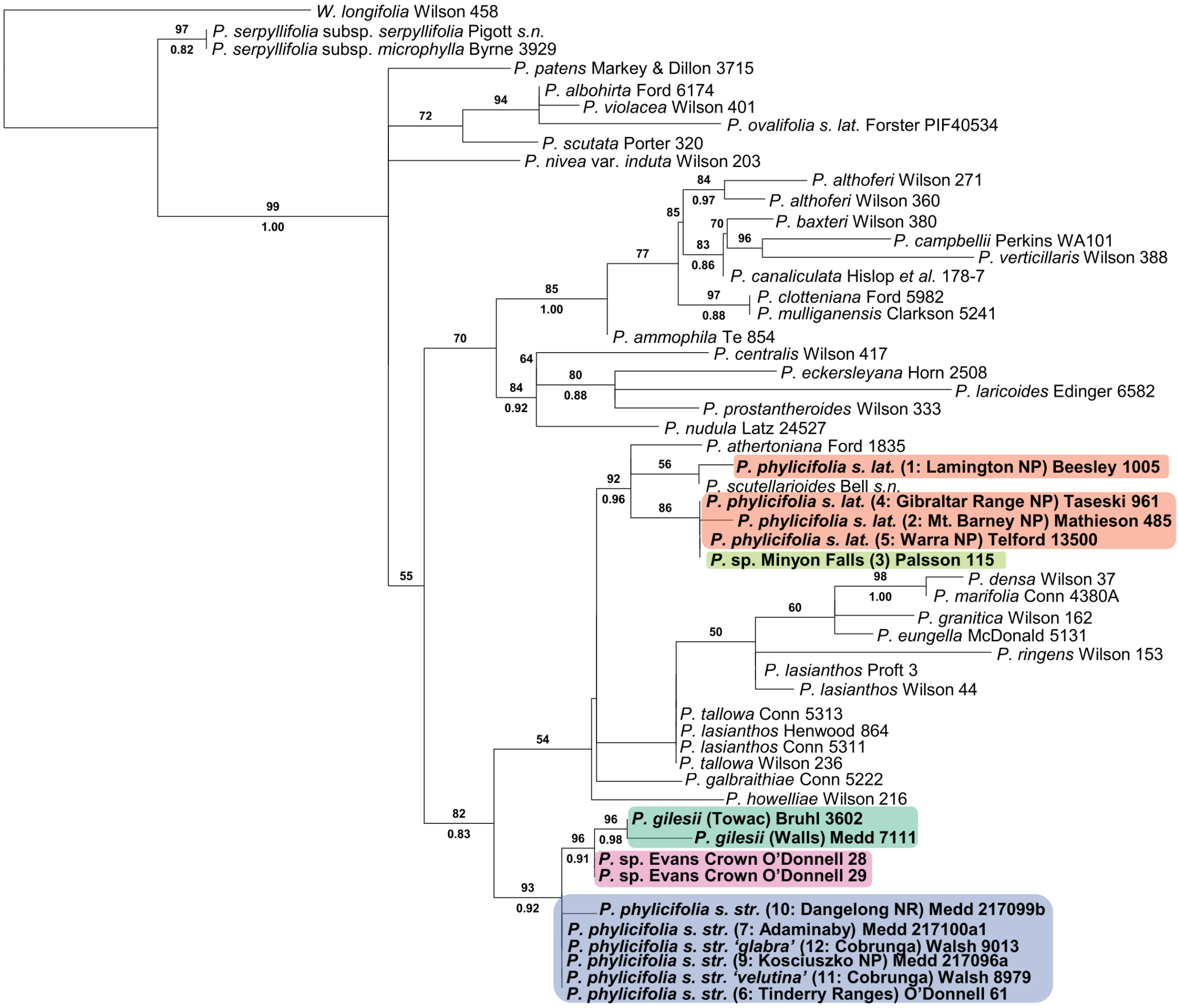

**Fig. 5.** Best scoring ML phylogram produced by IQ-TREE from analysis of chloroplast *trn*H-*psb*A sequence data for *Prostanthera* including one species of *Westringia* as an outgroup representative. Bayesian analysis posterior probabilities ≥0.8 from MrBayes and ML bootstrap values ≥50% are reported. *Prostanthera gilesii*, *P. phylicifolia s. str.*, *P. phylicifolia s. lat.*, *P.* sp. Minyon Falls and *P.* sp. Evans Crown are highlighted. Labels are species/phrase names, followed by primary collector and collection number. Labels for accessions of *P. phylicifolia* and *P. sp.* Minyon Falls include localities and population codes as outlined in Figure 2.

Analysis of the ETS region (Figure 4) recovered a tree not markedly different from that of the concatenated analysis, while analysis of the *trn*H-*psb*A region recovered a tree which differed substantially in topology and resolution (Figure 5). Analysis of the *trn*H-*psb*A region recovered all southern accessions of *P. phylicifolia.*, *P. gilesii* and *P.* sp. Evans Crown as a moderately supported clade (BS = 93%; PP = 0.92). Within this clade, *P. gilesii* and *P.* sp. Evans Crown were recovered as sister taxa with moderate-to-strong support (BS = 96%; PP = 0.91), sister to an unresolved polytomy consisting of all southern samples of *P. phylicifolia*. Both specimens of *P. gilesii* were recovered as a distinct clade with moderate-to-strong support (BS = 96%; PP = 0.98). This clade was recovered as sister to a larger, poorly-supported clade (BS = 54%; PP = N/A), which contained *P. howelliae, P. densa, P. marifolia, P. granitica, P. eungella, P. galbraithiae* B.J.Conn, *P. tallowa* B.J.Conn & T.C.Wilson, *P. lasianthos, P. athertoniana, P. scutellarioides,* and all northern samples variously identified as *P. phylicifolia* or *P. scutellarioides*. Northern samples identified as either *P. phylicifolia* or *P. scutellarioides* were recovered as a moderately-supported clade (BS = 92%; PP = 0.96) with *P. scutellarioides* and *P. athertoniana.* Relationships within this clade differed between ML and Bayesian analyses. Bayesian analyses recovered all northern accessions as a polytomy with weak support (PP = 0.82) distinct from *P. scutellarioides* and *P. athertoniana.* ML analyses recovered *P.* sp. Minyon Falls and accessions from Warra National Park, Mt. Barney National Park and Gibraltar Range National Park as a moderately-supported polytomy (BS = 86%), while the accession from Lamington National Park was recovered in a poorly-supported clade with *P. scutellarioides* (BS = 56%).

## Discussion

The results of this study support a close relationship between *P. gilesii*, *P. phylicifolia s. str.* and *P.* sp. Evans Crown and their placement within "Clade C". Furthermore, our results highlight that *P. phylicifolia* as currently circumscribed is not monophyletic. Morphologically and geographically disparate populations variously identified as *P. phylicifolia* or *P. scutellarioides* demonstrably represent more than two distinct taxa.

All southern populations of *P. phylicifolia* from Victoria through to south of Canberra share a cohesive morphology characterised by white corollas with porrect adaxial lobes, purple and yellow throat markings, distinctly elongated anther appendages that either match or exceed the length of the anther cell, and leaves that are narrow-ovate to oblong with an obtuse apex. The morphology and locality shared by these populations is congruent with that identified for *P. phylicifolia* in Mueller's (1858) protologue and they are shown here to be closely related, although recovered across two distinct clades that each have a distinct geographic distribution. Populations occurring along the Victorian Alps and Snowy Mountains form a 'western' clade, while populations from the Tinderry Mountains south to Dangelong Nature Reserve form an 'eastern' clade. The biogeographic pattern examined here might be of further use to studies examining how topography and orogeny may have influenced speciation processes in south-eastern Australia. Analyses here did not support substantive genetic differentiation between glabrous and velutinous individuals and as both forms have been found to grow adjacently within populations, recognition of distinct varieties is unwarranted. This result highlights that caution must be taken when delimiting species of *Prostanthera* based solely on differences in indumentum. The contrasting levels of resolution between phylogenies recovered here indicate that further close study of this group at the population level is necessary to assess the extent of molecular and morphological divergence between the western and eastern clades As the taxonomic status of these clades is still uncertain, we have elected to refrain from designating a lectotype for *P. phylicifolia s. str.* here. For the meantime, application of *P. phylicifolia s. str.* should be restricted to these southern populations, excluding all populations from northern New South Wales and southern Queensland.

Similar to *P. phylicifolia s. str., P. gilesii* and *P.* sp. Evans Crown are compact shrubs that exhibit leaves that are narrow-ovate to oblong with recurved margins and an obtuse apex, flowers with white corollas, purple and yellow throat markings, and anther appendages that either match or exceed the length of the anther cell. While *P. gilesii* and *P.* sp. Evans Crown are consistently recovered as sister taxa, they are separated by over 120 km and occupy different substrates (basalt for *P. gilesii* vs. granite for *P.* sp. Evans Crown) (Conn and Wilson 2015). On the basis of these differences, there is evidence to suggest a lack of contemporary gene flow between them. Nevertheless, it is unclear from the results presented here whether *P. gilesii* and *P.* sp. Evans Crown represent distinct taxa independent from *P. phylicifolia s. str.* If evidence for contemporary gene flow between the three taxa can be presented, it might follow that *P. gilesii* and *P.* sp. Evans Crown should be subsumed into *P. phylicifolia*. However, if further study were to unequivocally demonstrate that each taxon represents an independently evolving lineage, *P. gilesii* would retain its critically endangered status, its range would be restricted to the two known subpopulations within one catchment, and *P.* sp. Evans Crown would merit recognition at the specific rank. If *P.* sp. Evans Crown is a distinct species, it is likely that it would be of critical conservation concern on the basis of its restricted distribution and small population size. Within *Prostanthera*, a close study of the sister species *P. densa* and *P. marifolia* (Conn *et al.* 2013) demonstrated clear boundaries between the two species by incorporating population-level genetic data and statistical tests of morphological variation. Similar integrative taxonomic studies are required here to elucidate the molecular and morphological relationships and boundaries between *P. phylicifolia s. str*., *P. gilesii* and *P.* sp. Evans Crown before firm taxonomic and conservation conclusions can be made.

**Fig. 6.** Photographs of *P.* sp. Minyon Falls and *P. ovalifolia s. lat*. A = *P.* sp. Minyon Falls; B = *P. ovalifolia s. lat.* (Nightcap National Park), demonstrating similarities in floral morphology and differences in vegetative morphology. Images: M. Colpus (A); J. J. Bruhl (B).

Populations that have been previously assigned as *P. phylicifolia s. lat.* or *P. scutellarioides* from northern New South Wales and southern Queensland are readily morphologically distinguishable from southern populations deemed as *P. phylicifolia s. str.* and molecular evidence here corroborates this distinction. These populations represent an assemblage of taxa that are only broadly related to *P. phylicifolia s. str.* or *P. scutellarioides*, several of which are possibly undescribed. While molecular data indicate that several putative species may be present, it is difficult to distinguish clear morphological discontinuities between them. Superficial variation between putative entities in this group can be seen in lamina length and width, however it is unclear whether this variation in vegetative traits is simply the result of phenotypic plasticity. This group requires a rigorous assessment of genetic and morphological variation to ascertain an accurate understanding of extant diversity.

*Prostanthera* sp. Minyon Falls (Figure 6A) bears no strong resemblance to species it was recovered with in nuclear and concatenated analyses (Figure 6B), but most closely resembles species it was recovered with in chloroplast analyses (Figures 1D, E) *Prostanthera* sp. Minyon Falls most closely resembles accessions collected on the Tweed Volcano, namely the Lamington National Park accession which was recovered as sister to the Warra National Park accession within a clade containing *P. howelliae* and *P. eungella* in concatenated and nuclear analyses. This taxon has linear leaves with revolute margins, apices that are acute to apiculate and flowers that are axillary, similar to other taxa recovered in “Clade C” after Wilson *et al.* (2012). Analysis of the chloroplast dataset recovered this accession in a clade including other “Clade C” taxa, as hypothesised based on morphological characters. In contrast, nuclear and concatenated analyses recovered this accession as sister to *P. ovalifolia* (Figure 6B)—a species previously recovered within “Clade J” after Wilson *et al.* (2012).

This pattern of results is repeated in our analyses in *P. athertoniana,* a narrowly restricted species from north-eastern Queensland. Of the accessions identified as *P. ovalifolia,* one was collected from Whian Whian State Conservation Area, which is adjacent to Minyon Falls. Similar to *P. phylicifolia*, *P. ovalifolia* is another species with contentious taxonomy, and accessions from this locality are variously identified as *P. ovalifolia* or *P. lanceolata* Domin. Recent genetic studies of this group have shown accessions considered under either name to be firmly placed within “Clade J” (Palsson *et al.* unpublished data). Like other members of “Clade J”, these accessions can be characterised by leaves that are narrow-ovate to ovate with entire to minutely toothed margins and flowers in a terminal botryoid (Wilson *et al.* 2012). While *P.* sp. Minyon Falls can be readily distinguished from these accessions on the basis of leaf morphology and inflorescence type, they all share mauve–purple flowers with corollas that are less strongly zygomorphic, lateral lobes directed outward and anther appendages that are reduced to absent (Wilson *et al.* 2012, 2017) (Figures 6A, B). This combination of floral characters corresponds with a generalist pollination syndrome found to be more frequently visited by an unspecialised pollinator assemblage consisting of flies and beetles (Wilson *et al.* 2017). This suggests that sympatric species exhibiting this floral type are likely to share pollen if they flower synchronously.

The different placement of *P.* sp. Minyon Falls in nuclear and chloroplast regions suggests that hybridisation may have occurred between sympatric species of *Prostanthera*. Cytonuclear discordance is commonly observed in phylogenetic studies and is often attributed to either incomplete lineage sorting or hybridisation (Lee-Yaw *et al.* 2019; Nge *et al.* 2021). Hybridisation in *Prostanthera* is documented in cultivation in the horticultural literature (Althofer 1978) and has been hypothesised to explain aberrant collections and populations (Conn 1998); however, there has been no genetic evidence to date to substantiate these hypotheses. If hybridisation can be confirmed, this study may represent the first genetic evidence of hybridisation in *Prostanthera* in wild populations. While it is unclear whether the cytonuclear discordance observed here is the result of a recent hybridisation event, ancestral introgression, or incomplete lineage sorting, we have identified strong candidate taxa to investigate this phenomenon further. As cytonuclear discordance was observed across multiple taxa in this study, it is probable that more densely sampled phylogenies of *Prostanthera* would reveal further instances of this phenomenon. If hybridisation is shown to be responsible for this pattern, future studies of *Prostanthera* must consider and test for this in order to avoid taxonomic over-inflation. Genomic technologies are now readily accessible and have demonstrated their efficacy in resolving questions of hybridisation and admixture (Sansaloni *et al.* 2010; Steane *et al.* 2011; Rutherford *et al.* 2018; Alwadani *et al.* 2019; Collins *et al.* 2021). Population-scale studies that incorporate genomic data from *P.* sp. Minyon Falls and other species found within the region may be able to elucidate the affinities of this taxon and the genetic processes at play.

The results of this study and other studies of *Prostanthera* (Conn *et al.* 2013; Conn *et al.* 2016) indicate that the *trn*H-*psb*A region provides poor resolution within the genus, which casts further doubt on the utility of the region as a DNA barcode. Similar to previous molecular studies of *Prostanthera* (Conn *et al.* 2013; Conn *et al.* 2016), the *trn*H-*psb*A chloroplast marker demonstrated substantially less resolution than the ETS nuclear marker (Figs. 4, 5, Table 3). The region had been originally identified as a good candidate for use as an efficient DNA barcode, with some success with its implementation (Pang *et al.* 2012; Hoveka *et al.* 2016; Loera-Sánchez *et al.* 2020). Previous studies stressed that the region should be analysed in combination with at least one to three additional chloroplast regions and a rapidly evolving nuclear region (e.g. ITS, ETS). When compared with other variable chloroplast regions, the *trn*H-*psb*A region has been shown to yield results that are not markedly different from comparable regions and caution has been advised in its use for barcoding purposes (Byrne and Hankinson 2012). Chase *et al.* (2007) noted that the region is highly variable in length (<300bp to >1000bp) even between closely related taxa, and exhibits high rates of insertions and deletions, making alignments difficult and prone to error. Other studies incorporating analysis of the region have demonstrated unresolved results like those obtained in this study (Whitlock *et al.* 2010; Singh *et al.* 2012; Chandrasekara *et al.* 2021). In a study of some species of Gentianaceae, the region was found to exhibit intraspecific inversions, potentially resulting in overestimation of divergence in conspecific taxa (Whitlock *et al.* 2010; Chandrasekara *et al.* 2021). Continued use of this region in studies of *Prostanthera* is unlikely to yield robust results.

## Conclusion

Molecular evidence here has clarified the identity of *P. phylicifolia s. str.* Our results indicate that application of the name *P. phylicifolia* should be restricted to populations south of Canberra through to Victoria. These populations are unrelated to morphologically and geographically disparate northern populations variously identified as *P. phylicifolia* or *P. scutellarioides*. Rigorous morphological and genetic assessment will be required before taxonomic conclusions can be made.

In this study we have identified *P. phylicifolia s. str.* and *P.* sp. Evans Crown as close relatives of the critically endangered *P. gilesii*. *Prostanthera* sp. Evans Crown is most closely related to *P. gilesii,* and it is possible that it represents a new species of *Prostanthera.* As phylogenies estimated here exhibited variable topologies and support levels, we recommend further analysis of a more comprehensive sample set at the population scale, using high-throughput technologies that have been shown to be effective in resolving questions of historical gene flow and genetic divergence between putative taxa (Sansaloni *et al.* 2010; Steane *et al.* 2011; Collins *et al.* 2021; Joyce *et al.* 2021).

Our results highlight how broad species concepts and unresolved taxonomic issues have impeded an accurate understanding of diversity within *Prostanthera. Prostanthera* is already the most speciose Australian genus of Lamiaceae, and we have shown that it is highly likely that the genus is far more diverse than currently recognised.

Furthermore, we have demonstrated that *Prostanthera* is subject to confounding evolutionary processes such as incomplete lineage sorting and hybridisation. Tools better equipped to handle complex situations such as these will be crucial to achieving a clearer understanding of the evolutionary history of *Prostanthera.*

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Declaration of funding

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