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Nicotiana paulineana, a new Australian species in *Nicotiana* section *Suaveolentes*

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Abstract. *Nicotiana* is found predominantly in the Americas and Australia, but also has representatives in Africa and the Pacific Islands. All native Australian *Nicotiana* species belong to section *Suaveolentes*. The number of species in this section is uncertain and subject to revision. An example of this uncertainty is the taxonomic status of a South Australian *Nicotiana* accession colloquially termed 'Corunna'. Here, we report sequences for nuclear and plastid markers for *N.* sp. Corunna (D.E. Symon 17088) and accessions of two other Australian species, *N. burbidgeae* and *N. benthamiana*. Phylogenetic comparison of these sequences with those of other members of *Nicotiana* places all three taxa in *N.* section *Suaveolentes* and shows that 'Corunna' represents a distinct phylogenetic lineage in a well supported clade along with *N. goodspeedii*, *N. maritima*, *N. amplexicaulis* and *N. suaveolentes*. Phenetic analysis of floral characters also supports recognition of *N.* sp. Corunna (D.E.Symon 17088) as a distinct species, which we describe here as *Nicotiana paulineana* Newbigin & P.M.Waterh., sp. nov. The enlarged molecular dataset described here contributes to a better understanding of taxonomic relationships within the section.

Keywords: *Nicotiana*, *Suaveolentes*, phylogenetic tree, plastid genome, classification, new species.

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Introduction

The nightshade or Solanaceae family is widely distributed across all temperate and tropical continents. The family contains many of the world's most important agricultural species, such as potatoes, tomatoes, eggplants and tobacco. It also includes several important model species used in plant research, including *Nicotiana benthamiana* Domin and *Petunia* Juss. (Bombarely *et al*. 2012; Vandenbussche *et al*. 2016; Bally *et al*. 2018). The Solanaceae consists of ~100 genera and 2800 species and relationships within the family have been the subject of repeated phylogenetic revision (D'Arcy 1979; Olmstead *et al*. 2008).

Nicotiana L. is one of the larger genera in the Solanaceae that includes mainly annual, non-woody plants, and includes various species commonly referred to as 'tobacco plants'. The genus *Nicotiana* includes 86 species in 13 sections distributed across tropical and temperate regions, with most being native to South and North America (Knapp *et al*. 2004). With currently ~35 species, section *Suaveolentes* Goodsp. is a monophyletic group of ancient allopolyploid origin and the largest of the *Nicotiana* sections (Leitch *et al*. 2008; Clarkson *et al*. 2010). Unlike other *Nicotiana* sections, *Suaveolentes* contains no American taxa and is native to Australia (26 recognised species), the Pacific (three species) and Africa (one species). Australian *Suaveolentes* taxa are widespread across the continent, especially in the arid zone (Knapp *et al*. 2004; Chase *et al*. 2018*a*).

The number of species in section *Suaveolentes* is currently subject to revision (Chase *et al*. 2018*a*). Misidentification of available *N.* section *Suaveolentes* seed and herbarium material is common, with estimates ranging from 23% to at least 50% (Marks *et al*. 2011*a*; Chase *et al*. 2018*a*). For instance, several Western Australian accessions previously described as *N. umbratica* N.T.Burb. appear to represent a recently described species, *N. karijini* M.W.Chase & Christenh. (Chase and Christenhusz 2018), and several inland South

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Australian tobaccos previously attributed to *N. maritima* H.-M.Wheeler belong to the novel taxa *N. yandinga* M.W. Chase & Christenh. and *N. fauciciola* M.W.Chase & Christenh. (Chase *et al*. 2018*b*, 2018*c*).

As well as misidentified material, there are also undescribed species in *N.* section *Suaveolentes* (Chase *et al*. 2018*a*). One example is an accession related to *N. goodspeedii* H.-M.Wheeler that was collected by the noted Australian botanist D. E. Symon in 2004 and is currently named *Nicotiana* sp. Corunna (D.E.Symon 17088) Symon after the property (Corunna Station, near Iron Knob in South Australia) where it was found. A subsequent morphological study of *N.* sp. Corunna (D.E.Symon 17088) by Marks (2010), using plants grown from seed collected by Symon, concluded that it was sufficiently distinctive to be recognised as a new species. Here, we resolve the taxonomic status of *N.* sp. Corunna (D.E.Symon 17088) by further characterising plants grown from Symon's original seed. On the basis of plastid and nuclear DNA sequences, we show that *N.* sp. Corunna (D.E.Symon 17088) is a distinct species, which we have called *N. paulineana.* We also provide sequence data for *N. burbidgeae* and *N. benthamiana*, two Australian species that have rarely appeared in phylogenetic treatments of *N.* section *Suaveolentes* (Chase *et al*. 2003; Clarkson *et al*. 2010).

Materials and methods

Plant material and DNA sequencing

DNA sequences for the Nicotiana species shown in Table S2 were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank). Although *N. suaveolens* Lehm. and *N. exigua* H.-M.Wheeler are considered conspecific (Horton 1981), the sequences lodged in GenBank under these names for *matK*, *ITS* and the two *GS* paralogs are not identical, and thus the original species names were retained and have been treated here as independent taxa. The voucher material for these sequences should be re-examined to confirm their identities.

Twelve species of *Nicotiana*, including the undescribed *N.* sp. Corunna (D.E.Symon 17088) and *N. burbidgeae*, were propagated for at least two generations, confirmed to have the morphological features diagnostic of the taxa, and used for DNA extraction. Total genomic DNA was extracted from leaf tissues using the CTAB method (Clarke 2009) and amplified with a high-fidelity *Taq* polymerase and the primers described in Table S1 of the Supplementary material, using a touchdown cycling technique and annealing temperatures of 50–55°C. For species without existing sequence information, the nuclear DNA regions amplified were the internal transcribed spacer of rRNA (*ITS*), the long and short forms of the chloroplast-expressed glutamine synthetase (*GSL* and *GSS* respectively; Clarkson *et al*. 2010), RNA-dependent RNA polymerase 1 (*RDR1*; Bally *et al*. 2015) and alcohol dehydrogenase C locus (*ADHC*); and the plastid region amplified was maturase K (*matK*). Because nuclear and chloroplast DNA sequences were not available for *N.* sp. Corunna (D.E.Symon 17088) or *N. burbidgeae*, regions of ribosomal *ITS* and *matK* from these accessions, and also from *N. benthamiana*, were amplified and sequenced (Table S2). Cycle sequence reactions, performed with BigDye Terminator (ver. 3.1, Applied Biosystems) at suggested cycling conditions, were purified with an ethanol and EDTA precipitation. After purification, the amplified fragments were run on a Life Technologies 3500 Genetic Analyser at the Central Analytical Research Facility Genomics Laboratory at the Queensland University of Technology. Analysis of output chromatograms and further preliminary sequence editing was conducted using Geneious (ver. R11, Biomatters, New Zealand, see www.geneious.com/; Kearse *et al.* 2012).

Sequence alignment and phylogenetic analyses

Gene sequences were aligned (Fig. S1 of the Supplementary material) using MUSCLE (ver. 3.8.31, see http://www.drive5.com/muscle/; Edgar 2004), followed by manual adjustments in BioEdit (ver. 7.2.6, see https://bioedit.software.informer.com/). For concatenation, the sequences were appended in the following order: *ITS*, *matK*, *GSL*, *GSS*, *RDR1* and *ADHC*. The Akaike information criteria in ModelFinder in IQ-TREE (ver. 1.5.4, see http://www.iqtree.org/; Kalyaanamoorthy *et al*. 2017) and jModelTest2 (ver. 2.1.10, see https://github.com/ddarriba/jmodeltest2/; Darriba *et al*. 2012) were used to estimate the best-fit substitution models. Model selection and the parameters used are described in Table S3. Phylogenetic analyses of individual and concatenated gene sequences were based on maximum likelihood implemented by a rapid and effective stochastic algorithm in IQ-TREE (Trifinopoulos *et al*. 2016) including partition files. Additional phylogenetic trees for concatenated data of all six genes were built through Bayesian inference as implemented in MrBayes (ver. 3.2.6, see https://github.com/NBISweden/MrBayes/; Ronquist *et al*. 2012) including partition files. The final consensus trees were displayed using FigTree (ver. 1.4.3, see https://github.com/rambaut/figtree/releases/tag/release_1_3/).

Morphological assessment of flowers and phenetic analysis

Marks *et al*. (2011*a*) reported measurements of 21 floral character states from a range of *N.* section *Suaveolentes* taxa, with each measurement being based on a minimum of 10 biological replicates. Measurements for selected taxa were extracted from those reported in this paper and data matrices of floral characters were subject to principal-component analysis (PCA) using R (ver. 3.5.0, R Foundation for Statistical Computing, Vienna, Austria) and the package factoextra (ver. 1.0.3, see https://CRAN.R-project.org/package=factoextra/).

Results

Phylogenetic analyses

A maximum-likelihood analysis (Fig. 1) was performed using the concatenated *ITS* and *matK* sequences for *N. burbidgeae*, *N. benthamiana* and *N.* sp. Corunna (D.E.Symon 17088), and equivalent sequences from other *Nicotiana* taxa and from the Australian genus *Anthocercis* Labill. (Solanaceae: Anthocercideae) as the designated outgroup (Clarkson *et al*. 2010). The trees produced with *ITS* or *matK* sequences alone are shown in Fig. S1 and S2. For the concatenated tree, the total number of nucleotides used was 2213, of which 406 were

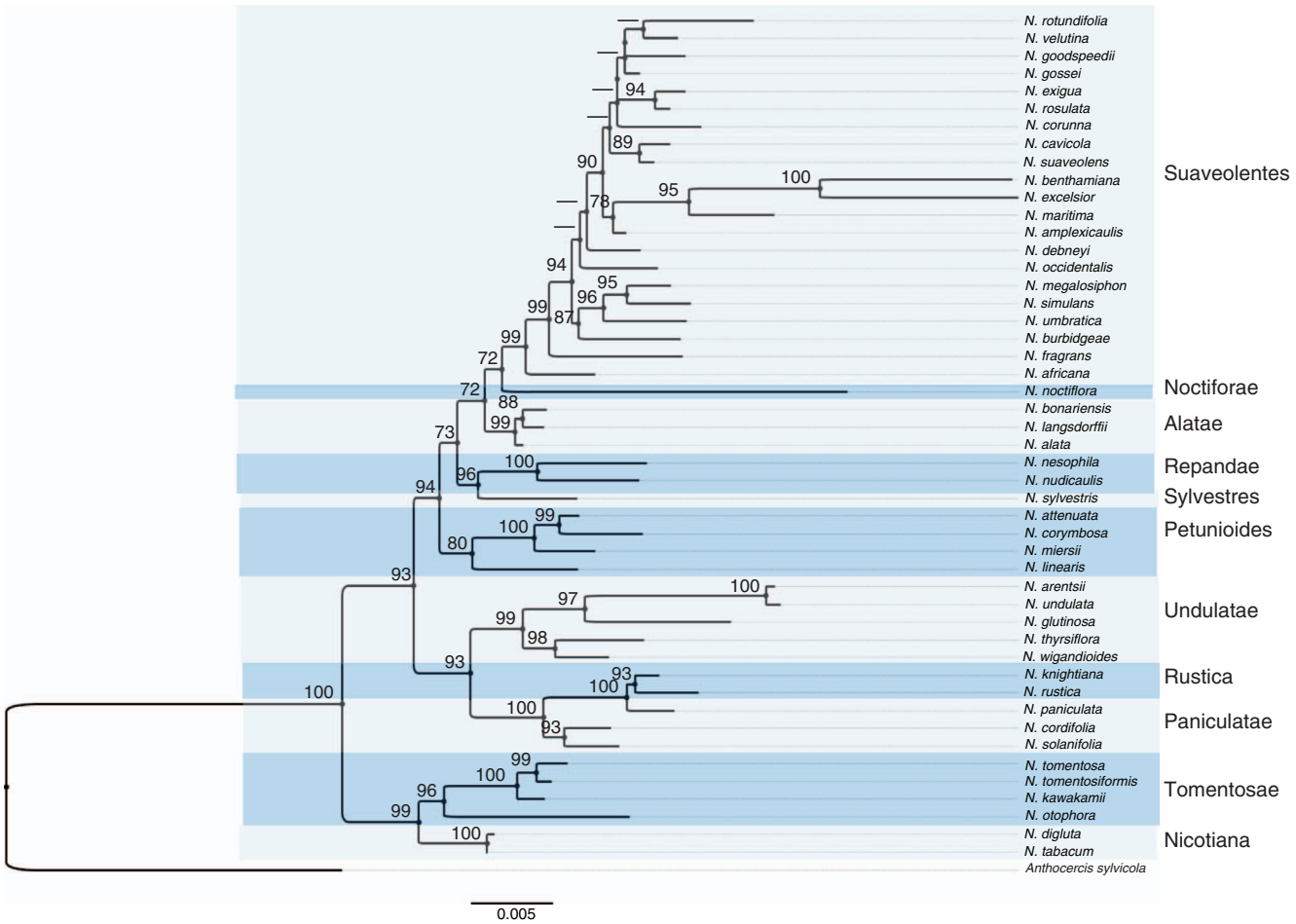


Fig. 1. Phylogeny of *Nicotiana* species and the *N.* sp. Corunna (D.E.Symon 17088) accession inferred from maximum-likelihood analysis of combined *ITS* and *matK* sequences. Bootstrap support values >70% are shown above branches. Branches with ≤70% bootstrap support values are annotated with an en-dash (–). Sections of *Nicotiana* are shown on the right-hand side corresponding to the shaded boxes.

variable. *Nicotiana* formed a well supported clade made up of several lineages and the branching pattern shown in Fig. 1 was consistent with previous analyses of this genus (e.g. see Clarkson *et al*. 2010). The branching pattern of the *ITS* tree was like that of the concatenated tree but the *matK* tree had fewer resolved nodes. All trees placed members of *N.* section *Suaveolentes* in a well supported lineage that included *N. benthamiana*, *N. burbidgeae* and *N.* sp. Corunna (D.E. Symon 17088). *Nicotiana benthamiana* was sister to *N. excelsior* (J.M.Black) J.M.Black (bootstrap support (BS) = 100%) and *N. burbidgeae* along with *N. umbratica* were on early diverging branches among Australian representatives of the section (Fig. 1). All trees showed that *N.* sp. Corunna (D.E. Symon 17088) and *N. goodspeedii* were on separate branches, as were *N. suaveolens* and its synonym *N. exigua*.

To further confirm *N.* sp. Corunna (D.E.Symon 17088) as a distinct species, additional nuclear sequences were obtained. Species in the allopolyploid *N.* section *Suaveolentes* retain both parental copies of the nuclear-encoded, chloroplast-expressed glutamine synthetase, and the paralogs, called *GSL* and *GSS*, have previously been used to determine phylogenetic relationships (Clarkson *et al*. 2010). As well as *N.* sp. Corunna (D.E.Symon 17088), *GSL* and *GSS* sequences were obtained for *N. burbidgeae*, *N. benthamiana* and *N. rosulata* (S.Moore) Domin and added to the existing *N.* section *Suaveolentes* sequence dataset (Table S2). Regions of a further two genes, *RDR1* and *ADHC*, were either sequenced from these species or retrieved from GenBank. Most species in *N.* section *Suaveolentes* appear to have retained only one of the parental copies of these genes (Kelly *et al*. 2013; Bally *et al*. 2015, 2018). Consistent with this, there were no polymorphisms observed in the amplified *ADHC* and *RDR1* products for any accession. The sequences of all six gene regions (*ITS*, *matK*, *GSL*, *GSS*, *RDR1* and *ADHC*) were used to generate phylogenetic trees for each individual gene region (Fig. S1–S6) and for a concatenation of all six (Fig. 2); their counterpart sequences from *Anthocercis gracilis* Benth., *Anthocercis sylvicola* T.D.Macfarl. & Ward.-Johnson, *Symonanthus bancroftii* (F.Muell.) Haegi or *N. tabacum* L. and *N. sylvestris* Speg. were used as outgroups, depending on availability. As previously encountered in phylogenetic trees of members of the *N.* section

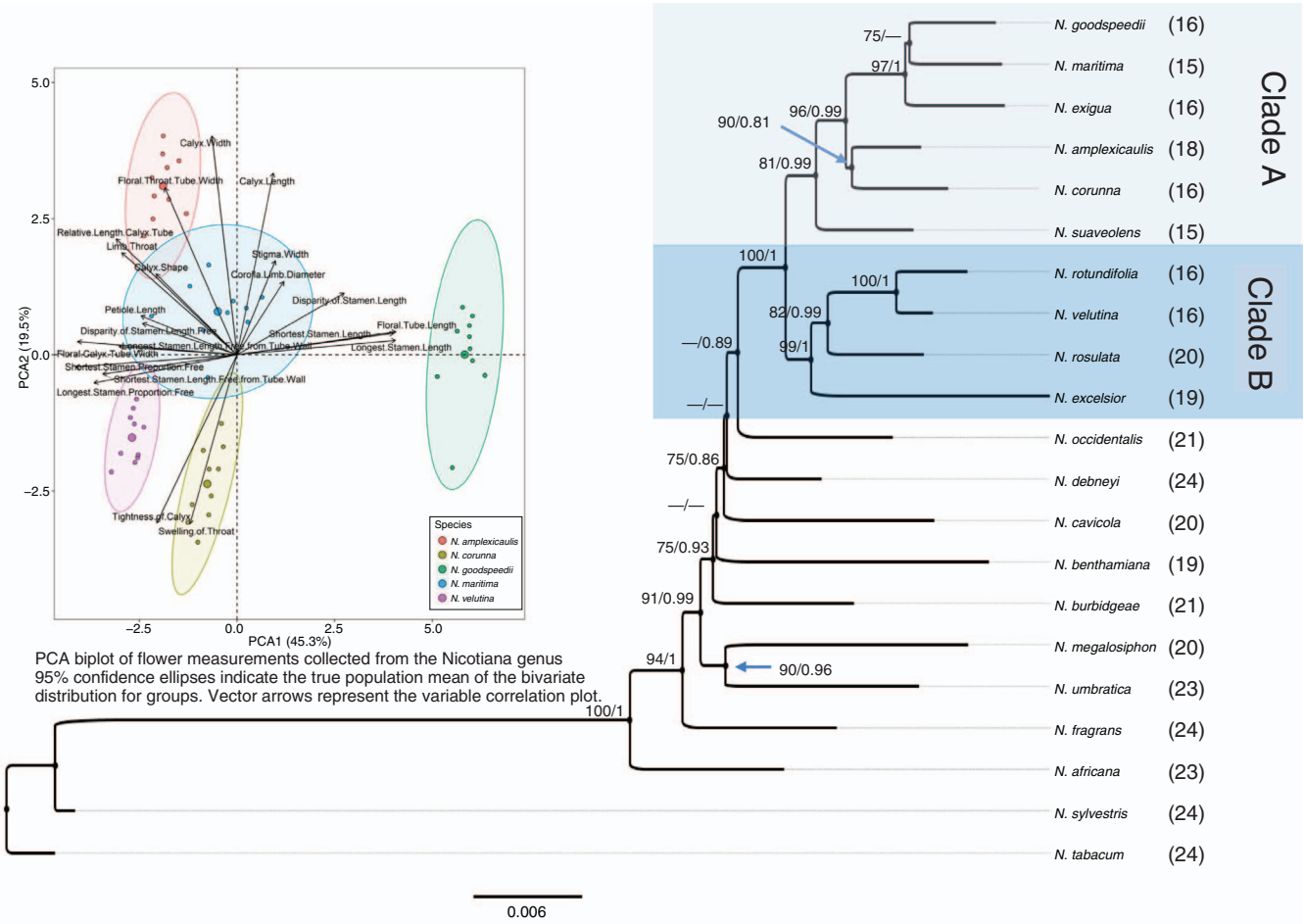


Fig. 2. Relationships of taxa in *Nicotiana* section *Suaveolentes* and relative position of the *N.* sp. Corunna (D.E.Symon 17088) accession. A. PCA biplot of flower measurements collected from selected *Nicotiana* species. The 95% confidence ellipses indicate the true population mean of the bivariate distribution for groups. Vector arrows represent the variable correlation plot. B. Summary phylogenetic maximum-likelihood and Bayesian-inference tree from combined *ITS*, *matK*, *GSS*, *GSL*, *RDR1* and *ADHC* sequences with support values (bootstrap support and posterior probability values >70% or 0.7 respectively) are shown above branches. Branches with ≤70% /0.7 support value are annotated with an en-dash (–). Chromosome number is indicated in parentheses for each taxon.

Suaveolentes using different sequence datasets (Clarkson *et al*. 2010; Marks *et al*. 2011*a*), the patterns from the different gene-region sequences had several conflicting branches. Nevertheless, in each tree, with one exception, *N.* sp. Corunna (D.E.Symon 17088) was distinct from other members of *N.* section *Suaveolentes*. On the basis of the *ADHC* sequences *N.* sp. Corunna (D.E.Symon 17088) was not distinct from *N.burbidgeae*. Using the tree generated from the concatenated sequences, among *N.* section *Suaveolentes*, the African species *N. africana* Merxm. and the New Caledonian species *N. fragrans* Hook. were sister to a well supported clade that contained all the Australian members. Most nodes in the Australian *Suaveolentes* clade were poorly supported and only two clades, being composed of more derived species, were well resolved. Species in these two groups generally have fewer chromosomes than do other members of the section. In Fig. 2, these clades are labelled A (six species; BS = 81%, posterior probability (PP) = 0.99) and B (4 species; BS = 99%, PP = 1). Clade A and Clade B are well supported as sisters (BS = 100%, PP = 1) in the tree. *Nicotiana* sp. Corunna (D.E.Symon 17088) forms a clade with *N. goodspeedii*, *N. maritima*, *N. exigua*, *N. amplexicaulis* N.T. Burb. and *N. suaveolens*. Although both are placed in Clade A, *N. suaveolens* and its synonym *N. exigua* do not cluster together. Clade B contains *N. excelsior, N. rosulata*, *N. rotundifolia* Lindl., and *N. velutina* H.-M.Wheeler.

Phenetic analysis of floral characters

Because DNA-based phylogenies pointed to *N.* sp. Corunna (D.E.Symon 17088) being a distinct species, a phenetic analysis of flowers was performed to obtain further evidence for it being a new species and to find characters that could be potentially useful in its identification. A PCA biplot (Fig. 2, inset) was constructed using *N.* sp. Corunna (D.E.Symon 17088) and the three species (*N. goodspeedii*,

N. maritima and *N. velutina*) that overlap its geographic distribution (Fig. 3) and with its sequence-based cladistic sister, *N. amplexicaulis*. *Nicotiana* sp. Corunna (D.E.Symon 17088) flower characters formed a cluster that was well separated from *N. goodspeedii*, *N. velutina* and *N. amplexicaulis*, and predominantly separated from *N. maritima*. *Nicotiana* sp. Corunna (D.E.Symon 17088) was readily distinguished from *N. goodspeedii*, by its shorter stamens, smaller floral tubes and calyx, and thinner corollas; it was distinguishable from *N. velutina* by its shorter calyx length and narrower corolla limb diameter. A PCA that uses all the species described in Marks *et al*. (2011*a*) is shown in Fig. S7.

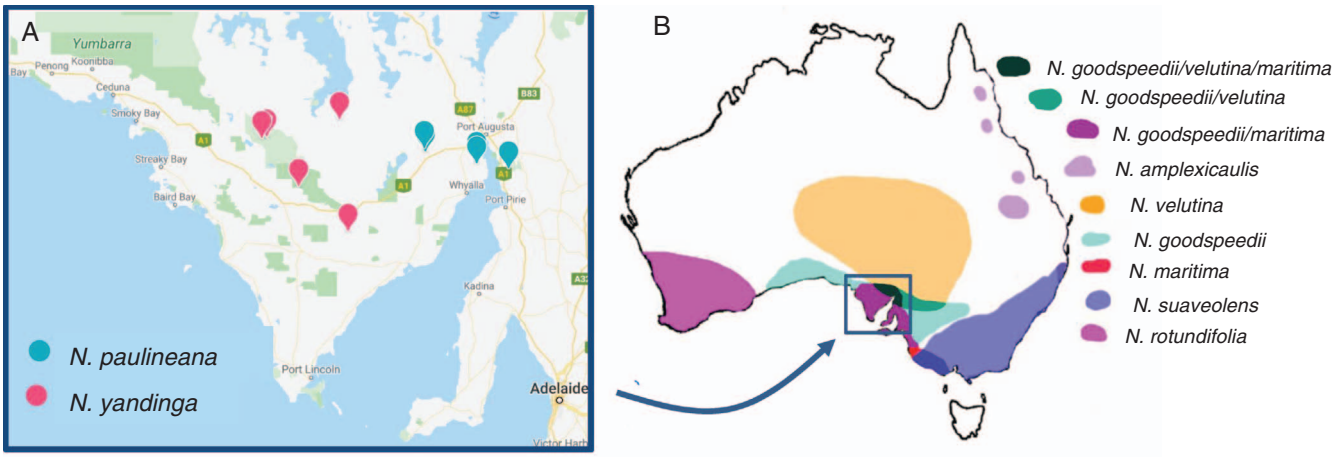
Discussion

The status of a potential new species, namely, *N.* sp. Corunna (D.E.Symon 17088) within *N.* section *Suaveolentes*, has remained unverified for many years, predominantly because of a lack of sequence information. This lack of information has been redressed by determining the *ITS*, *matK*, *GSL*, *GSS*, *RDR1* and *ADHC* sequences from *N.* sp. Corunna (D.E. Symon 17088) and comparing these sequences with their counterparts from members of the *N.* section *Suaveolentes*. The new sequence information identifies *N.* sp. Corunna (D.E. Symon 17088) as a distinct terminal taxon.

Three well recognised members of *N.* section *Suaveolentes* (*N. maritima*, *N. goodspeedii* and *N. velutina*) have distributions that intersect with *N.* sp. Corunna (D.E.Symon 17088); however, of the three, only *N. goodspeedii* has the same chromosome number (n = 16) as does *N.* sp. Corunna (D.E.Symon 17088) and none has flowers that are identical to those of *N.* sp. Corunna (D.E.Symon 17088), as demonstrated in the PCA. *Nicotiana maritima* has recently been recircumscribed following recognition of a new species, *N. yandinga* (Chase *et al*. 2018*b*), that occurs in the vicinity of *N.* sp. Corunna (D.E.Symon 17088) (Fig. 4A). However, *N. yandinga* has features, such as indumentum and a genome of 21 chromosome pairs (Chase *et al*. 2018*b*), that distinguish it from *N.* sp. Corunna (D.E.Symon 17088). Altogether, this provides strong evidence that *N.* sp. Corunna (D.E.Symon 17088) merits recognition as a new species that we name as *N. paulineana*, sp. nov. and describe below. This posthumously fulfils an ambition of the highly respected Australian plant taxonomist, David Symon. His obituary (Barker 2013) listed several projects



Fig. 3. *Nicotiana paulineana* showing phyllotaxis, leaf form, flower structure, capsule and seed. Photograph inset: flower showing deeply cleft corolla lobes and approach herkogamy.



C

Collection date	Longitude	Latitude	Locations of Corunna / *N. paulineana*
26/8/2004	–32.682	137.118	Corunna Station
23/8/2010	–32.649	137.109	Baxter Hill Creek
9/9/2010	–32.751	137.684	Near Port Augusta
10/9/2010	–32.793	137.686	Near Port Augusta
26/12/2010	–32.838	138.038	Mount Remarkable National Park

Fig. 4. Distribution of *Nicotiana* species in Groups A and B of Fig. 2B. A. Distribution of *N. paulineana* and *N. yandinga* based on records in the Atlas of Living Australia (ala.org.au). B. Distribution of *N. amplexicaulis*, *N. velutina*, *N. goodspeedii*, *N. maritima*, *N. suaveolens* and *N. rotundifolia* based on Burbidge (Burbidge 1960), Horton (Horton 1981), Chase (Chase *et al.* 2018*b*), and records in the Atlas of Living Australia (ala.org.au). Overlapping distributions are denoted by colours (black, green and purple) and, in the magnified region (A), the locations of *N. yandinga* and *N. paulineana* are shown as red and blue pins respectively.

that he regarded as unfinished, one of which was obtaining evidence for the recognition of *N.* sp. Corunna (D.E.Symon 17088) as a new species.

Taxonomy

Nicotiana paulineana Newbigin & P.M.Waterh., sp. nov.

Type: Cultivated. Victoria. Melbourne University, Parkville, glasshouse on Natural Philosophy Building, 25 June 2007, *C.E.Marks 299* (holo: MELU D106463!).

Plants glabrous, erect, annual, 0.4–1 m high. Initially single-stemmed, commonly developing several branched stems. Seedlings with cotyledons 6–7 mm long. Basal leaves rosulate, petiolate, attenuate, 10–30 cm long. Eglandular trichomes with one single gland cell on the calyx. Panicles loosely decompound, major branching long, rapidly ascendant. Calyx 5–7 mm long, appressed to tube. Corolla slightly zygomorphic, lobes emarginate, spreading; corolla limb lobes pure white inside, with a green to yellow vein running down the back of each lobe; floral tube broadening above calyx, 15–25 mm long exclusive of limb, 2–3 mm wide, often purplish. Stamens all included, 4 anthers at or close to one level, near mouth of corolla, anther of 5th stamen 2–3 mm lower; filaments inserted in lower half of corolla. Capsule not constricted or thickened, no seeds retained. Seeds reinform, sinuous seeds testa, 0.9 mm long, brown. Chromosome number 16 pairs.

Notes

This species can be distinguished from *Nicotiana goodspeedii* and *N. maritima* by its narrower leaf shape and more deeply cleft corolla lobes (Fig. 3) and from *N. maritima* by its lack of a woolly indumentum

The holotype is a flowering specimen taken from the plant used by Marks *et al.* (2011*b*) to assess the chromosome number. Additional notes on the specimen lodged at The University of Melbourne Herbarium (MELUD106464) say ‘Hydroponically grown for chromosome count, actual plant used for count. . .’, ‘cultivated from seed supplied by David Symon (AD) with provenance’, ‘grown from Symon 17088’. David Symon indicated to Claire Marks that this seed lot came from Corunna Station and has the provenance described for *D.E. Symon s.n.* (AD 169037), a specimen grown from a wild collection from Corunna Station.

Distribution and habitat

The five known collection locations of *N. paulineana* are all from South Australia within a 50-km radius south of Port Augusta, and its habitat is primarily vegetated natural and

semi-natural terrestrial vegetation in coastal South Australia. All collections have been made in association with drainage lines and usually below rocky outcrops. Recorded growing in association with the native species *Allocasuarina* sp., *Atriplex cinerea*, *Austrostipa nitida*, *A. scabra* subsp. *scabra*, *Callitris glaucophylla*, *Cassinia* sp., *Cheilanthes lasiophylla*, *Dodonaea* sp., *Enchylaena tomentosa* var. *tomentosa*, *Eremophila glabra* subsp. *glabra*, *Erodium crinitum*, *Eucalyptus camaldulensis*, *E. socialis*, *Exocarpos* sp., *Geranium* sp., *Goodenia havelandii*, *Heliotropium asperrimum*, *Hydrocotyle trachycarpa*, *Isolepis congrua*, *Lemooria burkittii*, *Levenhookia dubia*, *Millotia perpusilla*, *Myoporum* sp., *Nicotiana maritima*, *N. occidentalis*, *Olearia muelleri*, *Oxalis perennans*, *Parietaria cardiostegia*, *Philotheca linearis*, *Plagiobothrys plurisepaleus*, *Plantago turrifera*, *Ptilotus obovatus*, *Pycnosorus pleiocephalus*, *Ranunculus hamatosetosus*, *R. sessiliflorus* var. *sessiliflorus*, *Rhodanthe pygmaea*, *Rumex brownii*, *Scaevola spinescens*, *Sida* sp., *Vittadinia* sp., *Wahlenbergia gracilenta* and *Zygophyllum* sp. Some sites are noted to have numerous weeds present, including *Carrichtera annua*, *Conyza* sp., *Echium plantagineum*, *Galenia pubescens*, *Hypochaeris glabra*, *Lactuca* sp., *Marrubium vulgare*, *Solanum elaeagnifolium*, *Trifolium angustifolium*, *T. arvense*, *Verbascum* sp. and *Vulpia myuros* f. *myuros*, and the presence of these weeds indicates a degradation of habitat for this localised species.

Etymology

This species is named after Professor Pauline Yvonne Ladiges AO FAA, a botanist distinguished for her studies on the systematics, biogeography and ecology of Australian plants, particularly the eucalypts, and who has greatly contributed to the study of Australian *Nicotiana*.

Other specimens examined

SOUTH AUSTRALIA: Mt Remarkable N[ational] P[ark], Mambray Creek, opposite campsite no. 44 on other side of creek, 26 Dec. 2010, *C.J. Brodie 1938* (AD); Baxter Hill Creek, 23 Aug. 2010, *M. Jusaitis s.n.* (AD 227148); W side of the Range [S Gawler Ranges], 26 Aug. 2004, *D.E.Symon 17116* (AD); pot grown from seed collected at base of waterfall on Corunna Stn above old ?tourist campsite on ?W side of the Range [S Gawler Ranges], 17 Sep. 2004, *D.E. Symon s.n.* (AD 169953); cultivated [in Adelaide] from seed from *D.E.Symon 17088*, 5 Oct. 2010, *D.E. Symon 17165* (AD); pot grown, originally from base of waterfall on Corunna Stn above old ? tourist campsite on ?W side of the Range [S Gawler Ranges], no date, *D.E. Symon s.n.* (AD 169037); upper reaches of Waterfall Gully, Cultana Defence Training Area, S of Port Augusta, 9 Sep. 2010, *T.S. Te & M. Jusaitis 980* (AD); ~2 km NE of Tregolana Hill in Cultana Defence Training Area, S of Port Augusta, 10 Sep. 2010, *T.S. Te & M. Jusaitis 990* (AD).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Declaration of funding

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