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Taxonomy of the *Nicotiana megalosiphon* species complex (Solanaceae; *Nicotiana* section *Suaveolentes*): analyses of RADseq data identifies a new cryptic species

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ABSTRACT

The *Nicotiana megalosiphon* Van Heurck & Müll.Arg. species complex has been shown to be composed of several morphologically cryptic species similar to *N. simulans* N.T.Burb. Using phylogenetic and population genetic approaches (maximum likelihood, co-ancestry, admixture proportions, Bayesian species delimitation and coalescent methods), we demonstrate that there is an additional undescribed species in this complex. The species limits of *N. latifolia* M.W.Chase & Christenh., *N. latzii* M.W.Chase, R.W.Jobson & Christenh., *N. megalosiphon*, *N. sessilifolia* (P.Horton) M.W.Chase & Christenh. and *N. simulans*, previously circumscribed based solely on a phylogenetic approach, are confirmed in the new analyses and a new species, *N. palssonae* M.W.Chase & Christenh., is described. A map of species distributions and a key to the species of the *N. megalosiphon* species complex are provided.

Keywords: admixture analysis, Bayesian species delimitation, coalescent methods, cryptic species, flora of eastern Australia, *Nicotiana simulans*, Nicotianoideae, wild tobacco.

Introduction

Nicotiana section *Suaveolentes* Goodsp. comprises all native Australian species of the genus that occur throughout the continent except for Tasmania. Cauz-Santos *et al.* (2024) demonstrated that after arrival in Australia *c*. 6 million years ago (Ma), the distribution was concentrated in mesic habitats in the northern monsoon region and along the east coast. However, the species subsequently moved into the Pilbara where the number of clades increased dramatically and adaptations to aridity developed, after which the species dispersed widely across the continent in several episodes over the past 5 Ma. Our research on this group was initiated due to the descending chromosome series and variable genome sizes, both occurring during the diploidisation phase while these allotetraploid species were adapting to a variety of habitats throughout the Eremaean Zone (Chase *et al.* 2023*b*). While studying these aspects of *N.* sect. *Suaveolentes*, we realised that in previous approaches to the taxonomy of this section (Horton 1981), the number of species had been underestimated at 16. Symon (1984, 1998), Clarkson and Symon (1991), Symon and Kenneally (1994) and Symon and Lepschi (2007) added 5 species to the section, bringing the total to 21 native species recorded for Australia by 2007. An additional 30 species have been described (reviewed in Chase *et al.* 2022), through elevation of subspecies and reinstatement of names in synonymy but mostly through the description of previously unrecognised species (reviewed in Chase *et al.* 2023*a*). New species occur throughout the range of the section, but Western Australia has yielded the highest number of new species.

Phylogenetic relationships for *N.* section *Suaveolentes* are described in Chase *et al.* (2022). The focus of this paper is a group of species related to *N. megalosiphon* Van Heurck & Müll.Arg. and *N. simulans* N.T.Burb. that we refer to as the *N. megalosiphon*

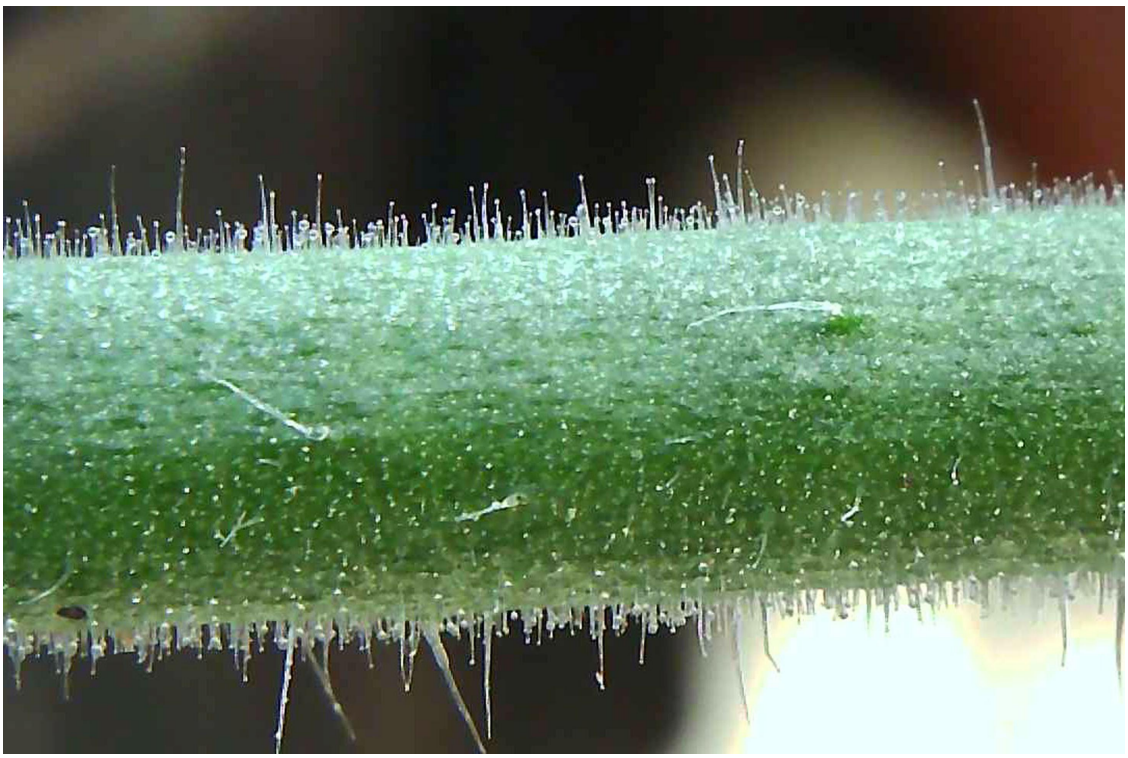

Fig. 1. Vestiture of species in the *Nicotiana megalosiphon–simulans* clade, comprising mixed long to short hairs with small apical glands.

species complex. These species form part of a larger clade in which the chromosome number is mostly $n = 20$ (Chase *et al.* 2023*b*) and the vestiture comprises a mixture of long, tiny, gland-bearing multicellular and short, gland-tipped hairs, with a few intermediate-length hairs, especially on the lower stems and petioles (Fig. 1). Different types of vestiture are characteristic of each of the major clades in the section. The long hairs on the upper stems may break off, leaving stout bases that cause the stems to feel rough to the touch. The *N. megalosiphon* species complex does not occur in Western Australia but the sister clade, the *N. stenocarpa* Wheeler species complex (also $n = 20$ with the same vestiture type (Chase and Christenhusz 2018), is largely concentrated there. As far as we can determine, the species we studied are the only species of the *N. megalosiphon* species complex.

We evaluate species limits of the *N. megalosiphon* species complex to determine whether there is an additional undescribed species. During examination of specimens in several Australian herbaria (BRI, CANB and NSW), some specimens appeared to be *N. simulans* but these differed slightly morphologically and more importantly, the ecological characterisitcs deviated from those of *N. simulans*. Our phylogenetic analyses initially used a *RAxML* approach to determine interspecific relationships of the species, but as described in Cauz-Santos *et al.* (2022), we constructed a coancestry heatmap and individual admixture proportions with *NGSadmix* to evaluate population structure and detect admixture. We also used a Bayesian species delimitation approach to estimate the most appropriate number of species for recognition. We built a multilocus, coalescent species tree using the optimal species model defined in the delimitation analyses to estimate the ages of species divergences in the *N. megalosiphon* species complex.

Materials and methods

Plant material

We sampled 36 individuals, 33 of which were from the *N. megalosiphon* species complex (4 from viable seeds recovered from herbarium specimens up to 27 years old) and 3 of *N. walpa* M.W.Chase, Dodsworth & Christenh. (Chase *et al.* 2021), the latter being an outgroup in the phylogenetic analysis (Supplementary Table S1). This sampling reflects the distribution of this species complex (Fig. 2) in New South Wales, Northern Territory, Queensland and South Australia (Fig. 2).

DNA extraction, library preparation and sequencing

Total genomic DNA was isolated from ~20 mg of silica-dried or fresh leaf tissue, pre-treated for 20 min in a buffer of cold sorbitol (100 mM tris-HCl, 5 mM EDTA, 0.35 M sorbitol, pH 8.0) under a cetyltrimethylammonium bromide

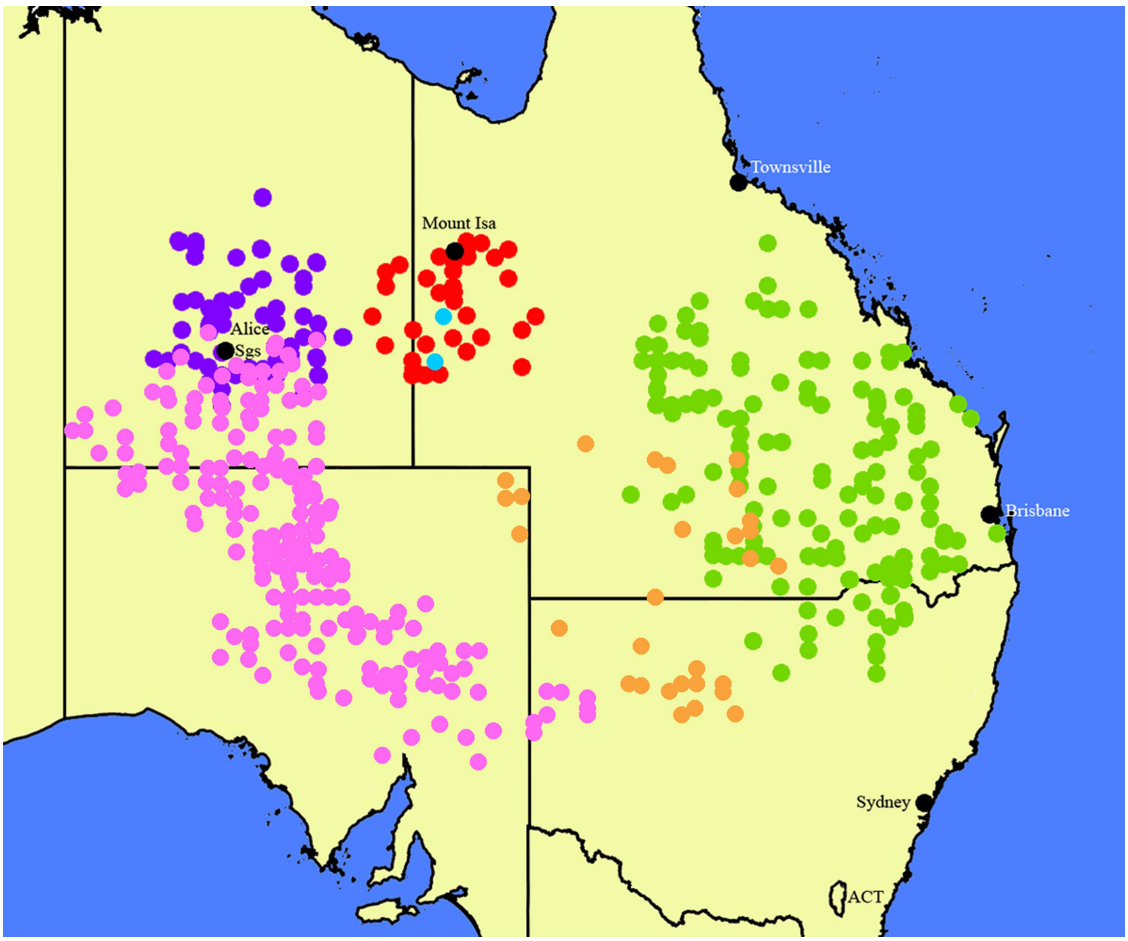

Fig. 2. Map of the species in the *N. megalosiphon* species complex. Blue, *N. sessilifolia*; pink, *N. simulans*; red, *N. latifolia*; turquoise, *N. latzii*; green, *N. megalosiphon*; orange, *N. palssonae*. Created by M. Christenhusz from data downloaded from the Australian Virtual Herbarium website and our specimens that have not yet been databased in Australia.

(CTAB) procedure and purified according to the manufacturer's instructions for the NucleoSpin gDNA clean-up Kit (Machery–Nagel, Düren, Germany).

Single-digestion RADseq libraries were prepared according to Paun *et al.* (2016) including digestion with PstI and use of index barcodes distinct by at least three nucleotide positions. Libraries were sequenced as pair-end reads of 125 bp at the VBCF NGS Unit (see www.vbcf.ac.at/ngs).

SNP calling from RADseq data

The raw reads were processed in the *BamIndexDecoder* (ver. 1.03, included in Picard *Illumina2Bam* package, see http://gq1.github.io/illumina2bam/) and demultiplexed in sublibraries according to index barcodes. We subsequently used process_radtags in *Stacks* (ver. 1.47, see https://catchenlab.life.illinois.edu/stacks/; Catchen *et al.* 2013) and inline barcode information to demultiplex the individual samples. This last process was performed in conjunction with quality filtering to remove low quality reads, allowing a maximum of one mismatch in the barcodes and cut-site sequences (Supplementary Table S2).

The mappings were performed in *BWA MEM* (ver. 0.7.17, see https://github.com/lh3/bwa; Li and Durbin 2009), using the genome of *N. benthamiana* (ver. 1.0.1, see https://solgenomics.net/organism/Nicotiana_benthamiana/genome; Bombarely *et al.* 2012) as a reference. For the mapping, we used the option −M to flag shorter split hits as secondary and the resulting aligned SAM file was sorted by reference coordinates. Read groups were added using the Picard *Toolkit* (see http://broadinstitute.github.io/picard/). Mapping quality was improved with a realignment around indels in the *Genome Analysis Toolkit* (*GATK*, ver. 3.8, see https://gatk.broadinstitute.org/hc/en-us; McKenna *et al.* 2010), thinning data per interval to a maximum of 100,000 reads.

Variant calling was performed in *GATK*, starting with generation of a gVCF file for each sample using the GNVCF mode of HaplotypeCaller, followed by the processing of all samples using the GenotypeGVCFs module. We used *VCFtools* (ver. 0.1.15, see https://vcftools.github.io/;

Danecek *et al.* 2011) to retain only variants present in at least 50% of the individuals (Supplementary Table S1) and performed filtering in the VariantFiltration module of *GATK* using the following criteria: (1) depth of coverage (DP) < 500; (2) variant confidence (QUAL) < 30.00; (3) variant confidence divided by the unfiltered depth (QD) < 2; (4) Phred-scaled *P*-value for the Fisher's exact test to detect strand bias (FS) > 60; (5) a root mean square of mapping quality across all samples (MQ) < 40; (6) u-based z-approximation from the rank sum test for mapping qualities (ReadPosRankSum) < −8.0; and (7) u-based z-approximation from the rank sum test for the distance from the end of the reads with the alternate allele (MQRankSum) < −12.5.

After first filtering in *GATK*, 1,656,211 variable sites were retained. Subsequently, an additional filtering was performed in *VCFtools* to retain only SNPs with a minor allele frequency of at least 0.055 (i.e. present in at least four haplotypes), an average depth greater than 20 and 20% maximum missing data. To avoid further use of any pooled paralogs, we filtered positions with maximum observed heterozygosity greater than 0.65 using the populations pipeline from Stacks. The final filtered file included 103,282 SNPs among the *Nicotiana* accessions included that was used for *RAxML* (maximum likelihood) analysis.

Phylogenomic analysis

To perform the phylogenetic analysis, we converted the final filtered VCF file to PHYLIP format using *PGDspider* (ver. 2.1.1.0, see https://software.bioinformatics.unibe.ch/pgdspider/; Lischer and Excoffier 2012) and used the asc-bias.py script (see https://github.com/btmartin721/raxml-ascbias) to remove invariant sites. A maximum likelihood tree was produced with *RAxML* (ver. 8.2.12, see https://github.com/stamatak/standard-RAxML; Stamatakis 2014) using the Lewis (2001) correction for ascertainment bias. We identified the best ML tree for 36 accessions using the general time reversible model of nucleotide substitution and the CAT approximation of rate heterogeneity (GTRCAT), and performed 1000 rapid bootstrap replicates. The tree was finally visualised and annotated in *R* (ver. 4.4.2, R Foundation for Statistical Computing, Vienna, Austria, see https://www.r-project.org/) with *ape* (ver. 5.3, see http://ape-package.ird.fr/; Paradis and Schliep 2019), *biostrings* (ver. 2.72.1, H. Pagès, P. Aboyoun, R. Gentleman and S. DebRoy, see https://bioconductor.org/packages/Biostrings), *ggplot2* (ver. 2.3.5.1, H. Wickham, W. Chang and M. H. Wickham, see https://CRAN.R-project.org/package=ggplot2; Wickham 2016), *ggtree* (ver. 3.20, see https://doi.org/doi:10.18129/B9.bioc.ggtree; Yu *et al.* 2017) and *treeio* (ver. 3.20, see https://doi.org/10.18129/B9.bioc.treeio; Wang *et al.* 2020).

Admixture and genetic clustering analyses

To identify admixture proportions and population structure for the 33 accessions comprising the *N. megalosiphon* species complex, we obtained genotype likelihoods using the indel-realigned.bam files and genotype-free method implemented in *ANGSD* (ver. 0.930, see https://github.com/ANGSD/angsd; Korneliussen *et al.* 2014). The analysis only kept sites with data for at least 50% of individuals, a minimum mapping quality of 20 and the *GATK*-based genotype likelihood model used to estimate major and minor allele frequencies. Filtering resulted in 1,215,636 high-confidence ($P < 1 \times 10^{-6}$) variable positions with a shared minor allele by three or more individuals.

Subsequently, we selected unlinked sites from the genotype likelihoods obtained in *ANGSD* (distance of 10,000 bp between variants, resulting in 39,293 positions) and estimated individual admixture proportions using *NGSadmix* (see https://www.popgen.dk/software/index.php/NgsAdmix; Skotte *et al.* 2013). We analysed $K = 1$–10 as the number of clusters, initialising the EM algorithm with 10 seeds and considering only variants with minor allele frequency higher than 0.055.

The clusters produced by use of the *K* values were evaluated with the Evanno method (Evanno *et al.* 2005) available in *CLUMPAK* (see http://clumpak.tau.ac.il/bestK.html; Kopelman *et al.* 2015) and the final plot was produced in *R*.

Species delimitation and species tree

Bayesian species delimitation was performed with a matrix of 19 individuals (16 of the *N. megalosiphon* complex and 3 of *N. walpa*) with at least 2 individuals per group identified in the models. This method cannot manage the 36 accessions used in the *RAxML* analysis, therefore we eliminated some individuals. We also explored the *NGSadmix* results and selected individuals without evidence of admixture. We used *VCFtools* to filter the VCF file, selecting unlinked biallelic SNPs (>50,000-bp distance on the same contig) and removing all missing data.

We evaluated six models for the *N. megalosiphon* complex using *N. walpa* as outgroup. The first model treated *N. megalosiphon* in the broadest possible sense and subsequently split the *N. megalosiphon* complex in up to seven groups. Bayesian species delimitation was performed in *SNAPP* (ver. 1.2.5, see https://www.beast2.org/snapp/; Bryant *et al.* 2012) and marginal likelihood values were obtained using 24 initialisation steps. The analysis was conducted with 1 million chain-lengths for each mode, storing a tree every 1000 generations and excluding the first 10% as burn in. The optimal model of species assignment was obtained by calculating the Bayes factor based on the marginal likelihood values.

We used the optimal model obtained in the species delimitation to construct a coalescent species tree in *SNAPP* (ver. 1.2.5). The analysis was based on the same dataset of unlinked SNPs (5475), with 10 million as the chain length using the same procedures for keeping trees. We used the

ESS values from the log-file to check convergence of the run in *Tracer* (ver. 1.6, see https://github.com/beast-dev/tracer/releases/tag/v1.6; Rambaut *et al.* 2018). We calculated posterior probabilities using *TreeAnnotator* (ver. 1.8.3, see https://www.beast2.org/treeannotator/; Drummond *et al.* 2012) and visualised the *SNAPP* trees as a cloudogram with *DensiTree 2* (ver. 2.3.1.0, see https://github.com/rbouckaert/DensiTree; Bouckaert and Heled 2018) that produced divergence times and re-scaled the trees. Divergence estimates were based on the total length of investigated sites in the included loci and the total number of polymorphic sites across the length using 5×10^{-9} as the rate of substitution per site per generation (Schiavinato *et al.* 2020) and 1 year as generation time (these species are all ephemerals or short-lived perennials that only rarely survive the summer).

Results

Phylogenomic analysis (*RAxML*)

Except for the position of *N. simulans*, the *RAxML* analysis produced a generally well supported tree (Fig. 3) with well differentiated relationships of all species concepts. One accession of *N. palssonae* M.W.Chase & Christenh. (18016) is only weakly supported as a member of the cluster assigned to this species.

Admixture and genetic clustering analyses

We initially used these analyses to select accessions that did not exhibit admixture, which would interfere with subsequent analyses (i.e. the coalescent species tree in *SNAPP*). The Structure plots ranged from $K = 2$–9 (Fig. 4) with $K = 7$ optimal and some accessions did not consistently exhibit the genetic identities that we assigned to species concepts based on *RAxML* results (Fig. 3). Several accessions of *N. palssonae* and *N. megalosiphon* exhibited admixture, some even outside the zone of geographical overlap and some geographical population structuring was present in several species, including *N. palssonae* and *N. megalosiphon*. The two accessions from northern New South Wales (18161, 18175) assigned to *N. megalosiphon* in the *RAxML* tree in some cases could be assigned to the *N. latzii* M.W.Chase, R.W.Jobson & Christenh. cluster. Two accessions of *N. palssonae* from the eastern part of the range in New South Wales appeared as unique genotypes in the $K = 7$ plot. An accession assigned to *N. palssonae* in the *RAxML* tree (but weakly supported) (18016) exhibited admixture in some of the structure plots with *N. megalosiphon*.

The 16 accessions of the *N. megalosiphon* species complex used in the Bayesian species delimitation were evaluated for the optimal number of species to be recognised (Fig. 5*a*) that was model 5 (with the best Bayes score). This analysis split the *N. megalosiphon* species complex into six clusters (species) and *N. walpa*, the outgroup. The next best result was model 6 that split the accessions of *N. megalosiphon* from New South Wales and Queensland, a result we will discuss below.

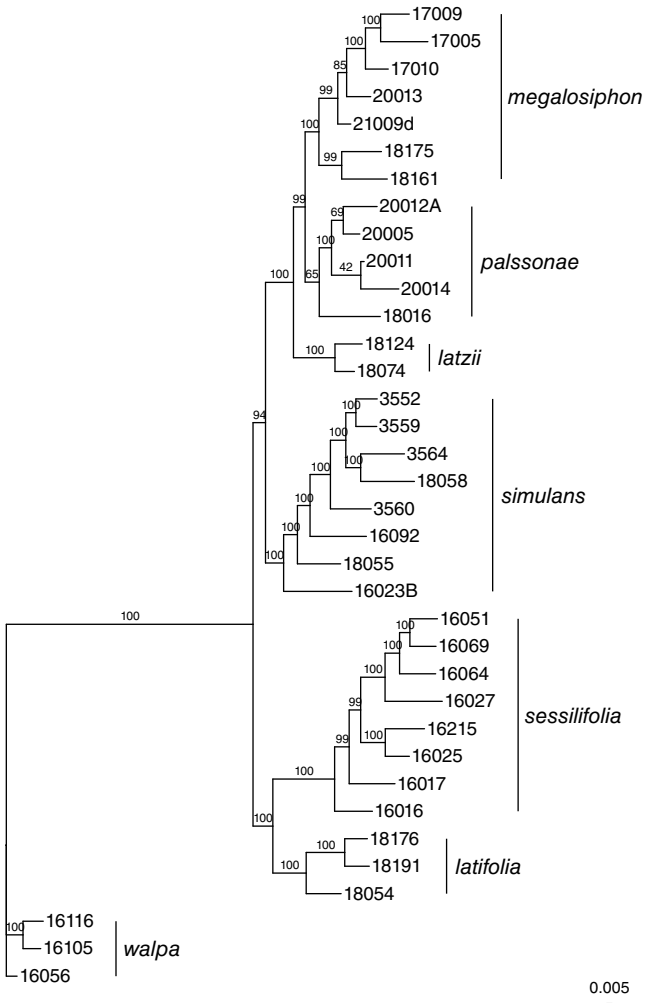

Fig. 3. *RAxML* tree of the *N. megalosiphon* species complex. Numbers at nodes are bootstrap percentages.

The coalescent species tree (Fig. 5*b*) generally agreed with the *RAxML* tree, except for the position of *N. simulans* that was weakly supported as sister to *N. sessilifolia* (P.Horton) M.W.Chase & Christenh. However this pair was well supported as a member of a clade with *N. latifolia*. *Nicotiana palssonae* was sister to *N. latzii* instead of *N. megalosiphon* as in the *RAxML* tree. When we calculated the divergence estimates, the *N. megalosiphon* species complex began to diverge *c*. 200,000 years ago with *N. palssonae* and *N. latzii* splitting only 60,000 years ago.

Discussion

The *N. megalosiphon* species complex began to radiate in north-eastern Australia only *c*. 200,000 years ago, with the

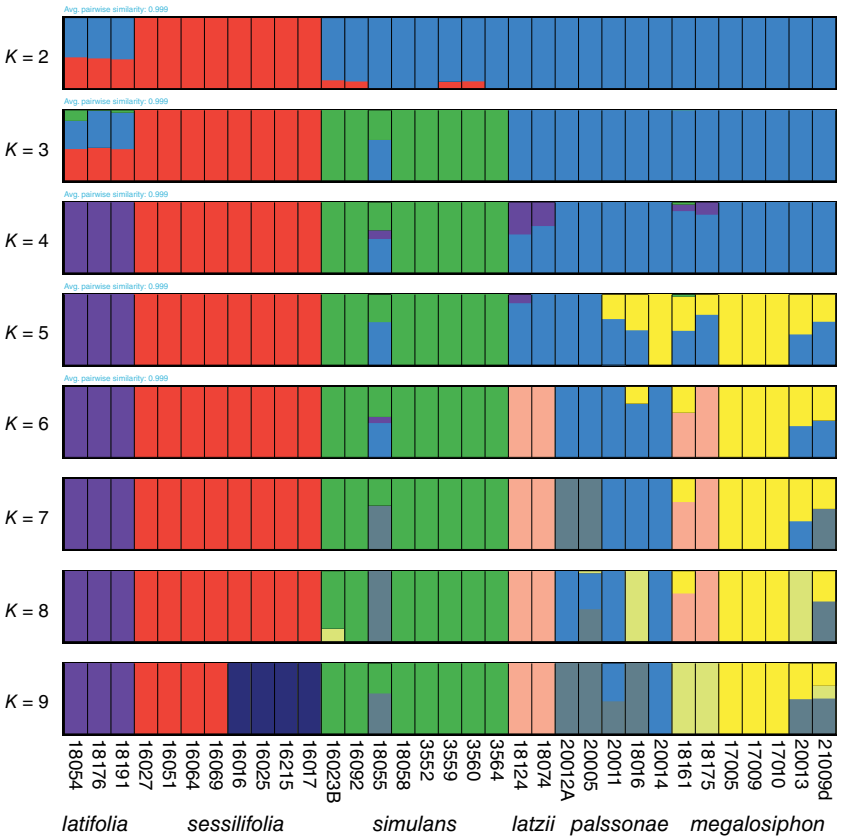

Fig. 4. Structure plots for *K* = 2–9. *K* = 7 is the most likely.

first split in the Bayesian species tree (Fig. 5*b*) representing ancestors of the *N. latifolia*–*N. sessilifolia*–*N. simulans* and *N. latzii*–*N. palssonae*–*N. megalosiphon* clades. Biogeographically, this represents a split between the western and eastern parts of the overall range. However, if the *RAxML* tree is correct, the clade originated in the western part of the range and spread eastward from there. The relationship of *N. simulans* to the other species is different in the Bayesian species and *RAxML* trees, making a more detailed scenario for how divergences progressed ambiguous.

On morphological grounds, we are content with the assignments of accessions in the *RAxML* tree, but we note problematic accessions from a genetic standpoint in the Structure results (Fig. 3). One of these is the *Prendergast 354* specimen (secondary voucher *Chase & Christenhusz 18016*) that falls with *N. palssonae* in the *RAxML* tree (Fig. 2) but with low support and is distinct in some of the Structure plots. Morphologically, this accession is most like *N. palssonae* and we include this in the latter but note that the accession is problematic. We acknowledge that our sampling for the genetic studies only includes one accession from the area in which *N. palssonae* and *N. megalosiphon* overlap, and this individual exhibits admixture in some cases. However, several other accessions of *N. palssonae* and *N. megalosiphon* exhibit admixture, including some outside the area of geographical overlap. Despite this evidence for some degree of gene flow, the two species remain morphologically distinct. Some species are known to have porous genomes (Lexer *et al.* 2009) but nonetheless maintain species boundaries and distinct morphological characteristics, as may be the case here. We would need multiple samples per population and more populations, especially from the area of geographical overlap, to determine the situation more conclusively. Despite these deficiencies, we rely upon the morphological distinctiveness of these two species to justify description of *N. palssonae*.

Our original attention to accessions of what we name *N. palssonae* was drawn by herbarium collections from outside the southern Northern Territory/South Australia that had been identified as *N. simulans* (not *N. megalosiphon*, with which these accessions were rarely identified). When we use the term ‘cryptic species’ we refer to the similarity of *N. palssonae* to *N. simulans*, not *N. megalosiphon*, the most morphologically divergent and easily diagnosed species in this species group. The specimens of both *N. latzii* and *N. palssonae* generally agreed with the concept of *N. simulans* (for which the type is from South Australia) but these differed sufficiently to elicit our interest. The first living material of the newly recognised species, *N. palssonae*, were plants that Ruth Palsson collected when searching for *N. velutina* Wheeler near Broken Hill during the COVID-19 years. Palsson did not find *N. velutina* but did yield material of a *N. simulans*-like accession that had ecological characteristics that differed from those of accessions

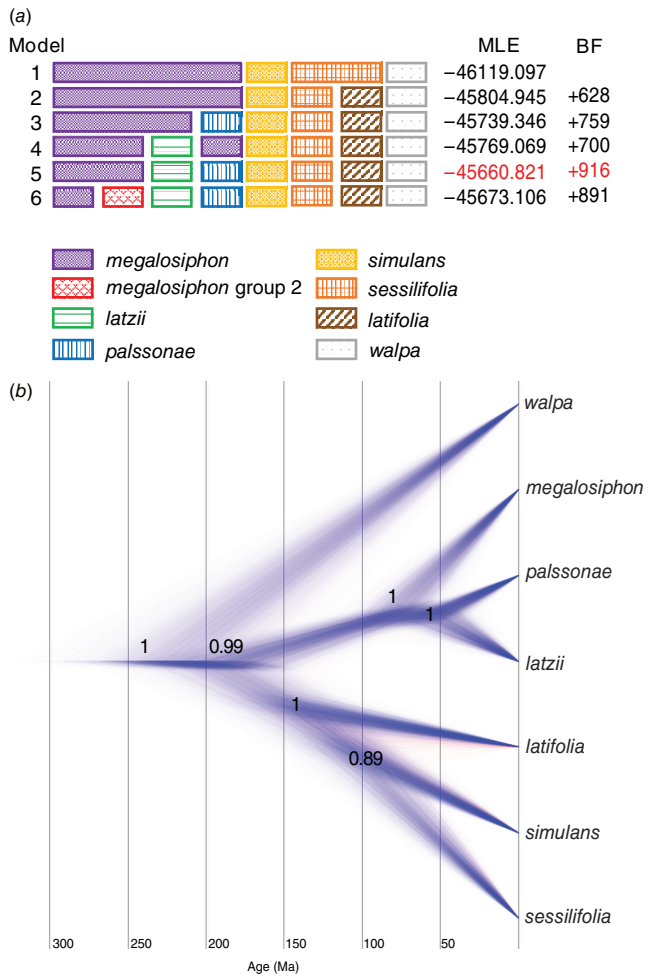

Fig. 5. Model evaluation for recognition of genetic entities in the *N. megalosiphon* species complex using Bayesian species delimitation (*a*). Bayesian species tree for the *N. megalosiphon* species complex with age estimates (*b*).

from South Australia. *Nicotiana simulans* generally occurs in the open from the gibber plains in South Australia to the southernmost Northern Territory, whereas the new species occurs in sheltered sites under mulga (*Acacia aneura* F.Muell. ex Benth. species complex) and various eucalypt species from north-western New South Wales and north-easternmost South Australia to south-western Queensland. In years with plentiful rainfall, the species has occurred commonly and been collected frequently. The other species in this complex are typically associated with sheltered sites, although in years with the highest rainfall, these can also be found growing in more open habitats. The Western Australian and Northern Territory counterpart is the *N. stenocarpa* complex (including the species used here as outgroup, *N. walpa*), the species of which also mostly occur in shade (usually under mulga). Most authors have referred these specimens to *N. simulans*. *Nicotiana palssonae* and *N. simulans* also differ morphologically (leaf shape and generally long petioles, often with a slight wing in the latterand abruptly attenuate bases in *N. palssonae*). *Nicotiana latzii*, *N. palssonae* and *N. simulans* were readily distinguishable upon cultivation together but herbarium material of these species can be challenging to distinguish.

Morphologically, *N. sessilifolia* (Fig. 6) is easily distinguished from *N. megalosiphon* (Fig. 7) based on the shorter floral tubes and broadly winged petioles with auriculate bases. *Nicotiana latifolia* (Fig. 8) can be considered to have such broadly winged petioles as to appear sessile, distinguishing this species from both *N. megalosiphon* and *N. sessilifolia*. In addition, the latter two species have symmetrical perianth limbs with the four longer stamens of the same length, as opposed to the oblique floral tubes and didynamous stamens in *N. palssonae* (Fig. 9) and *N. simulans* (Fig. 10). *Nicotiana latzii* (Fig. 11) has symmetrical limbs and the four longer stamens of the same length, and differs from *N. palssonae* and *N. simulans* in these floral characteristics and the short, broadly winged petioles. Oblique floral tubes and didynamous stamens appear to revert or appear independently in many clades in *N.* sect. *Suaveolentes* but this condition makes a useful species character in many cases even though difficult to discern in herbarium material. The other often conspicuous floral trait of *N.* sect. *Suaveolentes* is the anther cups (in which the stamens are positioned) that are absent in all these species and their sister clade (including *N. walpa* and *N. stenocarpa*).

Nicotiana megalosiphon has a major split between the New South Wales and Queensland accessions (the latter 18175 and 18161; Fig. 3), and there is a case for recognising two entities on morphological grounds (longer floral tubes that remain pale green externally even during anthesis). The McPherson Range in Queensland on the border with New South Wales appears to be responsible for this north–south disjunction that has been noted in several animal and plant species complexes (Cracraft 1991; Crisp *et al.* 1995; Chapple *et al.* 2011; Simpson *et al.* 2018). The McPherson Range is an east–west spur of the predominantly north–south Great Dividing Range. The range has montane forests that represent a barrier to lowland and dry-forest plant species such as *Nicotiana*. Currently we refrain from recognising the New South Wales plants as a new species but further study with more extensive sampling may prove this to be warranted.

The techniques we used to genetically distinguish the species that we initially recognised as distinct on morphological, ecological and geographical grounds provide new dimensions to the issue of species delimitation. These also provide an objective basis for species delimitation that is independent of the other considerations (such as morphological interpretations) typically used in species circumscription. In situations such as that here, these techniques can provide clear results that support conclusions made on morphological grounds. These analyses confirm our previous delimitations (Chase and Christenhusz 2021; Chase *et al.* 2023*a*) for *N. latifolia, N. latzii, N. megalosiphon* and *N. sessilifolia* based solely on a *RAxML* analysis.

Fig. 6. (*Caption on next page*)

Fig. 6. Illustration of *Nicotiana sessilifolia* based on living plants cultivated at the Royal Botanic Gardens, Kew, grown from seeds from *Chase & Christenhusz 16046* (NT), collected at the Old Telegraph Station, Alice Springs, Northern Territory. Illustrated by Deborah Lambkin. (*a*) Floral limb, front view. (*b*) Corolla split open to reveal positions of stamens. (*c*) Flower, side view. (*d*) Carpel, style and stigma. (*e*) Pubescence on leaf surface near midvein. (*f*) Pubescence on leaf margin. (*g*) Pubescence on upper stem and pedicel. (*h*) Pubescence on lower stem. (*i*) Upper stem leaf. (*j*) Lower stem leaf. (*k*) Mature capsule. (*l*) Dehisced capsule with calyx removed. (*m*) Habit. Scale bars: *a–d, k, l*, 2.0 cm; *e–h*, 7.0 mm; *i, j*, 4.0 cm; plant in (*m*) is 110 cm tall. Reproduced from Chase *et al.* (2023*a* in *Australian Systematic Botany*) with permission.

Key to species of the *Nicotiana megalosiphon* species complex

At this stage of our studies, these are the only known members of this species complex. As for all species in *N.* sect. *Suaveolentes*, these species are problematic to distinguish if the material is in poor condition or fragmentary. Examining the leaves on the lower halves of the stems to determine whether there is a petiole, and if so, whether this is winged with an auriculate base, is important. The length of the floral tube is also a useful character and this is not significantly affected by the conditions under which the plants grew. In a season with plentiful rainfall, these species can all produce plants of 1.5–2.0 m tall, in which case identification is much easier, but if the plant is less than 50 cm tall, accurate identification will be difficult based on the morphological features and geographic locality should be emphasised. Establishing certainty regarding whether the specimen is a member of this species complex, for which the most distinctive feature is the mixture of hair types: long often multicellular hairs with a swollen base and short, small gland-tipped hairs especially on the upper stems and leaves, is also important (Fig. 1). The swollen bases of the long hairs (Fig. 1) remain on the stems and make these rough to the touch, especially when the stems are dry. As mentioned above, discerning the stamen condition (didynamous or not) and floral symmetry (oblique or symmetrical tubes) is difficult in herbarium specimens.

1 Leaves with a winged petiole and auriculate base.....2
Leaves with or without a winged petiole, without an auriculate base.....4
2 Floral tube (above the calyx) 1.0–2.0 cm long, oblique with didynamous stamens, South Australia and Northern Territory.....*N. simulans*
Floral tube (above the calyx) longer than 2.0 cm, not oblique, longer four stamens of same length, Northern Territory, Queensland, only north-easternmost South Australia.....3
3 Petiole so broadly winged as to appear sessile, easternmost Northern Territory and Queensland.....*N. latifolia*
Petiole not broadly winged, Northern Territory.....*N. sessilifolia*
4 Petiole absent, western Queensland.....*N. latzii*
Petiole present, eastern Queensland and New South Wales.....5
5 Floral tube (above the calyx) longer than 4.0 cm, not oblique, longer four stamens of same length, northern New South Wales and eastern Queensland.....*N. megalosiphon*
Floral tube (above the calyx) 1.6–2.5 cm long, oblique, stamens didynamous, south-western Queensland, northern New South Wales and north-easternmost South Australia.....*N. palssonae*

Taxonomy

Nicotiana palssonae M.W.Chase & Christenh., **sp. nov.**

Type: AUSTRALIA. New South Wales: Broken Hill, Living Desert State Park, open mulga woodland, 302 m, 31°53′30″S, 141°25′58″E, 14 Oct. 2005, *Palsson 419* (holo: NE 110953; iso: CANB, NSW).

Diagnosis

Nicotiana palssonae is closely related (Fig. 3, 5) and similar in morphology to *N. megalosiphon* in lacking a winged petiole and auriculate leaf base but *N. palssonae* has a much shorter floral tube than *N. megalosiphon.* This species has the longer four stamens of two lengths (didynamous), whereas in *N. megalosiphon* all four stamens are of the same length. The new species is most morphologically similar to *N. simulans* but the latter often bears both petiolate and sessile leaves, whereas *N. palssonae* rarely has sessile leaves. The ecological characteristics are different and the two species are disjunct (see below).

Erect, herbaceous annual *herbs* forming a minimal rosette but with numerous large leaves in the basal portion of the stems, the main stem with major branches in the lower half of the inflorescence but with only a few small branches in the upper half. *Leaves* without petiole wings or with only narrowly winged petioles, the wing up to 1.0–1.5 cm wide, sometimes bullate, blades 8.2–12.8 × 1.5–4.8 cm (including petiole), broadly ovate to lanceolate, the apex blunt to acute in the basal leaves, becoming acuminate in those higher up, upper leaf base gently attenuate, in some cases slightly auriculate, margins entire, undulate, often basally bullate, uppermost leaves often sessile. *Vestiture* composed of short, dense, gland-tipped hairs and longer multicellular, non-glandular hairs on all surfaces. *Inflorescence bracts* sessile, linear lanceolate, ~0.5–2.3 cm long, the apex acuminate. *Calyx* 1.4–1.6 × 0.2 cm, one lobe slightly longer and one shorter than the others, the tips acuminate, slightly flaring to clasping, slightly wider and longer in fruit, extending 0.5 cm beyond and surrounding the capsule, the calyx slightly enlarging at maturity. *Flowers* white, outwardly to upward facing, upper part of the floral tube 0.2 cm longer than the lower part. *Corolla tube* 2.0–2.5 cm long (from tip of the calyx), oblique, 0.2 cm in diameter, with no throat cup, the *limb* 1.6–1.8 cm across, the lobes slightly cleft, cleft

Fig. 7. Illustration of *Nicotiana megalosiphon* based on living plants cultivated at the Royal Botanic Gardens, Kew, grown from seeds from with *Chase & Christenhusz 17009*, collected atTelleraga-Millie Road, bridge over Mehi River, New South Wales. Illustrated by Deborah Lambkin. (*a*) Carpel, style and stigma. (*b*) Corolla, opened out. (*c*) Flower, lateral view. (*d*) Corolla, dorsal view. (*e*) Capsule, with calyx. (*f*) Dehisced capsule, calyx removed. (*g*) Stem leaf. (*h*) Stem pubescence. (*i*) Calyx hairs. (*j*) Leaf margin pubescence. (*k*) Habit. Scale bars: *a–f*, 2.0 cm; *g*, 3.0 cm; *h–j*, 1.0 cm; plant in (*k*) is 90 cm tall. Reproduced from Chase and Christenhusz (2021 in *Curtis's Botanical Magazine*) with permission.

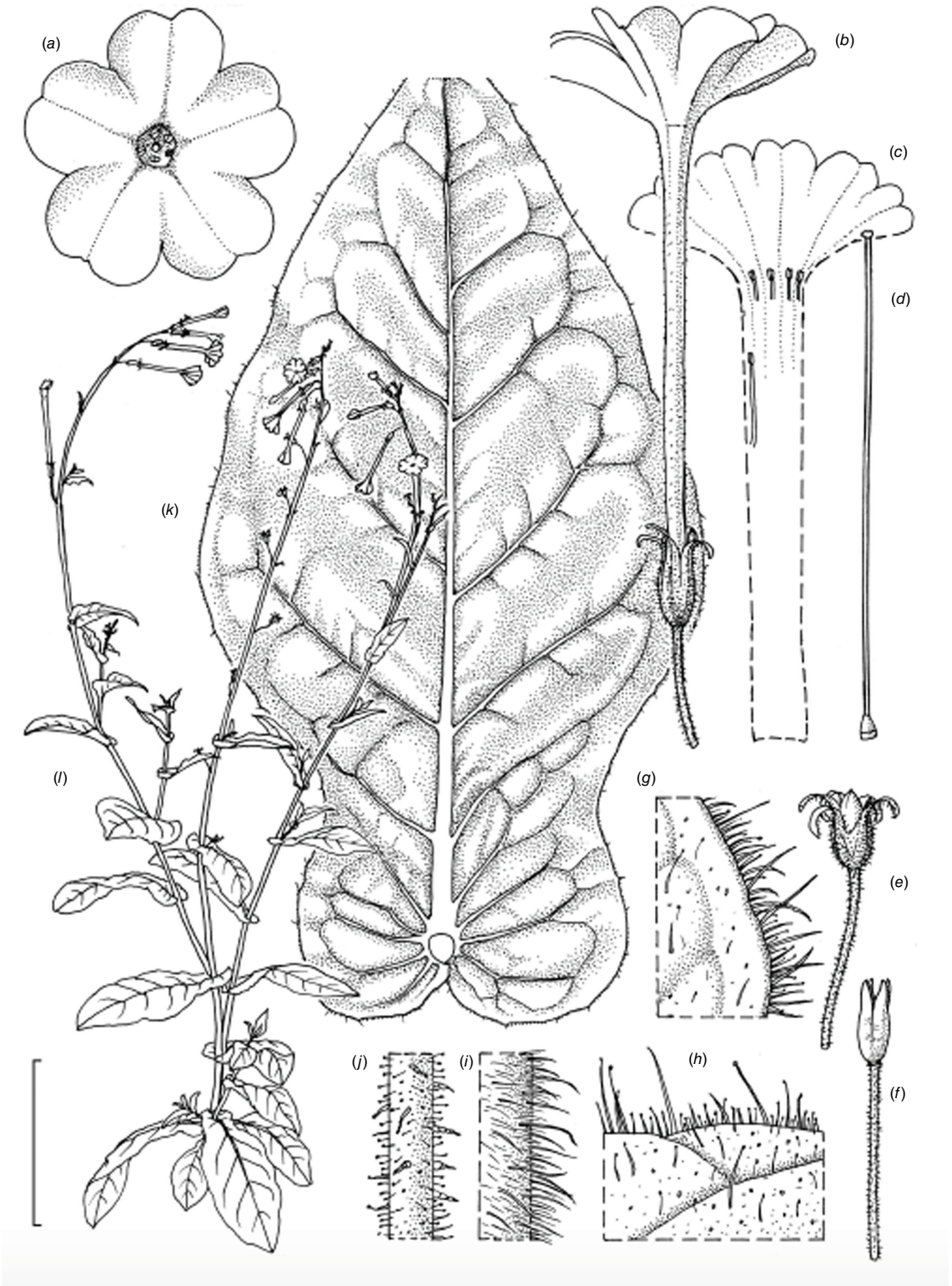

Fig. 8. Illustration of *Nicotiana latifolia* based on living plants cultivated at the Royal Botanic Gardens, Kew, grown from seeds from *Chase & Christenhusz 18177* (BRI), Cloncurry, on the banks of the Cloncurry River, Queenland. Illustrated by Deborah Lambkin. (*a*) Floral limb, dorsal view. (*b*) Flower, lateral view. (*c*) Corolla split open to reveal positions of stamens. (*d*) Carpel, style and stigma. (*e*) Mature capsule. (*f*) Dehisced capsule with calyx removed. (*g*) Pubescence on leaf margin. (*h*) Pubescence on leaf lamina. (*i*) Pubescence on lower stem. (*j*) Pubescence on upper stem. (*k*) Stem leaf. (*l*) Habit. Scale bars: *a–f*, 2.0 cm; *g–j*, 1.0 cm; *k*, 3.0 cm; plant in (*l*) is 90 cm tall. Reproduced from Chase *et al.* (2023*a* in *Australian Systematic Botany*) with permission.

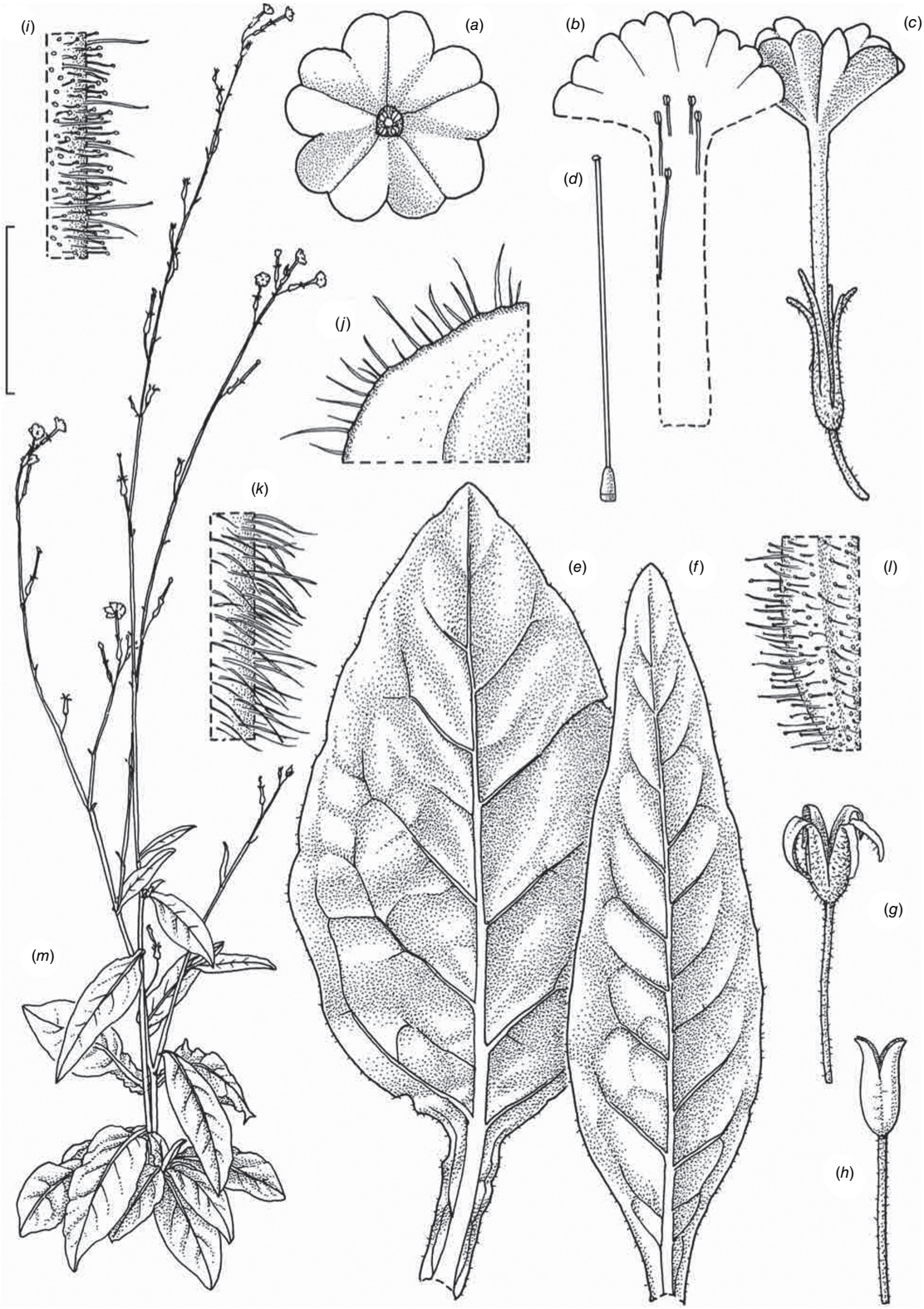

Fig. 9. Illustration of *Nicotiana palssona*e based on living plants cultivated at the Royal Botanic Gardens, Kew, grown from seeds from *Chase & Christenhusz 20014* (NSW), Living Desert State Park, Broken Hill, New South Wales. Illustrated by Deborah Lambkin. (*a*) Floral limb, dorsal view. (*b*) Corolla split open to reveal positions of stamens. (*c*) Flower, lateral view. (*d*) Carpel, style and stigma. (*e*) Lower leaf on stem. (*f*) Upper leaf on stem. (*g*) Mature fruit. (*h*) Dehisced capsule with calyx removed. (*i*) Pubescence on upper stem. (*j*) Pubescence on leaf margin. (*k*) Pubescence on lower stem. (*l*) Pubescence on calyx. (*m*) Habit. Scale bars: *a–f*, 2.0 cm; *g–j*, 1.0 cm; *k*, 3.0 cm; plant in (*m*) is 130 cm tall.

Fig. 10. Illustration of *Nicotiana simulans* based on living plants cultivated at the Royal Botanic Gardens, Kew, grown from seeds from with a secondary voucher of *Chase & Christenhusz 24005* (*k*) and original specimen, *Conran 437* (AD), Paralana Springs, South Australia. (*a*) Plant 116 cm tall. (*b*, *c*) Two stem leaves to illustrate leaf variation of leaves on a single plant. (*d*). Floral limb, dorsal view. (*e*) Flower, lateral view. (*f*) Corolla split open to reveal positions of stamens. (*g*) Carpel, style and stigma. (*h*) Mature fruit. (*i*) Dehisced capsule with calyx removed. (*j*) Leaf margin pubescence. (*k*) Pubescence on upper stem. (*l*) Pubescence on lower stem. Scale bars: *b*, *c*, 3 cm; *d*–*i*, 2 cm; *j*–*l*, 1 cm; plant in (*a*) is 85 cm tall.

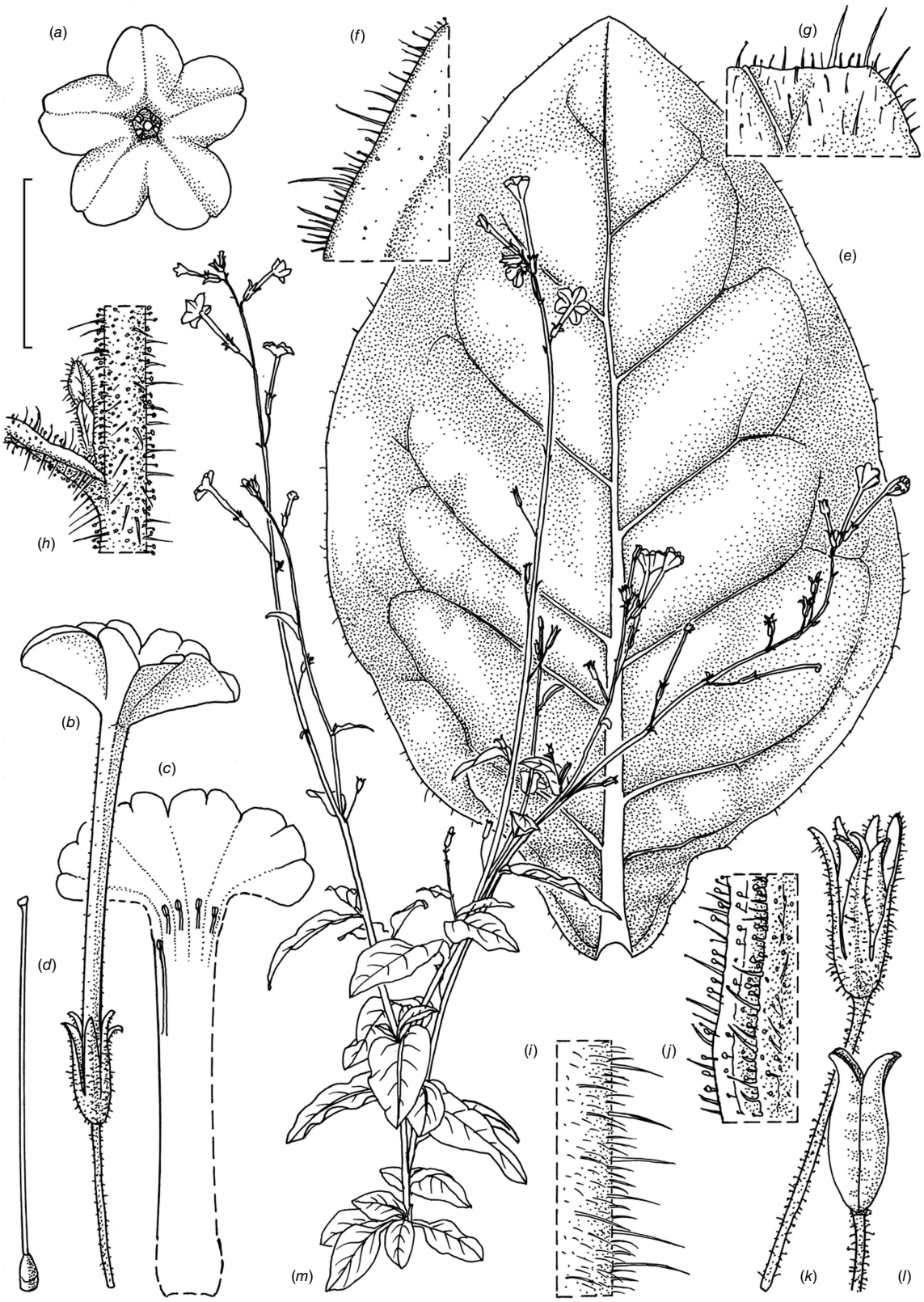

Fig. 11. (*Caption on next page*)

Fig. 11. Illustration of *Nicotiana latzii* based on living plants cultivated at the Royal Botanic Gardens, Kew, grown from seeds from *Latz 21442* (NT A0110082), Ethabuka Station, Queensland. Illustrated by Deborah Lambkin, vouchered as *Chase & Christenhusz 18074* (K). (*a*) Floral limb, dorsal view. (*b*) Flower, lateral view. (*c*) Corolla split open to reveal positions of stamens. (*d*) Carpel, style and stigma. (*e*) Stem leaf. (*f*) Pubescence on leaf margin. (*g*) Pubescence on leaf surface. (*h*) Pubescence on upper stem with leaf base and axillary shoot. (*i*) Pubescence on lower stem. (*j*) Pubescence on calyx. (*k*) Dehisced mature fruit. (*l*) Capsule with calyx removed. (*m*) Habit. Scale bars: *a–e*, 2.0 cm; *f–j*, 5.0 mm; *k, l*, 1.0 cm; plant in (*m*) is 77 cm tall. Reproduced from Chase *et al.* (2023*a* in *Australian Systematic Botany*) with permission.

0.1 cm deep, sinus 0.4 cm deep, lobes 0.6 cm long; four *stamens* near throat of the floral tube in two pairs, didynamous, the lower pair 0.1–0.2 cm longer than the upper pair and the fifth ~0.7 cm deeper in the tube. *Fruit* a capsule splitting in four lobes, 0.8–1.1 cm long at maturity (Fig. 8).

Distribution

Occurring in south-western Queensland and north-easternmost South Australia through north-western New South Wales. The species is found in the north-western portion of the Murray–Darling and adjacent Lake Eyre Basins (Fig. 2).

Habitat and ecological characteristics

Generally occurring in shaded sites under the shade of mulga, eucalypt, *Casuarina* or *Callitris* species but also in the open during seasons of high rainfall.

Phenology

Collected in flower in September and October.

Etymology

Named for Ruth Palsson, botanist associated with the Beadle Herbarium, University of New England (NE), who collected this species several times, including the type material.

Chromosome number

Unknown.

Notes

Collections of this species have been confused with *N. simulans* but rarely with *N. megalosiphon*, with which *N. palssonae* has a partial range overlap. As far as we can determine, these never occur microsympatrically despite having generally similar habitat preferences. The species differs from *N. simulans* in leaf shape (lacking the winged petiole and often auriculate leaf base of the latter) and from *N. megalosiphon* in the smaller flowers (floral tubes 2.0–2.5 cm long *v.* 3.3–5.0 cm). The holotype is represented in the phylogenetic analysis (Fig. 4) by a secondary specimen grown from seeds collected at the same time as the type by Palsson and vouchered as *Chase & Christenhusz 20014* (K, NE, CANB). The other accessions (Fig. 3, 4) with secondary vouchers at K are *Chase & Christenhusz 18016 (Prendergast 354)*, *Chase & Christenhusz 20005 (Palsson 321)*, *Chase & Christenhusz 20010 (Palsson 410)* and *Chase & Christenhusz 20012 (Palsson 412)*.

Selected specimens examined

AUSTRALIA. New South Wales: Yandama station, ~40 m W of Milparinka, −29°41′S, 141°25′E, 1 Apr. 1910, *Collier s.n.* (NSW48817); 44 miles [~70.8 km] W of Wilcannia, −31°42′S, 142°46′E, 11 Jan. 1959, *Filson 1357* (CANB335359); 21 km north on Tilpa Road, N from Cobar-Wilcannia Road, −31°25′S, 145°53′E, 12 Aug. 1973, *Cunningham 916* (CANB 834574); 500 m inside the gate on Pooncarie Road, Kinchega National Park, −32°31′12″S, 142°10′12″E, Sep. 1983, *Fox 8309018* (NSW 575645); Nocoleche Nature Reserve, W side of Paroo River, ~15.4 km due S of Shearer's Quarters, −29°59′20″S, 144°7′9″E, 18 Jan. 2017, *Albrecht 14779* (CANB 897287); Narran Lake NR, Kurrajong Road, 142 m, −29°44′43″S, 147°33′13.2″E, 23 May 2020, *Palsson 321* (NE110374); South of Bourke, Kidman Way, ~8 km north of intersection with Wilga Downs Rd, 205 m, −30°57′14″S, 145°53′51″E, 29 Sep. 2020, *Palsson 410* (NE 110888); Gundabooka National Park, Ben Lomond Gorge, 192 m, −30°34′18″S, 145°42′47″E, 8 Sep 2020, *Palsson 412* (NE 110890). Queensland: Warrego, 15.5 km SE of Wynburn turn-off, on old Quilpie-Charleville road, 230 m, −26°52′24.9″S, 144°35′33.3″E, 22 Sep. 1990, *Prendergast 354* (MSB 87373; BRI AQ0501528, K).

Supplementary material

Supplementary material is available online.

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Data availability. New RADseq data used in this study have been submitted to GenBank.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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