MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the master's Thesis:

Phylogenomics of *Nicotiana* section *Suaveolentes* (Solanaceae), with a focus on *N. benthamiana*

verfasst von / submitted by

Aljaž Jakob

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of Master of Science (MSc)

Wien, 2021 / Vienna 2021

Studienkennzahl lt. Studienblatt/ degree program code as it appears on the student record sheet	UA 066 832
Studienrichtung lt. Studienblatt/ degree program as it appears on the student record sheet	Masterstudium Botanik
Betreut von / Supervisor	Assoz. Prof. Dipl.-Ing. Dr. Ovidiu Paun

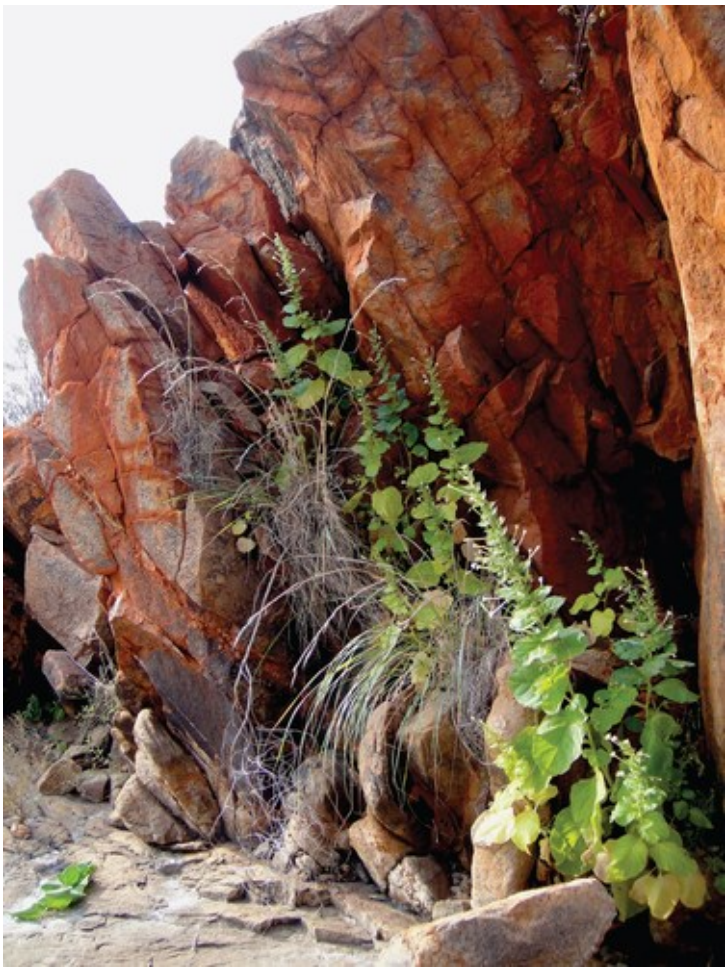

Figure 1: Breakaway southwest of Shaw River on the Mable Bar Road, Pilbara (WA) with *Nicotiana benthamiana*. Photograph: Maarten Christenhusz. Modified after (Chase & Christenhusz, 2018d).

CONTENT

ABSTRACT

Nicotiana sect. *Suaveolentes* is a paleopolyploid section, native to Australia, Africa and the South Pacific. This group is suitable for studying longer-term effects triggered by whole genome doubling. The group is around 6 mil years old, and it is expected to have had an ancestral chromosome number *n* = 24. As a result of chromosomal rearrangements and loss of DNA, at present the species of the group have a series of chromosome reductions with between 24 and 15 chromosome pairs. The species in this section show also variable ecologies, including some with adaptations to harsh, extreme habitats in the central Australian deserts. The group contains *Nicotiana benthamiana*, a useful and important model organism much used in plant virology and biotechnology. *Nicotiana benthamiana* has a wide geographical distribution, and morphological and immunological diversity. The natural origin of the lab line is near the Granites Gold Mine in the Northern Territory, Australia. In this study, phylogenetic relationships were investigated for 260 accessions of 47 described and undescribed taxa, and additionally population structure and biogeography of *N. benthamiana* were explored using restriction site associated DNA sequencing (RADseq) data, making use of an available reference genome of *N. benthamiana*. The resulting phylogenetic tree is robust and well supported, providing a good framework for future study of post-allopolyploidization chromosomal evolution and adaptation to extreme habitats. *Nicotiana benthamiana* was found to have four geographical subpopulations. The laboratory strain was found to be closely related to the two accessions from Judbarra/Gregory National Park in the Northern Territory.

ZUSAMMENFASSUNG

Nicotiana sect. *Suaveolentes* stellt eine palaeopolyploide Sektion dar, welche in Australien, Afrika und in der südlichen Pazifikregio heimisch ist. Diese Gruppe eignet sich für die Erforschung langfristiger, durch Genomduplikation ausgelöster, Effekte. Das Alter dieser Sektion wird auf etwa sechs Millionen Jahre geschätzt, die anzestrale Chromosomenanzahl beträgt *n* = 24. Als Folge von Chromosomenumlagerungen und DNA-Verlust hat sich die Chromosomenanzahl der Arten dieser Sektion auf 24 bis 15 Chromosomenpaare in mehreren Chromosomenreduktionsereignissen reduziert. Die Arten dieser Sektion variieren in ihren ökologischen Präferenzen, manche haben auch spezielle Anpassungen an die extremen Lebensräume der zentralaustralischen Wüsten entwickelt. Eine Art dieser Sektion, *N. benthamiana*, gilt als wichtiger Modellorganismus in pflanzenvirologischen und biotechnologischen Forschungsfeldern. *Nicotiana benthamiana* zeigt sowohl eine weite geographische Verbreitung als auch eine hohe morphologische und immunologische Diversität. Der natürliche Ursprung des Labor Stamms liegt nahe der Granites Goldmine in Northern Territory, Australien. Die vorliegende Studie untersucht phylogenetische Beziehungen zwischen 260 Proben von 47 beschriebenen sowie unbeschriebenen Taxa und behandelt zusätzlich die Populationsstruktur sowie Biogeographie von *N. benthamiana* basierend auf Restriktionsstellen-assoziierter DNA-Sequenzierung (RADseq) mithilfe des verfügbaren Referenzgenoms von *N. benthamiana*. Der daraus resultierende Stammbaum zeigt sich robust und gut statistisch unterstützt. Demnach stellt er eine gute Grundlage für zukünftige Studien der Chromosomenevolution nach einer Allopolyploidisierung in Zusammenhang mit extremen Umweltbedingungen dar. Weiters wurde festgestellt, dass *N. benthamiana* in vier geographische Subpopulationen untergliedert werden kann und, dass der Labor Stamm eine enge Verwandtschaftsbeziehung zu zwei Proben aus dem Judbarra/Gregory Nationalpark in Northern Territory aufweist.

ACKNOWLEDGEMENTS

Prof. Mark W Chase of the Royal Botanical Gardens, Kew, UK is gratefully acknowledged for the excellent introduction to the taxonomy of this group as well as for his support and valuable discussion on the evolution of this section of the genus *Nicotiana*, and guidance, hospitality and giving me the opportunity to observe living plants in the Royal Botanic Gardens, Kew, UK, as well as the valuable herbarium specimens. Plant material needed for the RAD library were collected by Prof. Chase together with his Australian collaborators.

Assoc. Prof. Ovidiu Paun is gratefully acknowledged for support, discussions and guidance in the methodology of plant phylogenomics and many other topics. Prof. Rosabelle Samuel is gratefully acknowledged for valuable discussions and encouragement. Juliana Baar, Gabriel Vignolle and Daniela Paun are acknowledged for their technical support while preparing the RAD libraries. Anna-Sophie Hawranek is gratefully acknowledged for help with the German translation of the thesis abstract.

Financial support to prepare the RAD libraries funded by research grants P 26548-B22, P33028-B of the Austrian Science Fund (FWF) awarded to Prof. Rosabelle Samuel. Finally, prize (Dr. Anton Oelzelt-Newin Foundation) from the ÖAW (Austrian Academy of Sciences) to Prof. Rosabelle Samuel which financially supported this Master thesis work. The author also acknowledges the Public Scholarship, Development, Disabled and Subsidy fund of the Republic of Slovenia (https://www.srips-rs.si/), which granted the Ad Futura scholarship to the author for his master studies.

1. INTRODUCTION

1.1 Introduction to phylogenomics

The goal of phylogenetics and phylogenomics is the reconstruction of an accurate tree, representing the evolutionary relationships within a given group. Phylogenetic methods traditionally rely on few markers to provide data for the reconstruction of phylogeny. The markers had to be carefully chosen with respect to the rates of change occurring within them. Certain markers, such as plastid *rbcL* and nuclear ribosomal DNA (Savolainen & Chase, 2003), are known to be highly conserved and are especially suited for study of distantly related organisms. However, such genes do not provide enough information for closely related organisms. For these, more rapidly evolving regions are necessary, such as the internal transcribed spacer (ITS) and plastid *ndhF* and *matK* (Baldwin *et al.*, 1995; Sang, 2002). For closely related species and at the population level, the information contained in single genes is often not sufficient to reconstruct phylogenetic relationships in a satisfactory manner (Sang, 2002). In such cases, multiple genes can be used to magnify resolution. However, it often happens that gene trees, that is, trees constructed from a single gene, do not produce congruent results. Several evolutionary processes can cause this often-observed incongruence between gene trees (Degnan & Rosenberg, 2006, 2009; Mallet *et al.*, 2016):

i) instances of speciation involving some level of hybridisation: species of hybrid origin, introgression between closely related species and ancient gene flow (or past introgression).

ii) incomplete lineage sorting (ILS), where two alleles or more are present in an ancestor and this polymorphism is retained past speciation events and then becomes sorted in descendants in a pattern inconsistent with their phylogenetic relationships.

iii) lateral gene transfer, which is rare in higher organisms.

The answer to the problem of gene-tree incongruences are methods, accounting for ILS (multispecies coalescent model: Degnan & Rosenberg, 2009), for introgression (phylogenetic networks: Wu, 2010), for both (Yu *et al.*, 2013) or the use of a large amount of data, in essence concatenating data from a large portion of the genome that hopefully represents the true phylogenetic signal and decreases the stochasticity in the analysis. However, the results from

analyses of these large datasets, although well supported, may not always uncover the true species tree (Smith *et al.*, 2020).

1.2 Genus *Nicotiana*

Nicotiana L. (tobacco) is a genus of the nightshade family (Solanaceae). Solanaceae is an economically important family, containing many food crops, as well as pharmacological and ornamental plants. Certain species of the genus *Nicotiana* are widely used for their nicotine content, most notably *Nicotiana tabacum* L., whereas others are used as ornamentals (*N. sylvestris, N. alata, Nicotiana × sanderae*).

The closest relatives of *Nicotiana* are the species in the tribe Anthocercideae, a small group endemic to Australia and New Caledonia (Garcia & Olmstead, 2003; Särkinen *et al.*, 2013). These two groups constitute the subfamily Nicotianoideae, which is the sister clade to subfamily Solanoideae. Both subfamilies have the chromosome base number x = 12 (Särkinen *et al.*, 2013). These two subfamilies split from each other around 24 Ma, and the split between *Symonanthus,* sister to all other Anthocercideae, and *Nicotiana* is dated to 15 Ma (Särkinen *et al.*, 2013; Clarkson *et al.*, 2017). Humans used *Nicotiana* for psychoactive purposes at least twice, once in the Americas and once in Australia (Symon, 2005; Ratsch *et al.*, 2010). Both in America and Australia, several species of *Nicotiana* have been used, with varying levels of nicotine concentration and content of other psychoactive and toxic alkaloids, like nornicotine. In Australia, the related *Duboisia hopwoodii* was also used in certain areas, when the usual *Nicotiana* species were scarce. Although the use of native tobaccos has dwindled in Australia, a few species with exceptionally high nicotine content are still used, in particular by the indigenous population (Ratsch *et al.*, 2010)

1.3 Section *Suaveolentes*

Nicotiana is divided into eight diploid sections; *Sylvestres, Alatae, Noctiflorae, Petunioides, Undulatae, Paniculatae, Trigonophyllae* and *Tomentosae* and five allopolyploid sections; *Suaveolentes, Repanadae, Nicotiana, Rusticae* and *Polydicliae* (Knapp *et al.*, 2004). Sect. *Suaveolentes* is the most species-rich section in the genus, as recent research as well as this study indicate that around a half of all *Nicotiana* species may belong to this section (Chase *et al.*, 2018a). All sections except for *Suaveolentes* are endemic to the Americas. The diploid sections hold only a small proportion of species diversity (Knapp *et al.*, 2004).

In contrast, section *Suaveolentes* has a wide distribution, and although its origin is presumed to have been in South America, it spread outside of this ancestral range and is currently found predominantly in the arid and semi-arid regions of Australia, with additional representatives in the South Pacific and one in Africa. Unexpectedly, it is absent from the Americas.

Section *Suaveolentes* was first hypothesized to be of hybrid origin by Goodspeed (Goodspeed, 1945, 1947), who also proposed multiple origins of the section to explain the wide chromosome number diversity observed. However, newer studies indicated a single origin of the section (e.g., Kovarik *et al.*, 2008; Kelly *et al.*, 2013). The pollen parent of their common ancestor was a member or ancestor of section *Sylvestres*. The identification of the maternal progenitor has proven difficult. It is likely that the maternal parent was from either sect. *Noctiflorae* or *Petunioides* (Kelly *et al.*, 2013). According to Clarkson *et al.*, (2017), sect. *Noctiflorae*, *Alatae* and *Petunioides* might have diverged at similar times or only slightly before the section *Suaveolentes* had formed. This can explain the difficulties in ascertaining the maternal parent, as it could have been poorly diverged from related species giving rise to the other sections.

Nicotiana sylvestris has 12 pairs of chromosomes, as do members of sections *Noctiflorae* and *Petunioides* (Knapp et al., 2004), the most likely paternal sections to *Suaveolentes*. This would predict the basal chromosome number in the section *Suaveolentes* to be 24, a number found in some of the species in the section: *Nicotiana forsteri*, *N. monoschizocarpa* and *N. heterantha* (Marks *et al.*, 2011a). Most species in the section have undergone chromosome number reductions, taxa included in this study have chromosome numbers n = 24-15 (Williams, 1975; Marks *et al.*, 2011a; Dodsworth, 2015). *Nicotiana wuttkei*, not included in this study, was reported to have an even lower chromosome number n = 14 (Clarkson & Symon, 1991).

In addition to chromosome number diversity between the species, there is some evidence that certain species, including *N. benthamiana*, contain several cytotypes. The existence of neopolyploids was also reported (Marks *et al.*, 2011a).

The phylogenetic relationships among species in sect. *Suaveolentes* are poorly understood. There have been several molecular phylogenetic studies including species of this section since the year 2000 (Aoki & Ito, 2000; Chase *et al.*, 2003; Clarkson *et al.*, 2004, 2010, 2017; Marks *et al.*, 2011b), but these included a limited sampling of sect. *Suaveolentes* species, and the respective datasets did not have enough phylogenetic signal to yield well supported trees, resulting in uncertain and often conflicting trees.

The species of *Nicotiana* sect. *Suaveolentes* can produce both clesitogamous and chasmogamous flowers, depending on the available resources and the life stage of the plant (Burbidge, 1960; Horton, 1981). *Nicotiana heterantha* is known to regularly produce both types of flowers within the same inflorescence (Symon & Kenneally, 1994). Other species, most notably those that grow in exposed, harsher habitats (e.g., *N. simulans* and its close relatives) produce a few cleistogamous flowers and stay small during dry seasons or at sub-optimal sites but can produce normal flowers and grow much bigger during wetter seasons and at favourable sites (Chase & Christenhusz, 2018a,b). Cleistogamous flowers are always self-pollinating and do not require a pollinator, which can be an advantage in harsh habitats that lack pollinators, such as deserts. Normally, chasmogamous flowers are also capable of self-pollination, but the frequency of selfing varies greatly between species of the section (Chase & Christenhusz, 2018c; Chase *et al.*, 2018b,c). All species of sections *Suaveolentes* have palely coloured flowers, with most species being white, with greenish or pinkish markings on the outside of the floral tube. Certain species are light green or cream (*N. africana*, *N. hesperis*). The chasmogamous flower size in terms of corolla tube length range from less than 20 mm in *Nicotiana amplexicaulis* (Burbidge, 1960), up to several centimetres in *N. benthamiana*, *N. burbidgeae* and *N. excelsior* (Chase & Christenhusz, 2018d,e; Chase *et al.*, 2018c). The stamens and pistil are at the entrance to the tube or hidden in the tube in all species except *N. africana*, where they are exerted from the tube. The flowers are reported to present a sweet fragrance, which can be strong, emitted in the evening and early night. The colour, shape, fragrance and the timing of fragrance of the species included in this research suggest these flowers are pollinated by different species of night-flying hawkmoths (Raguso *et al.*, 2003, 2006; Adler *et al.*, 2012). However, no pollinator has yet been directly observed to visit the flowers (Marks *et al.*, 2011b). The most divergent species of the section, *N. africana*, is pollinated by sun birds. It has greenish flowers with exerted stamens and pistil and a wide floral tube (Marlin *et al.*, 2016).

1.4 *Nicotiana benthamiana*

Nicotiana benthamiana Domin (Figure 1) is an important model organism for plant virology and immunology, as the particular strain used for research possesses a loss of function mutation in RNA dependent RNA polymerase 1 (rdr1), which causes the plants to be highly susceptible to viruses. This characteristic made the strain attractive for multiple fields of study, such as research of cell biology, genetics, plant virology, biotechnology and pharming (Goodin *et al.*,

2008; Bally *et al.*, 2018; Chase & Christenhusz, 2018d; Derevnina *et al.*, 2019). This species was discovered in 1839 by Benjamin Bynoe, during the third voyage of HMS Beagle, famous for carrying Darwin on his formative voyage between 1831 and 1836. The type specimen was collected on the northwest coast of Australia, possibly in the vicinity of the Victoria river, but it was recognised and named as a species only 90 years later by Karel Domin. The type specimen is currently in the herbarium of the Royal Botanical Gardens, Kew. The specific epithet honours George Bentham, an English botanist and author of the first Australian flora (Goodin *et al.*, 2008).

Nicotiana benthamiana has a wide tropical distribution from the northern half of West Australia, Northern Territory and western Queensland (Figure 2, The Australasian Virtual Herbarium, Australian bureau of meteorology, http://www.bom.gov.au/jsp/ncc/climate_averages/climate-classifications/index.jsp?maptype=kpn#maps accessed 17.10.2020) and it has a characteristic habitat preference; it exclusively grows in highly protected sites on the south side of rocky outcrops, where it receives more moisture and is protected from strong sunlight (Chase & Christenhusz, 2018d).

The species appears to have highest population density in the westernmost part of its range in the Pilabra region with further increases in density along the border between Northern Territory and West Australia, and between Northern Territory and Queensland. Between those areas, the species was sampled less frequently, and the localities are further apart. This may represent real gaps in distribution, as is likely the case in central parts of Northern Territory, where the suitable habitat is rare or even absent (Mark Chase; personal communication 2020), or it may be that the gaps are artificial due to the lack of sampling effort in certain areas.

Chromosome numbers in *N. benthamiana* are n = 18 and 19. The strain used as a model organism has 19 chromosomes, but the first number reported was 18 (Kostoff D., 1943). Unfortunately, the provenance of the examined specimen in the first publication is not reported. Later, this number was revised from 18 to 19 (Goodspeed, 1945; Wheeler, 1945). It has now been revealed both cytotypes are present in natural populations (Chase et al., submitted), and there is a possibility that there may be a geographical aspect to this variation. The existence of several cytotypes also poses a question about how much, if any, gene flow exists between the populations with different chromosome numbers.

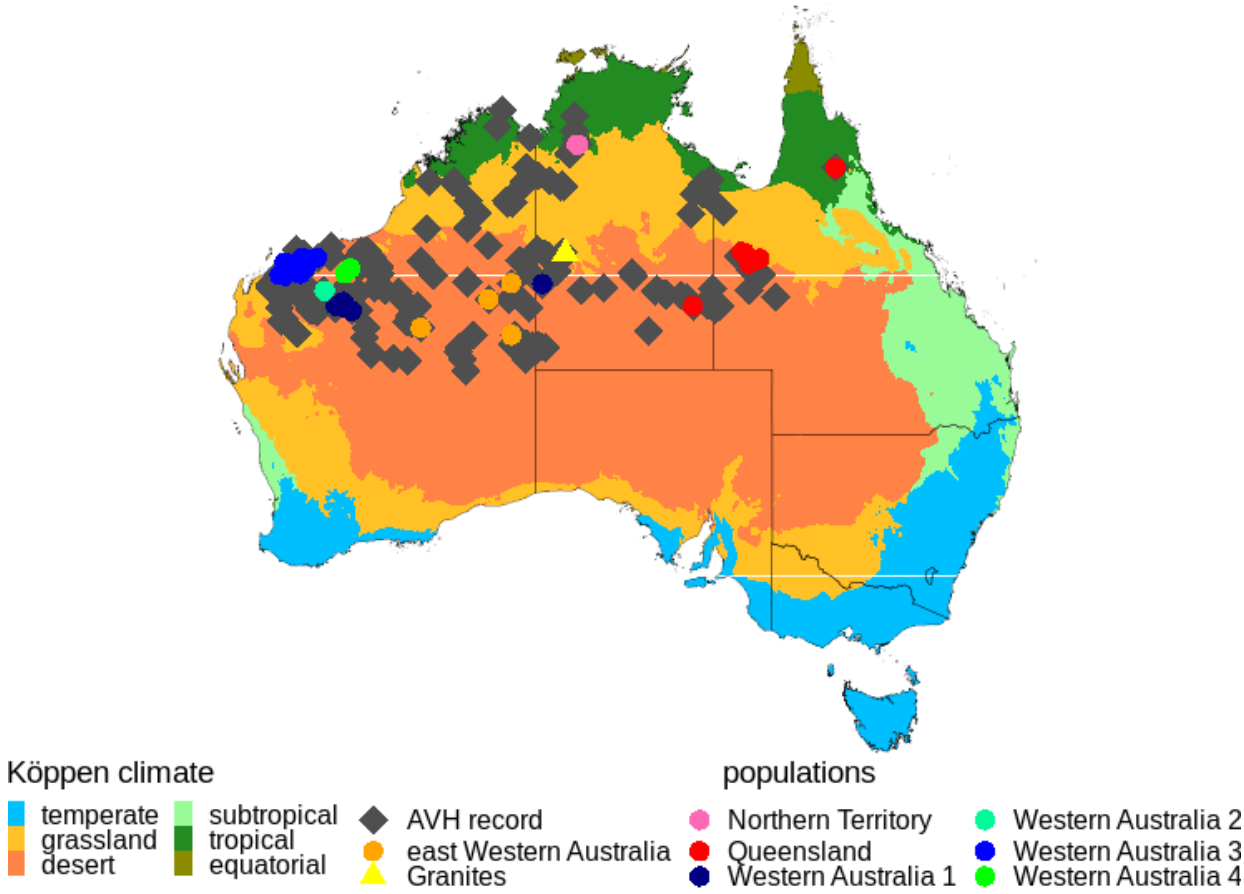

Figure 2: Distribution points of *Nicotiana benthamiana* on a map of climatic zones. Locations from the virtual herbarium of Australia (https://avh.chah.org.au/). Climate data from Australian bureau of meteorology (http://www.bom.gov.au/).

Despite the importance of *N. benthamiana* as a model organism, the geographical provenance of the laboratory strain was speculative for a long time. The material of the laboratory strain, known as LAB (Bally et al., 2015), is now known to have originated from a site in the vicinity of the Granites Gold Mine in the Tamani Desert, central western Northern Territory (Goodin *et al.*, 2008; Bally *et al.*, 2018), which was sent to Goodspeed and Wheeler at the University of California (Berkeley) to study (Goodspeed, 1954). How it spread from Goodspeed's laboratory is unknown, but in his monograph (1954) Goodspeed wrote that he received this material from Prof. J. Clelend in 1939 at the Waite Institute in Adelaide (South Australia).

1.5 Polyploidy in plants

Whole genome duplication (WGD) played an important role in plant evolution and speciation and shaped the structure and function of modern plant genomes. There were several ancient

WGD events in the evolutionary history of vascular plants, as well as ongoing and recent polyploidisation-related speciation events (Wood *et al.*, 2009; Jiao *et al.*, 2011, 2012). These events contributed to the evolution of novel adaptation and diversification of plants. Additionally, polyploidy could have had an impact on survival during times of rapid and severe environmental changes, such as the K-T extinction event 65 million years ago (Soltis & Burleigh, 2009). This can have implications for the evolution of modern plants in the face of present threats, such as quickly changing climatic conditions due to human influence (Fawcett *et al.*, 2009; Soltis & Burleigh, 2009).

Nicotiana is a good model system to study effects of polyploidisation. Due to its economic importance, there is a wide range of available resources and the age of allopolyploids ranges from the contemporary man-made hybrids to the six million years old section *Suaveolentes*, making this genus a suitable model for studying effects of polyploidy over a wide range of time scales (Soltis *et al.*, 2016).

1.6. Usefulness of restriction-site associated DNA sequencing (RADseq) for species delimitation and phylogeny

RADseq is a useful platform for de novo detection of many homologous single-nucleotide polymorphisms (SNPs, Baird *et al.*, 2008). It is a method for reducing the representation of the genome and relies on restriction enzymes that cut the DNA around a particular sequence that they recognize. The DNA can be digested with one enzyme (single-digest RADseq), or two different enzymes (double-digest RADseq). The second method avoids random shearing by reducing the fragment size via digestion and gives a more precise and repeatable means of size selection (Peterson *et al.*, 2012).

The resulting fragments can be sequenced either from one end, or both ends, resulting in single-read or paired-end data. Paired-end, single digest libraries result in the greatest amount of data. The random shearing employed in the double-digest RADseq ensures that different fragments from the same locus have a different length. Multiple fragments can be recognized during processing using the first read at the digestion site as part of the same locus, and their pair reads can be assembled to a larger locus up to 800-1,000 bp long (Andrews *et al.*, 2016). The overall amount of data is related to the number of loci obtained, which in turn are dependent on the length of the recognition sequences of the restriction enzyme used (Davey & Blaxter, 2010).

With RADseq, it is possible to cost-efficiently sequence a significant portion of the genome, resulting in many thousands of SNPs (Andrews *et al.*, 2016).

The species of section *Suaveolentes* are closely related, and their relationships proved to be difficult to discern due to low divergence, resulting in inconsistent patterns across several studies using different markers (Aoki & Ito, 2000; Marks *et al.*, 2011b; Dodsworth, 2015; Clarkson *et al.*, 2017; Bally *et al.*, 2018). RADseq has proven to be a good method of acquiring large quantities of genomic data for population genetics, phylogenomics and biogeography. For example, in the European orchid genus *Dactylorhiza*, RADseq data was used to successfully infer evolutionary relationships of diploid species and the origin of alloploids in the genus (Brandrud *et al.*, 2020). Similarly, it was used for inferring the phylogenetic relationships within the tribe Shoreae of the family Dipterocarpaceae, providing the relationships of the genera and grounds for possible taxonomic changes, as well as evidence about floral evolution in the group (Heckenhauer *et al.*, 2018).

In other cases, RADseq has been used to infer phylogenetic relationships in the context of explosive adaptive radiations, for example for New Caledonian members of the genus *Diospyros* (Ebenaceae), enabling further studies of the drivers of adaptive radiation and adaptation to the diverse habitats of New Caledonia, in particular to volcanic and ultramafic soils (Paun *et al.*, 2016). The method also proved useful in species and population delimitations in the north American species group of the genus *Nyssa* (Nyssaceae, Zhou *et al.*, 2018), resolving the biogeography of the Ericaceae genus *Cassiope* (Hou *et al.*, 2016), and in the study of introgression in *Rhinanthus*, a genus in Orobanchaceae (Eaton & Ree, 2013).

Here I am exemplifying the usage of RADseq to robustly infer the phylogenetic relationships between the species of *Nicotiana* section *Suaveolentes* and investigate in more detail the population structure and the biogeography of *N. benthamiana*, making use of a publicly available reference genome as means to guarantee homology of the sequenced fragements.

2. MATERIAL AND METHODS

The sampling included 260 accessions, representing 46 putative species. Leaf tissue, vouchers and seeds have been collected by Prof. Mark Chase from Royal Botanic Gardens Kew, together

with other colleagues, during field visits to Australia. The accessions and associated information are provided in Annex 1.

2.1 DNA extraction and sequencing

Total DNA was extracted from silica-dried leaf tissue, using a cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990), with pre-treatment of the tissue with ice-cold sorbitol buffer. The plant material was silica gel dried upon collection and later stored at room temperature. Approximately 20 mg of dry material was transferred to 2 ml safe-lock tubes with five glass beads. The TissueLyser adapters were frozen for at least 30 min prior to use at -80 °C. The tubes were placed in the pre-cooled adapters, and the material was ground two to four times for 30 s at 30 cycles/min in a TissueLyser II (Qiagen, Hilden, Germany).

The tubes with ground material were filled with ice cold sorbitol buffer (100 mM tris-HCl, 5 mM EDTA, 0.35 M sorbitol, pH 8.0) with 0.4 % β-mercaptoethanol, mixed well by vortexing and incubated on ice for 20 min. The material was centrifuged for 10 min at 4 °C and 10,000 rpm.

The supernatant was removed, and the pellets were resuspended in 750 µl pre-heated 2x CTAB buffer (100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2 % CTAB, pH 8.0) at 65 °C and mixed by vortexing. The tubes were incubated for 20 min at 65 °C. Then the tubes were placed at room temperature and 750 µl of ice-cold SEVAG (1:24 isoamyl alcohol : chloroform) were added. The tubes were mixed by vortexing and placed on a shaker at 900 rpm and incubated for 60 min at room temperature. The tubes were then centrifuged for 10 min at room temperature and 10,000 rpm. The aqueous phase was transferred into new 2 ml tubes. The DNA was precipitated by adding two volumes of ice cold 99 % ethanol. The tubes were incubated overnight at -20 °C. The precipitated DNA was pelleted in a pre-chilled centrifuge for 20 min at 4 °C and 10,000 rpm. The supernatant was discarded, and the pellet was washed with 500 µl of ice-cold 70 % ethanol and then centrifuged for 10 min at 4 °C and 10,000 rpm. The supernatant was discarded, and the pellet was air dried. After drying, the DNA was eluted in 100 µl of elution buffer.

The DNA extracts were treated with 4 µl of RNAse A (Thermo Fischer, USA) for 30 min at 37 °C. The DNA was cleaned with NucleoSpin gDNA clean-up Kit (Machery-Nagel, Germany), according to the manufacturer's manual. The DNA was quantified with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer’s protocol.

Single-digest paired-end RAD-seq libraries were prepared according to a protocol established in previous studies (Paun *et al.*, 2016; Heckenhauer *et al.*, 2018; Brandrud *et al.*, 2020). The DNA was digested with enzyme PstI and processed in batches of 60 individuals per library. The inline and index barcodes were different from each other by at least three nucleotide positions. The libraries have been sequenced at the VBCF NGS Unit (www.vbcf.ac.at) on an Illumina Hiseq 2500 as pair-end reads of 125 bp.

2.2 Identification of RAD loci and SNP calling

The raw Illumina sequences were demultiplexed with the BamIndexDecoder v. 1.03 of Illumina2bam package (accessible at: http://gq1.github.io/illumina2bam/) according to index barcodes. The sequences were then further demultiplexed according to inline barcodes and quality-filtered (i.e., reads with uncalled bases and low-quality scores were removed, but barcodes and cut sites with maximum one mismatch were rescued) with process_radtags in Stacks 1.47 (Catchen et al., 2011).

The sequences were aligned to the reference genome of *Nicotiana benthamiana* v1.0.1 (Bombarely *et al.*, 2012, genome available at: https://solgenomics.net/organism/*Nicotiana _benthamiana*/genome) with bwa version 0.7.17-r1188 algorithm option mem (Li, 2013).

The individual mapping rates were investigated to check for mapping bias, due to differing phylogenetic distances to the reference. Option M was applied to flag shorter split hits as secondary. As *Nicotiana benthamiana* is a member of the investigated section and the parents that hybridized to form this paleopolyploid section were distantly related to each other, our approach is expected to map the homoeologous reads to their own subgenome and avoids paralogy issues.

The aligned .sam files were further sorted by reference coordinates with the SortSam mode in Picard 2.19 (available from http://broadinstitute.github.io/picard/). The read groups were added with AddOrReplaceReadGroups in Picard. The sequences were indexed with samtools 1.7 (available at: http://www.htslib.org/doc/samtools.html) prior to indel realignment. Indel realignment was performed in the Life Science Compute Cluster (LISC) of the University of Vienna with the Genome Analysis Toolkit v. 3.8 (GATK; McKenna *et al.*, 2010), thinning the data to a maximum of 100,000 reads per interval. The realigned mapped files were further processed

in Stacks 1.47. Stacks built a catalogue of loci and called SNs with the ref_map.pl pipeline with default settings.

Several whitelists of loci were exported from the catalogue with export_sql.pl and populations. All whitelists asked for retaining loci with data at least for half of the individuals and maximum observed heterozygosity 0.65 at variable positions to avoid further use of any pooled paralogs. The whitelists differed in the maximum number of permitted SNPs per locus from 5 to 40. The whitelists were provided to the tool populations in Stacks package, which output SNPs to vcf files, which were used in further analyses.

The raw vcf file was filtered in vcftools 0.1.17 (Danecek *et al.*, 2011). The filtering was done for minor allele frequency and missingness. The minor allele frequency was set for SNPs to be present in at least two individuals (MAF > 0.06). The data were filtered for missingness multiple times, in steps of 5% from 0 to 30%, the optimum was determined later to maximize the average bootstrap support. Filtered .vcf files were transformed in Phylip format with PGDspider 2.1.1.5 (Lischer & Excoffier, 2012) and cleaned of invariant sites by the script ascbias master (available at: https://github.com/btmartin721/raxml_ascbias).

A SNP dataset of putatively unlinked SNPs was produced from the STACKS catalogue, using export_sql.pl and populations. A whitelist of loci containing at most 25 SNPs was made using export_sql.pl, and then a vcf file of SNPs was made using only a single random SNP from each locus. These SNPs were assumed to be unlinked. The vcf dataset was further filtered to keep only data for individuals of *Nicotiana benthamiana* and *N. karijini*, minor allele frequency 0.055 and 2 % missingness.

2.3 Maximum likelihood tree

Maximum likelihood trees were calculated with software RAxML v. 8.2.11 (Stamatakis, 2014) at LiSC. The tree calculation with RaxML used an ascertainment bias correction of likelihoods (Lewis, 2001) and a general time reversible model of nucleotide substitutions (GTRCAT), with 1,000 rapid bootstrap replicates and disabled rate heterogeneity between sites. The analysis was performed on several datasets of SNPs with varying levels of missingness. The optimum missingness was then determined by comparing the overall average bootstrap support.

The best tree was visualised and annotated in R 3.6.3, using packages: ape 5.3 (Paradis & Schliep, 2019), biostrings (Pagès *et al.*, 2020) ggplot2 (Wickham, 2016), ggtree (Yu *et al.*, 2017)

and treeio (Wang *et al.*, 2020). The tree was rooted with the accessions of *Nicotiana africana*, as it was shown to be a well-supported sister to the rest of section *Suaveolentes* in phylogenetic analyses using both plastid and nuclear data (Chase *et al.*, 2003; Clarkson *et al.*, 2004, 2010, 2017).

2.4 Co-ancestry heatmap

To detect hybridisation and introgression among species, a co-ancestry heatmap was constructed, using the software fineRADstructure 0.3 (Malinsky *et al.*, 2018). The dataset for this step was a haplotype table of loci with at most 40 SNPs per locus, exported from the Stacks catalogue with export_sql.pl. In the first step, a co-ancestry matrix was calculated using command paint, with option -p 4, which set the ploidy assumption to tetraploidy. If diploidy was assumed, the program detected too many sites with more than two alleles, presumably originating from the presence of neopolyploid individual with ID number 17012. In the second step, a burn-in of 100,000 iterations and 1,000,000 sample iterations were used to assign individuals to populations and build a tree with the command finestructure. The results were visualized in R with the scripts provided in the fineRADstructure package.

2.5 Population structure and admixture

Nicotiana benthamiana shows a degree of morphological and cytological differentiation across its wide range (Bally *et al.*, 2018; Chase & Christenhusz, 2018d). To investigate genetic differentiation, population structure was inferred with NGSadmix v.32 (Skotte *et al.*, 2013), a method implemented in ANGSD 0.931(-3-g035a72f) (Korneliussen *et al.*, 2014).

The starting point for this analysis was the realigned bam files. These were supplied for filtering in the software ANGSD with quality filtering (mapping quality and base quality above 20), and filtering for SNPs with p-value below 10^{-6}, minor allele frequency of at least two individuals (> 0.06) and data available for at least half of individuals (missingness 50%). This generated beagle genotype likelihood files, which were then used in admixture analysis with NGSadmix. The analysis was performed ten times for each K value, ranging from one to ten. The best K was chosen based on the Evanno method (Evanno *et al.*, 2005), as implemented in Clumpak 1.1 (Kopelman *et al.*, 2015).

2.6 Gene flow

To search for ancient gene flow into *Nicotiana benthamiana* from other species, ABBA-BABA tests as implemented in the ANGSD package (Soraggi *et al.*, 2018) were conducted. The multipop model of calculation of ABBA-BABA D-statistic was used, which allows for multiple individuals of the same group to be used. *N. africana*, for which it was assumed had no introgression with any other *Nicotiana* species, was used as an outgroup. *Nicotiana. africana* is the only species of *Nicotiana* native to Africa.

Species of interest were identified previously with fineRADstructure and their overlapping geographical distribution. The species of interest are *N. amplexicaulis*, *N. forsteri*, *N. monoschizocarpa* and *N. velutina* for apparent introgression/hybridization and *N. karijini* as a distant, yet sympatric sister species.

2.7 Species delimitation

The species delimitation around *N. benthamiana* was investigated with a SNAPP 1.5.0 analysis, as implemented in the BEAST 2.6.1 package (Drummond *et al.*, 2012). These analyses tested whether the degree of within taxon differentiation is high enough to potentially grant certain populations a named rank or a separation into a new species. The analysis was performed on a dataset of 1,214 random single SNPs from loci with less than 25 SNPs. The SNAPP calculation of model marginal likelihoods and species trees was repeated 24 times per model, each run had chains of length 1,000,000 steps after a burn-in of 50 %, sampling frequency was every 1,000 steps, alpha = 0.3, and the other parameters kept as defaults.

The proposed models that were tested were constructed based on geographical data, information from maximum likelihood tree (Figure 4) and NGS admixture analysis (Figure 7). The sister species of *N. benthamiana*, *N. karijinii*, was used as the outgroup. Five models were tested:

I. M_0: the currently accepted species delimitation for *Nicotiana benthamiana*.
II. M_2: two distinct units for *N. benthamiana*: Queensland and all other accessions.
III. M_3: three distinct units for *N. benthamiana* corresponding to three geographical regions: western Western Australia, central regions (Northern Territory, eastern West Australia), and Queensland.

IV. M_4: four units for *N. benthamiana* corresponding to geographical regions: western Western Australia, eastern West Australia, Northern Territory, and Queensland.

V. M_7: a split according to seven well supported branches (BS 100) of *N. benthamiana* in the ML tree: eastern Western Australia, Northern Territory, Western Australia population 1, Western Australia population 2, Western Australia population 3, Western Australia population 4, and Queensland.

The best model was chosen through Bayes Factors. Bayes factor for each model was calculated with the formula:

$$BF = 2 \times (M_0 - M_i)$$

wherein M_0 and M_i are marginal likelihood estimates for the null hypothesis and each of the alternative models.

3. RESULTS

3.1 Phylogenetic analyses

There were on average 4 million (SD ±1.2 million) reads per accession. An average of 96.4 ± 3.8% reads could be mapped successfully to the *N. benthamiana* reference genome. The average coverage of the data was 6.5x (SD ±1.2x). Different analyses required different levels of filtering and are therefore based on different numbers of SNPs (Table 1).

Table 1: The sizes and properties of the datasets used for different analyses.

dataset	missingness	SNPs per locus	number of SNPs
maximum likelihood	15%	all	457382
population structure in ANGSD	50%	all	1020901
species delimitation	2%	1	1214

While exploring the relationship between missingness and bootstrap support, the optimum missingness was shown to be 15% with an average bootstrap percentage of 96 across the phylogenetic tree. It was apparent that datasets of no missing data and 5% missing data did not contain enough data to resolve the relationships reliably, whereas missingness of 20% and above did not improve bootstrap support.

The phylogenetic tree illustrates relationships between species of the section *Suaveolentes* anew. After *N. africana*, previously shown to be the sister species to the rest of the section (e.g., in Clarkson *et al.*, 2017), there is a grade of five basal species that are consecutively sister to the rest: *N. fatuhivensis*, *N. forsteri*, *N. monoschizocarpa*, *N. heterantha* and *N. umbratica* (Fig. 3b). Of these, all species present with more than one accession seem good species and are well supported (bootstrap support 100). However, the exact relation of *Nicotiana monoschizocarpa* is not clear, as the branch leading to the clade of *N. monoschizocarpa* and the rest of exclusively Australian species has very low support (61).

Next group to split is a large group containing two relatively distantly sister clades: the smaller *benthamiana* group, consisting of *N. benthamiana* and *N. karijini*, two distantly related sister species on long branches, and the larger *Nicotiana simulans* group. The *N. simulans* group contains a grade of three species: *N. burbidgeae*, *N. gascoynica* and *N. cavicola*, and two crown subgroups. The *N. simulans* subgroup contains three described taxa and several undescribed putative taxa. *Nicotiana simulans* and *N. megalosiphon* are sister species, and to them *N. sessilifolia* is sister, the last originally described as a subspecies of *N. megalosiphon* (Horton, 1981), but the nominal subspecies is more closely related to *N. simulans* than to *sessilifolia*. This *simulans* subgroup is sister to a group containing *N. stenocarpa* and likely at least three undescribed taxa. *Nicotiana stenocarpa* was considered a later synonym of *N. rosulata* (Burbidge, 1960; Horton, 1981), but the taxon as understood here is not particularly closely related to this species.

Relationships between different accessions of *N. benthamiana* show some structure roughly corresponding to geographical areas. The individuals from Queensland are most distant from the rest - the Queensland population forms a unique cluster outside the rest of the accessions. The two accessions from the northern Northern Territory are remarkably similar to the laboratory accession, together they form a cluster with the accessions from the Gibson Desert (eastern Western Australia). This cluster is embedded among four well supported clusters from the Pilbara region (western Western Australian), the interrelationships among which are poorly supported here. These geographical subpopulations are represented in Figure 2 as follows: Queensland localities (Q) are marked red, Northern Territory (NT) are marked pink, eastern Western Australia (eWa) are marked orange, and the four West Australian (WA) populations are in green and blue (Fig 3b). After the *N. simulans* and *N. benthamiana* clade, the

next group to split off is the *N. occidentalis* species aggregate, which currently contains three described taxa at the subspecies level (Burbidge, 1960; Horton, 1981; Chase & Christenhusz, 2018b) and several putative undescribed taxa. The next group is the *N. rosulata*/*N. rotundifolia* group (Fig. 3a). It contains *N. excelsior* and *N. truncata*, that are together sister to the clade of *N. ingulba* plus a clade containing both *N. rosulata* and *N. rotundifolia*, as well as two undescribed species. At the crown of the sections *Suaveolentes* tree are the *N. velutina* group (*N. amplexicaulis*, *N. gossei*, *N. velutina* and an undescribed species) and *N. suaveolens* group (*N. goodspeedii*, *N. yandinga*, *N. maritima*, *N. faucicola*, *N. suaveolens* and undescribed species, Fig.3a).

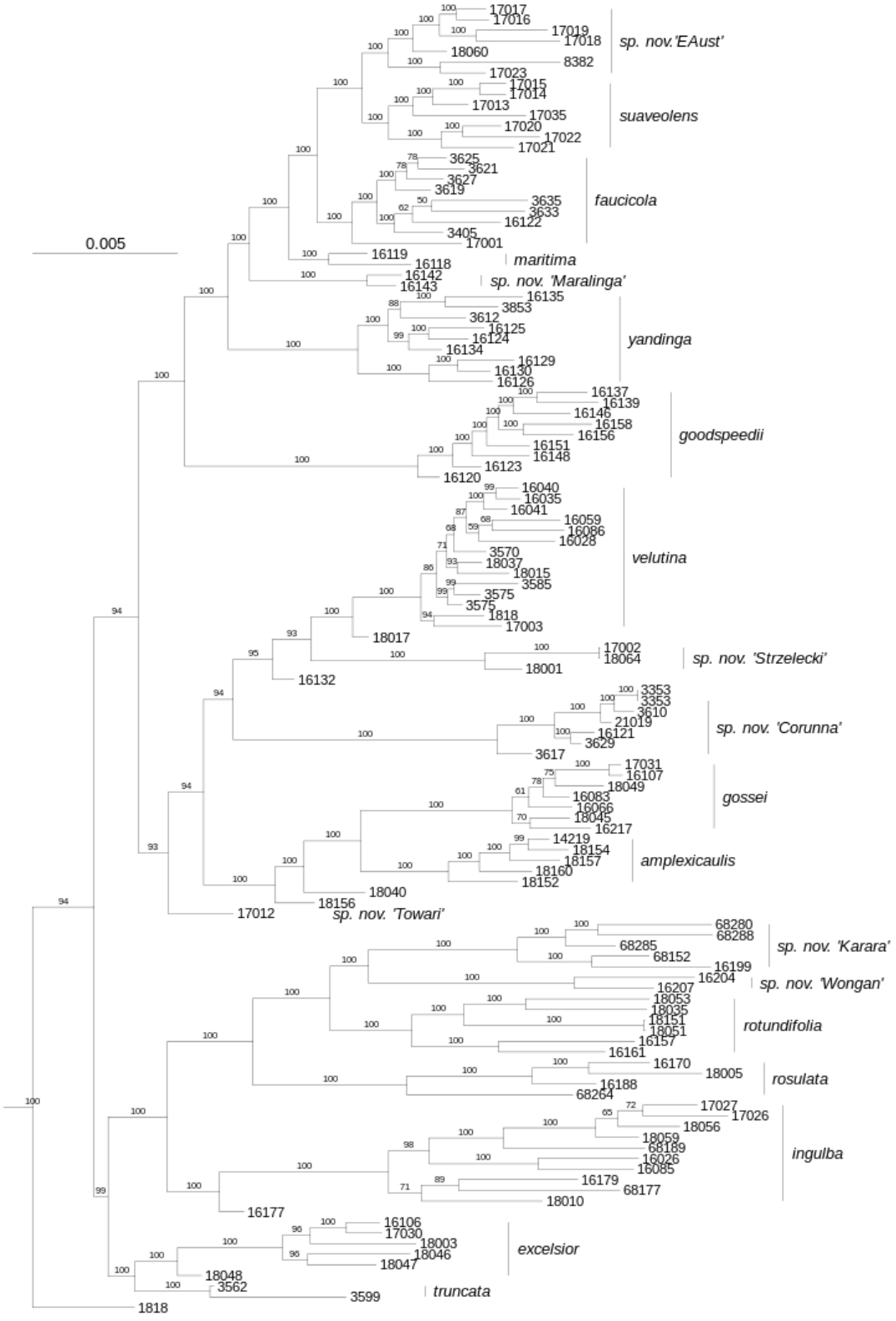

Figure 3a: Subtree A of the whole maximum likelihood tree. It contains *Nicotiana rosulata, N. velutina* and *N. suaveolens* groups.

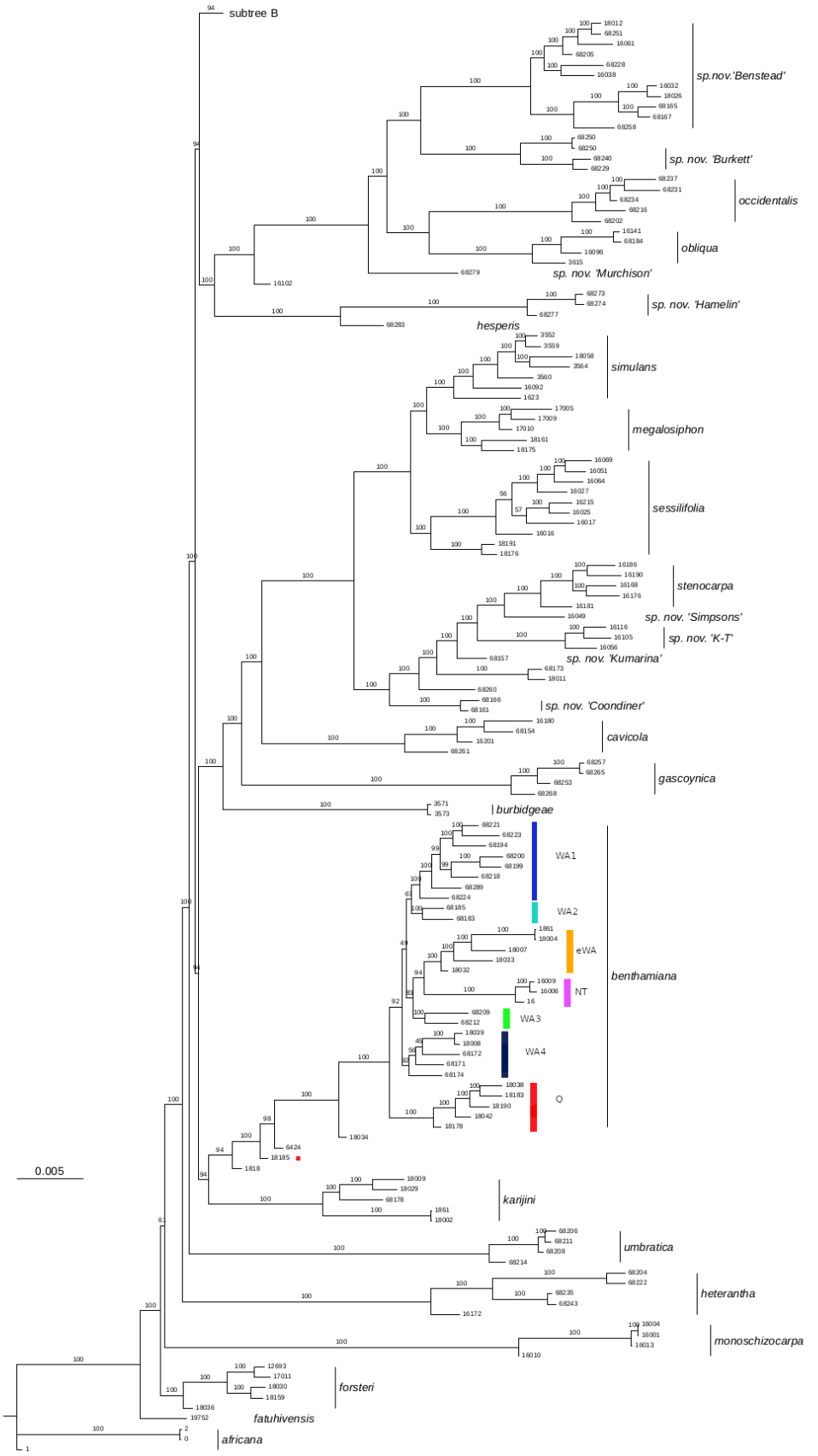

Figure 3b: The main portion of the maximum likelihood tree showing the root, the basal grade of species and *Nicotiana benthamiana, N. simulans* and *N. occidentalis* groups.

3.2 Genetic structure and patterns of gene flow in *Nicotiana* sect. *Suaveolentes* with an emphasis on *N. benthamiana*

The inbreeding coefficient F was calculated in vcftools.. This coefficient is an analogous measure to the population-estimate F_{IS}. However F is calculated per sample, and it takes into account the proportion of heterozygote positions and the amount of expected heterozygote positions. As F is calculated based on the variant sites included in the vcf file, the estimator should be regarded as a relative measure. Inbreeding coefficient F can take values between -1 (maximum heterozygosity) and 1 (maximum homozygosity). Most of the accessions included in the study have high inbreeding coefficients (between 0.5 and 1).

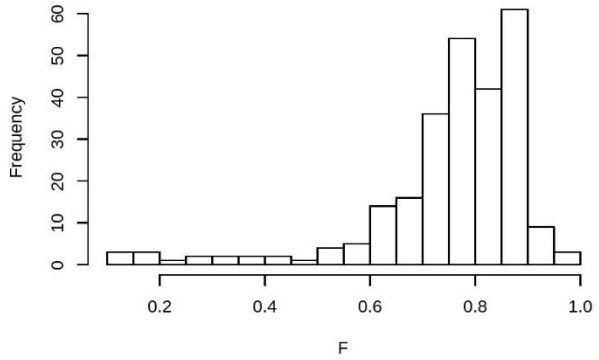

Figure 4: Histogram of the inbreeding coefficient F (vcftools) for all accessions.

The LAB strain has the highest coefficient, followed by the two Northern Territory accessions and eastern West Australian accessions. The lowest values for F are recorded for four Western Australian accessions and several accessions that have a putative hybrid origin.

3.2.1 Coancestry heatmap

Nicotiana africana, *N. fatuhivensis* and *N. forsteri* show a certain level of coancestry. *Nicotiana forsteri* exhibits lower levels of coancestry amongst its accessions, possibly explained by a larger population size or a wider distribution area. *Nicotiana forsteri* individual deb_18036 also shows a certain level of coancestry with *N. benthamiana* (Fig 5), as well as *N. benthamiana* individual showing coancestry from *N. forsteri* (at number 4 on fig. 5). *Nicotiana hesperis* and a closely related taxon (referred to as *Nicotiana sp. nov.* "Hamelin") do not cluster with the rest of *Nicotiana occidentalis* species group. In the latter group, a clear split into four separated, yet related populations are evident (Fig 5).

Table 2: The inbreeding coefficient F for the *Nicotiana benthamiana* accessions.

accession	population	F	accession	population	F
benTW16	LAB	0.95653	ben68172	West Australia 4	0.67720
ben16006	Northern Territory	0.92554	ben18190	Queensland	0.65028
ben18004	eastern West Australia	0.86416	ben68183	West Australia 2	0.63226
ben16009	Northern Territory	0.85592	ben18042	Queensland	0.59674
ben68209	West Australia 3	0.83324	ben68185	West Australia 2	0.59456
ben18007	eastern West Australia	0.83128	ben68224	West Australia 1	0.58448
ben18038	Queensland	0.78357	ben68221	West Australia 1	0.56791
ben68199	West Australia 1	0.77972	ben68171	West Australia 4	0.56757
ben68200	West Australia 1	0.77920	ben68174	West Australia 4	0.55845
ben68223	West Australia 1	0.77691	ben68218	West Australia 1	0.55475
ben18033	eastern West Australia	0.76359	ben18039	West Australia 4	0.50949
ben18183	Queensland	0.71178	ben18008	West Australia 4	0.43521
ben68212	West Australia 3	0.70434	ben18032	eastern West Australia	0.17031
ben68289	West Australia 1	0.69558	ben18178	Queensland	0.11960
ben18B61	eastern West Australia	0.67780	ben18185	Queensland	0.05812
ben68194	West Australia 1	0.67773			

The *simulans* group here lacks the basal grade. The three basal species of this group (*Nicotiana burbidgeae*, *N. gascoynica* and *N. cavicola*) do not show elevated levels of coancestry to any other species. The main *simulans* group reflects the relationships between the taxa in a similar manner to the RAxML tree, and the same holds true for the rest of the groups. It can be seen however, that coancestry between the species in the *suaveolens* group is somewhat lower than what can be seen in other groups, as well as coancestry levels between the accessions of the species is quite low (Fig 5).

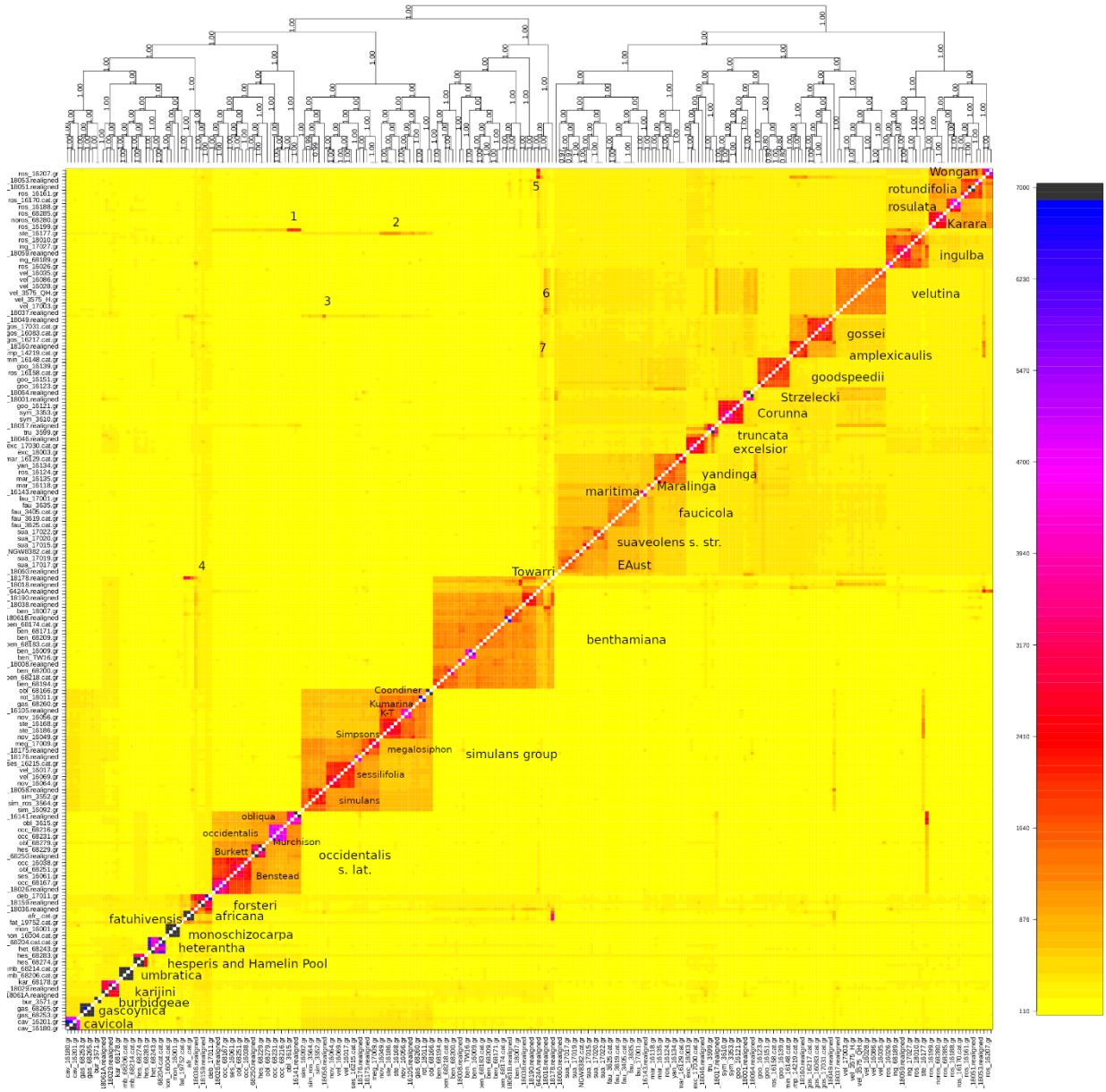

Figure 5: Coancestry heatmap, showcasing the shared ancestry between accessions. Species names are added for clarity. The numbers signify unexpected high levels of coancestry and are discussed in the text.

The parents of a possible neoallopolyploid taxon *Nicotiana sp. nov.* "Towarri" (accession sua_17012) are tentatively found to be *N. suaveolens* and *N. sp. nov.* Strzelecki. At point 1, the individual 16102 shows strong coancestry for both *N. ingulba* and *N. obliqua*, whereas at point 2, the individual 16177 shows coancestry for both *N. stenocarpa* and *N. ingulba*. The line at number 3 is of individual 18040, which has been excluded from further analyses due to very

high missingness. Number 4 is individual 18178, a possible hybrid between *Nicotiana benthamiana* and *N. forsteri*. Number 5 is accession number 6424A; showing coancestry for *N. benthamiana* and a taxon in *rosulata*/*rotundifolia* group and 6 is 18034, a mysterious accession, that here appears to have some coancestry with *N. benthamiana* and *N. velutina*, number 7 is 18185, a possible hybrid between *N. benthamiana* and *N. amplexicaulis* (Fig 5).

3.2.2 Admixture analysis

Admixture analysis contained all accessions of *Nicotiana benthamiana*. One of the accessions is a likely hybrid (ben_18185) between the Queensland population of *N. benthamiana* and *N. amplexicaulis*. In admixture analysis with both parents, it was split between the putative parents 50:50 (Fig 6).

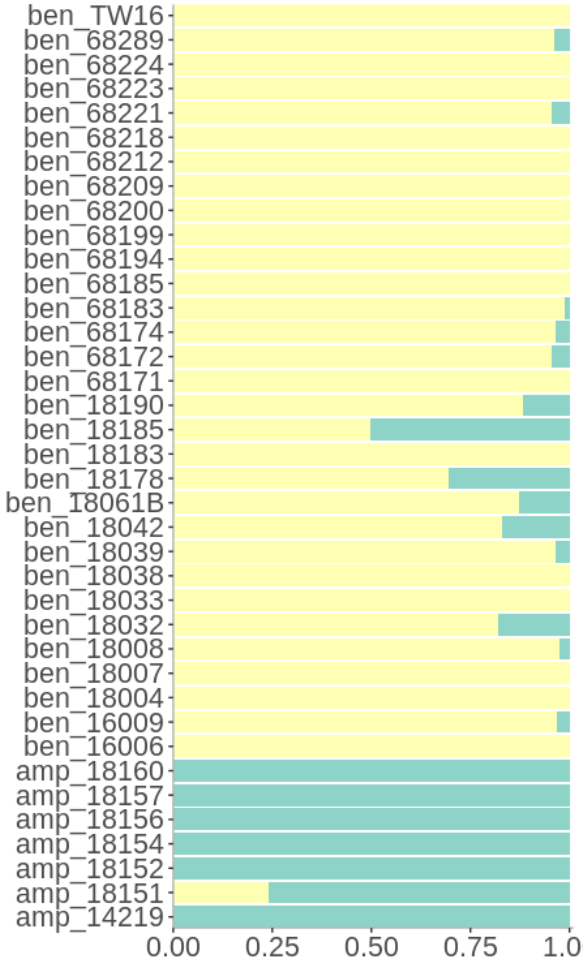

Figure 6: Admixture analysis with *Nicotiana benthamiana* and *N. amplexicaulis.* The most interesting individual is ben_18185.

In *Nicotiana benthamiana*, the best number of subpopulations is five: two from Western Australia, that do not correspond to the well supported branches in the RAxML tree (Fig 3b, green and blue coloured bars), a northern Northern Territory subpopulation including the LAB strain, Gibson Desert (eastern Western Australian) and Queensland populations. In the analysis with four populations, there is only one subpopulation in western Western Australia, and in the case of six subpopulations, the remnants of the recent hybridisation events are detected as the sixth population. The gene flow between the five populations in the best analysis seems to be relatively low. The Northern Territory population including LAB shows no signs of interaction with any other subpopulation (Fig 7).

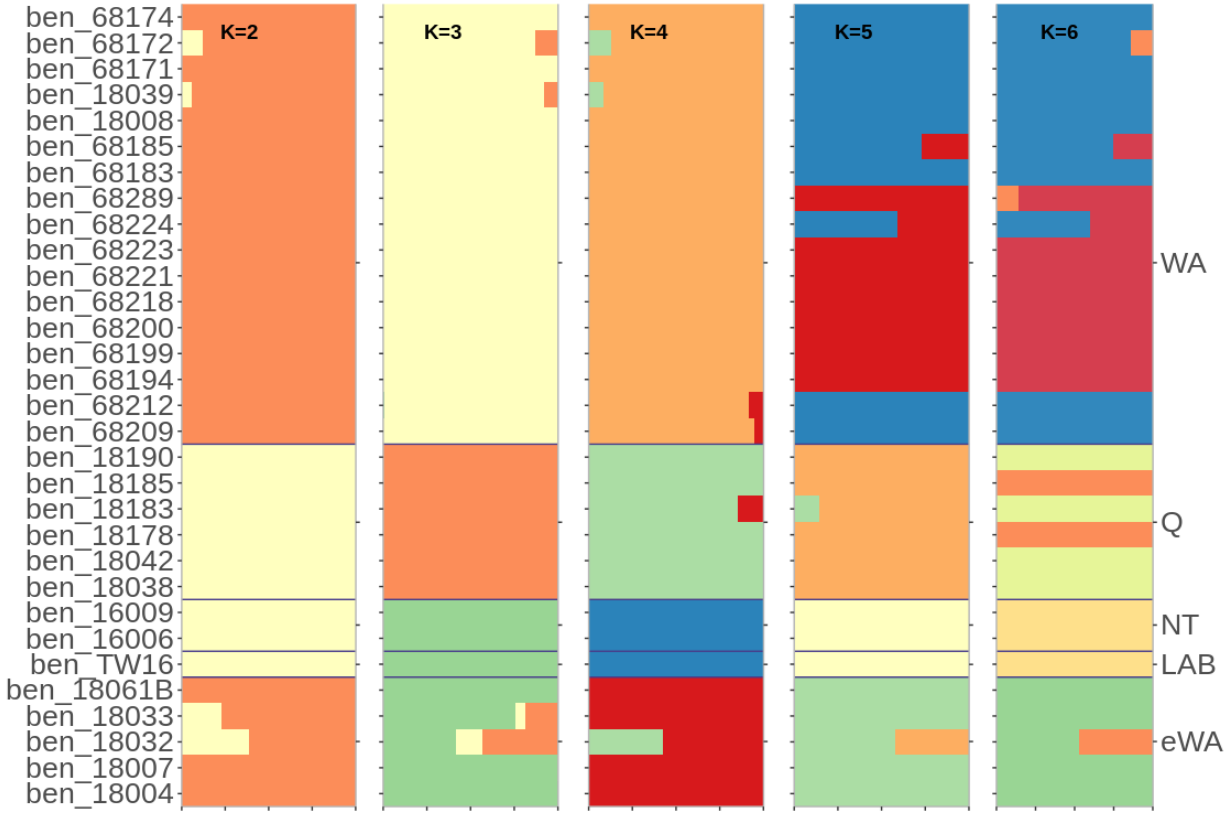

Figure 7: Detection of population structure with NGSadmix, K=2-6.

ABBA-BABA tests detected some aberrant patterns in gene flow (Table 3). While other methods strongly indicated towards gene flow between Queensland *N. benthamiana* and *N. amplexicaulis*, it was detected at very low levels. Direction of gene flow from *N. forsteri* (here designated as *debneyi*), where there appears to also be some introgression with Queensland *N. benthamiana* from other results, points here most strongly towards eWa population. Gene flow between NT population and *N. monoschizocarpa* was detected, as well as some between WA

population and *N. velutina* (Table 3). Among populations of *N. benthamiana*, Queensland is most isolated, in every pairing of populations, it is always the other population to which gene flow is directed (Table 3).

Table 3: D-statistics of ABBA-BABA tests for geneflow between *Nicotiana benthamiana* and other species and ABBA-BABA tests for geneflow between populations of *Nicotiana benthamiana.* Positive D values signify gene flow from column H3 into H2 (green), while negative show geneflow into H1 (pink). Gene flow from other species into populations of *N. benthamiana* are above the double line, below is gene flow among *N. benthamiana* populations.

D	H1	H2	H3	p-value
-0,29	NT	Q	monoschizocarpa	0
-0,20	NT	Q	debneyi	0
-0,19	NT	eWA	monoschizocarpa	0
-0,15	eWA	Q	monoschizocarpa	0
-0,12	NT	eWA	debneyi	0
-0,11	eWA	Q	debneyi	0
-0,02	Q	WA	amplexicaulis	0
0,01	eWA	WA	amplexicaulis	0
0,03	NT	eWA	amplexicaulis	0
0,03	eWA	Q	amplexicaulis	0
0,05	NT	WA	amplexicaulis	0
0,05	NT	Q	amplexicaulis	0
0,11	eWA	WA	debneyi	0
0,13	Q	WA	velutina	0
0,19	eWA	WA	monoschizocarpa	0
0,20	Q	WA	debneyi	0
0,31	Q	WA	monoschizocarpa	0
-0,30	NT	Q	eWA	0
-0,30	NT	Q	WA	0
-0,29	eWA	Q	NT	0
-0,23	eWA	Q	WA	0
0,31	Q	WA	eWA	0
0,37	Q	WA	NT	0

3.3 Is *Nicotiana benthamiana* a species complex?

Nicotiana benthamiana has been shown to be morphologically diverse (Bally et al., 2015, 2018). In this section results of species delimitation with a SNAPP analysis in the BEAST package are discussed.

The several models tested were already discussed and are presented in Table 5. Bayes factors of all the alternative models were very high with the highest support for a split of N.

benthamiana into seven distinct species. However this has been an exploratory analyses, that should be conducted with more data and accessions included, and also additional models.

Table 5: Marginal likelihoods of the models and their Bayes factors.

model	marginal L	bayes factor
M_0	-9115,5	0,0
M_2	-8777,1	-676,8
M_3	-8805,1	-620,9
M_4	-8701,1	-828,8
M_7	-8636,9	-957,3

4. DISCUSSION

4.1 A new phylogenetic tree

The results of the phylogenetic tree construction are not congruent with previous analyses, for example the ones found in Marks *et al.*, 2011b or Clarkson *et al.*, 2017. The new tree reveals the existence of several new clades, which may warrant description of new taxa or re-circumscription of current taxa. There are several accessions that do not group with the rest of the accessions of the same assigned species. Many of those, especially those that clearly group with another species in a clade are due to misidentification of material in the field due to the poor state of the specimens the material was collected from. In other cases, accessions were suspected to be hybrids. This has been further corroborated by coancestry heatmap (Figure 5), which has shown these accessions with ancestry in several clades.

4.2 Hybridisation and introgression

In certain cases, it would appear that the parents of the putative hybrids, or the sources of introgression, are not always from the same geographical area, for example accession ben_18185 is presumably a F1 hybrid (Fig. 5, 6) between *Nicotiana benthamiana* and *N. amplexicaulis* because of equal proportions of admixture from both parents (Fig. 5). *Nicotiana amplexicaulis* has a narrow distribution in eastern Queensland, with only two occurrences (one in Tablelands in North Queensland, sourced from virtual herbarium of Australia) not in or in the immediate vicinity of Central Highlands, and although *N. benthamiana* is widespread, its range extends to western Queensland and it does not occur in or east of the Great Dividing Range,

except for one locality in Tablelands in north Queensland (Australian Virtual Herbarium, https://avh.chah.org.au/). However, the putative hybrid accession does not hail from the Tablelands, and the main ranges of the two species are separated by several hundred kilometres, raising the question on how these hybrids came into existence. The pollinator of any of the species has not yet been observed, and the difference in flower size (2.5 cm – 5.5 cm in *Nicotiana benthamiana* (Chase & Christenhusz, 2018d) and less than 2 cm in *N. amplexicaulis* (Burbidge, 1960), would require a rather generalist pollinator to visit both species. A point of interest is also that Bally *et al.*, 2018 found the Queensland population of *Nicotiana benthamiana* has slightly smaller flowers than the populations of other populations, as well as the winged petiole, a character absent in other populations of *N. benthamiana*, but present in *N. amplexicaulis*. Accession ben_18185 has been grown in the greenhouse at Kew Gardens, and it is unique among the Queensland *N. benthamiana* accessions in having a slightly winged petiole and auriculate leaf bases, characters presumably derived through *N. amplexicualis*, in which the petioles are broadly winged with well-developed auriculate bases. The floral tubes were 3.2 cm long, typical in length for *N. benthamiana*, not intermediate between the two parental species.

A similar situation arises in accession ben_18178, which was assigned to *N. benthamiana*, but shows a strong coancestry with *N. forsteri* (Fig 5, point 4). The ranges of the species are not currently known to overlap, except with the already mentioned locality of *N. benthamiana* in Tablelands. During the last ice age, with the shifting of the climates it is possible that the range of the two species overlapped (Byrne *et al.*, 2008). *Nicotiana forsteri* also possesses broadly winged petioles with a well-developed auriculate base, as does accession ben_18178. The floral tube of *N. fosteri* is typically 08.–1.4, but this accession of *N. benthamiana* is 3.1 cm, again not particularly intermediate between the parental species.

The level of coancestry between accessions of certain species is much higher than in others. The species with remarkably high levels of coancestry are some of the more basal species, like *Nicotiana umbratica*, *N. monoschizocarpa*, *N. gascoynica* and *N. cavicola*. These species have small ranges and presumably low population (Horton, 1981; Chase & Christenhusz, 2018f,g; Chase *et al.*, 2018c), causing lower genetic diversity and more inbreeding.

The three species at the basalmost nodes, *N. africana*, *N. fatuhivensis*, and *N. forsteri*, appear to share some coancestry (Fig 5). It is possible that these three basal species retain a portion of

the ancestral polymorphisms that has been lost in the others. Ancestral polymorphism is a more likely explanation than hybridisation/introgression because *N. africana* and *N. fatuhivensis* are thousands of kilometers from the nearest other species.

Species with large ranges have much lower levels of coancestry compared to these species, including *N. benthamiana*, *N. suaveolens* group and *N. velutina* (Horton, 1981; Chase & Christenhusz, 2018d, Fig. 2). Whereas *N. velutina* and species in the *suaveolens* group show are relatively uniform levels of coancestry among accessions of the same species, possibly indicating a cohesive population structure and frequent outcrossing, the levels in *Nicotiana benthamiana* show some differentiation in the Queensland and Northern Territory accessions (Fig 5).

4.3 *Nicotiana benthamiana*

Nicotiana benthamiana has a very wide distribution, and there appears to be some level of cytological, morphological and other differentiation between geographically structured populations. This has been acknowledged in (Chase & Christenhusz, 2018d) and briefly explored in Bally *et al.* (2018).

Here, 31 accessions from across the range of the species have been studied (Fig. 2). The admixture analysis has shown that the best number of populations (K) is five (Fig. 6), three of which correspond to well supported groups (bootstrap 100%) in the likelihood tree (Fig. 3b). These populations are Queensland (Q, coded red), Gibson Desert (eastern Western Australia, eWa, coded orange) and Northern Territory (NT, coded pink). The other two populations are exclusively Western Australian (Fig. 2, 3b). In Figure 7, in histogram K=6, the sixth population (dark orange in K=6) is a very probable phantom population, and the method likely detected introgressed or hybrid individuals, as the three individuals; ben_18185, ben_18178 and ben_18032; with highest proportion of 6th population admixture also have the lowest inbreeding coefficients (Table3), and there is evidence for first two for hybridisation or introgression from other species. LAB accession (ben_TW16) is shown to belong to the same population as the two NT accessions (ben_16006 and ben_16009). This association has been observed in all analyses, in several different frameworks (RaxML, angsd, fineRADstructure, Figs. 3b, 7 and 5 respectively). LAB has been thus considered part of the NT population for purposes of further analyses.

Similarity between accessions ben_16006, ben_16009 and LAB indicate its origin somewhere in the north of the Northern Territory, roughly 550 km from the putative origin of the laboratory strain near the Granites Gold Mine (Cleland s.n., 1936, Adelaide Herbarium AD95711022; Bally et al., 2018).

The populations of *N. benthamiana* seem to be highly isolated from each other with little gene flow between them, sometimes comparable or only slightly higher to that between them and other species. In NGSadmix analysis (Fig 7), there are at most 5 individuals belonging to more than one population, and it is mostly those hypothesised to be hybrids.

Results from SNAPP indicate that it would be statistically consistent to view *Nicotiana benthamiana* as a group of species or subspecies, a view also held in (Chase & Christenhusz, 2018d). More detailed analyses with extended sampling are underway, formal taxonomical decisions will be taken depending on their results. Results from maximum likelihood phylogeny, ABBA-BABA and SNAPP (Figure 3b, Tables 3, 4 and 5) indicate the Queensland population to be the most isolated of the *N. benthamiana* populations. SNAPP analysis showed that all models splitting *N. benthamiana* are better than the uniform model.

The inbreeding coefficient is high (Figure 4), indicating frequent selfing and in some species with narrow distribution also small population sizes (e.g., *Nicotiana burbidgeae*, known only from several populations near Dalhousie Springs, SA [https://avh.chah.org.au/]). Although selfing is likely common in wild populations, and at least some species do not show reduced seed viability after selfing (Chase & Christenhusz, 2018d; Chase *et al.*, 2018b), the already mentioned introgressed accessions indicate that outcrossing must happen from time to time.

Among *N. benthamiana* accessions, the WA populations, whether have the lowest inbreeding coefficient (Table 2), except for putative hybrid accessions and accession ben_18032 from eWa, which in admixture analyses consistently shows as having some admixture from the Queensland (Fig. 7). The lower levels of inbreeding in WA are somewhat expected because the populations there are the biggest, and there is more suitable habitat in that area (Chase, pers. comm.). The extremely high F in LAB is also as expected because it came into laboratory cultivation from one collection and has been kept in laboratories for decades via seeds produced by selfing; however, NT accessions that have been determined to be the most similar also show very high F, indicating that this condition might precede cultivation in the laboratory. The rdr1 mutation (Bally et al., 2015) that makes *N. benthamiana* so useful as a model organism is under natural

condition deleterious, and populations with it can probably survive in nature only where the viral load is low. The occurrence of selfing in *N. benthamiana* likely contributed to increasing the frequency of the mutation rdr1. Additional populations of rdr1 defective *N. benthamiana* might be found in natural populations with high levels of inbreeding. Another candidate for new rdr1 deficient strains would be accession ben_18008 (F>0.85). This is the sample closest to Granites Gold Mine, the proposed origin of LAB, but it consistently grouped with the WA population (Figures 2, 3b, 5, 7).

This study provides a robust phylogenetic framework that enables further study of section *Suaveolentes* and hopefully contributed to the growing understanding of plant diversity and evolution. This section may become a good model for understanding of processes regarding polyploidy, radiation and adaptation in arid environments. Population genomics and biogeography of *Nicotiana benthamiana* were explored, revealing evidence for geographical isolation of populations and limited gene flow between them.

5. LITERATURE

Adler LS, Seifert MG, Wink M, Morse GE. **2012**. Reliance on pollinators predicts defensive chemistry across tobacco species. *Ecology Letters* **15**: 1140–1148.

Andrews KR, Good JM, Miller MR, Luikart G, Hohenlohe PA. **2016**. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Reviews Genetics* **17**: 81–92.

Aoki S, Ito M. **2000**. Molecular phylogeny of Nicotiana (Solanaceae) based on the nucleotide sequence of the matK gene. *Plant Biology* **2**: 316–324.

Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, Selker EU, Cresko WA, Johnson EA. **2008**. Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS ONE* **3**: 1–7.

Baldwin BG, Sanderson MJ, Porter JM, Martin F, Campbell CS, Donoghue MJ, Its THE, Of R, Baldwin BG, Source AV, *et al.* 1995. The its Region of Nuclear Ribosomal DNA : A Valuable Source of Evidence on Angiosperm Phylogeny Source : Annals of the Missouri Botanical Garden , Vol . 82 , No . 2 (1995), pp . 247-277 Published by : Missouri Botanical Garden Press Stable URL : http://. *Annals of the Missouri Botanical Garden* **82**: 247–277.

Bally J, Nakasugi K, Jia F, Jung H, Ho SY, Wong M, Paul CM, Naim F, Wood C.C, Crowhurst RN, Hellens RP. 2015. The extremophile Nicotiana benthamiana has traded viral defence for early vigour. *Nature Plants* **1**: 1–6.

Bally J, Jung H, Mortimer C, Naim F, Philips JG, Hellens R, Bombarely A, Goodin MM, Waterhouse PM. **2018**. The Rise and Rise of Nicotiana benthamiana : A Plant for All Reasons . *Annual Review of Phytopathology* **56**: 405–426.

Bombarely A, Rosli HG, Vrebalov J, Moffett P, Mueller LA, Martin GB. **2012**. A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. *Molecular Plant-Microbe Interactions* **25**: 1523–1530.

Brandrud MK, Baar J, Lorenzo MT, Athanasiadis A, Bateman RM, Chase MW, Hedrén M, Paun O. **2020**. Phylogenomic relationships of diploids and the origins of allotetraploids in Dactylorhiza (Orchidaceae). *Systematic Biology* **69**: 91–109.

Burbidge NT. **1960**. THE AUSTRALIAN SPECIES OF NICOTIANA L. (SOLANACEAE). *Australian Journal of Botany* **8**: 342–380.

Byrne M, Yeates DK, Joseph L, Kearney M, Bowler J, Williams MAJ, Cooper S, Donnellan SC, Keogh JS, Leys R, *et al.* 2008. Birth of a biome: Insights into the assembly and maintenance of the Australian arid zone biota. *Molecular Ecology* **17**: 4398–4417.

Chase MW, Christenhusz MJM. **2018a**. 894. NICOTIANA STENOCARPA. *Curtis's Botanical Magazine* **35**: 319–327.

Chase MW, Christenhusz MJM. **2018b**. 891. NICOTIANA OCCIDENTALIS SUBSPECIES OBLIQUA. *Curtis's Botanical Magazine* **35**: 295–303.

Chase MW, Christenhusz MJM. **2018c**. 883. NICOTIANA KARIJINI. *Curtis's Botanical Magazine* **35**: 228–236.

Chase MW, Christenhusz MJM. **2018d**. 890. NICOTIANA BENTHAMIANA. *Curtis's Botanical Magazine* **35**: 286–294.

Chase MW, Christenhusz MJM. **2018e**. 887. NICOTIANA EXCELSIOR. *Curtis's Botanical Magazine* **35**: 261–268.

Chase MW, Christenhusz MJM. **2018f**. 889. NICOTIANA UMBRATICA. *Curtis's Botanical Magazine* **35**: 278–285.

Chase MW, Christenhusz MJM. **2018g**. 885. NICOTIANA GASCOYNICA. *Curtis's Botanical Magazine* **35**: 245–252.

Chase MW, Christenhusz MJM, Conran JG, Dodsworth S, Medeiros de Assis FN, Felix LP, Fay MF. **2018a**. UNEXPECTED DIVERSITY OF AUSTRALIAN TOBACCO SPECIES (*NICOTIANA* SECTION *SUAVEOLENTES,* SOLANACEAE). *Curtis's Botanical Magazine* **35**: 212–227.

Chase MW, Conran JG, Christenhusz MJM. **2018b**. 884. NICOTIANA YANDINGA. *Curtis's Botanical Magazine* **35**: 237–244.

Chase MW, Conran JG, Christenhusz MJM. **2018c**. 892. NICOTIANA BURBIDGEAE. *Curtis's Botanical Magazine* **35**: 304–311.

Chase MW, Knapp S, Cox A V., Clarkson JJ, Butsko Y, Joseph J, Savolainen V, Parokonny AS. **2003**. Molecular systematics, GISH and the origin of hybrid taxa in Nicotiana (Solanaceae). *Annals of Botany* **92**: 107–127.

Clarkson JJ, Dodsworth S, Chase MW. **2017**. Time-calibrated phylogenetic trees establish a lag between polyploidisation and diversification in Nicotiana (Solanaceae). *Plant Systematics and Evolution* **303**: 1001–1012.

Clarkson JJ, Kelly LJ, Leitch AR, Knapp S, Chase MW. **2010**. Nuclear glutamine synthetase evolution in Nicotiana: Phylogenetics and the origins of allotetraploid and homoploid (diploid) hybrids. *Molecular Phylogenetics and Evolution* **55**: 99–112.

Clarkson JJ, Knapp S, Garcia VF, Olmstead RG, Leitch AR, Chase MW. **2004**. Phylogenetic relationships

in Nicotiana (Solanaceae) inferred from multiple plastid DNA regions. *Molecular Phylogenetics and Evolution* **33**: 75–90.

Clarkson JR, Symon DE. **1991**. Nicotiana Wuttkei (Solanaceae), a New Species From North-Eastern Queensland With an Unusual Chromosome Number. *Austrobaileya* **3**: 389–392.

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, *et al.* 2011. The variant call format and VCFtools. *Bioinformatics* **27**: 2156–2158.

Davey JL, Blaxter MW. **2010**. RADseq: Next-generation population genetics. *Briefings in Functional Genomics* **9**: 416–423.

Degnan JH, Rosenberg NA. **2006**. Discordance of species trees with their most likely gene trees. *PLoS Genetics* **2**: 762–768.

Degnan JH, Rosenberg NA. **2009**. Gene tree discordance, phylogenetic inference and the multispecies coalescent. *Trends in Ecology and Evolution* **24**: 332–340.

Derevnina L, Kamoun S, Wu C hang. **2019**. Dude, where is my mutant? Nicotiana benthamiana meets forward genetics. *New Phytologist* **221**: 607–610.

Dodsworth S. **2015**. Genome skimming for phylogenomics. *PhD Thesis*: 159.

Doyle JJ, Doyle JL. **1990**. A rapid total DNA preparation procedure for fresh plant tissue. *Focus* **12**: 13–15.

Drummond AJ, Suchard MA, Xie D, Rambaut A. **2012**. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**: 1969–1973.

Eaton DAR, Ree R. **2013**. Inferring phylogeny and introgression using RADseq data. *Systematic Biology* **62**: 689–706.

Evanno G, Regnaut S, Goudet J. **2005**. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology* **14**: 2611–2620.

Fawcett JA, Maere S, Van De Peer Y. **2009**. Plants with double genomes might have had a better chance to survive the Cretaceous-Tertiary extinction event. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 5737–5742.

Garcia VF, Olmstead RG. **2003**. Phylogenetics of tribe Anthocercideae (Solanaceae) based on ndhF and trnL/ F sequence data. *Systematic Botany* **28**: 609–615.

Goodin MM, Zaitlin D, Naidu RA, Lommel SA. **2008**. Nicotiana benthamiana: Its history and future as a model for plant-pathogen interactions. *Molecular Plant-Microbe Interactions* **21**: 1015–1026.

Goodspeed TH. **1945**. Cytotaxonomy of Nicotiana. *Botanical Review* **11**: 533–592.

Goodspeed TH. **1947**. On the Evolution of the Genus Nicotiana. *Proceedings of the National Academy of Sciences of the United States of America* **33**: 158–171.

Heckenhauer J, Samuel R, Ashton PS, Abu Salim K, Paun O. **2018**. Phylogenomics resolves evolutionary relationships and provides insights into floral evolution in the tribe Shoreeae (Dipterocarpaceae). *Molecular Phylogenetics and Evolution* **127**: 1–13.

Horton P. **1981**. A TAXONOMIC REVISION OF NICOTIANA (SOLANACEAE) IN AUSTRALIA. *Journal of the Adelaide Botanic Garden* **7**: 1–56.

Hou Y, Nowak MD, Mirré V, Bjorå CS, Brochmann C, Popp M. **2016**. RAD-seq data point to a northern origin of the arctic-alpine genus Cassiope (Ericaceae). *Molecular Phylogenetics and Evolution* **95**: 152–

160.

Jiao Y, Leebens-Mack J, Ayyampalayam S, Bowers JE, McKain MR, McNeal J, Rolf M, Ruzicka DR, Wafula E, Wickett NJ, *et al.* 2012. A genome triplication associated with early diversification of the core eudicots. *Genome Biology* **13**: 1–14.

Jiao Y, Wickett NJ, Ayyampalayam S, Chanderbali AS, Landherr L, Ralph PE, Tomsho LP, Hu Y, Liang H, Soltis PS, *et al.* 2011. Ancestral polyploidy in seed plants and angiosperms. *Nature* **473**: 97–100.

Kelly LJ, Leitch AR, Clarkson JJ, Knapp S, Chase MW. **2013**. Reconstructing the complex evolutionary origin of wild allopolyploid tobaccos (Nicotiana section Suaveolentes). *Evolution* **67**: 80–94.

Knapp S, Chase MW, Clarkson JJ. **2004**. Nomenclatural Changes and a New Sectional Classification in Nicotiana (Solanaceae). *Taxon* **53**: 73–82.

Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I. **2015**. Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources* **15**: 1179–1191.

Korneliussen TS, Albrechtsen A, Nielsen R. **2014**. ANGSD: Analysis of Next Generation Sequencing Data. *BMC Bioinformatics* **15**: 1–13.

Kostoff D. 1943. *Cytogenetics of the Genus Nicotiana.* State Printing House.

Kovarik A, Dadejova M, Lim YK, Chase MW, Clarkson JJ, Knapp S, Leitch AR. **2008**. Evolution of rDNA in Nicotiana Allopolyploids: A Potential Link between rDNA Homogenization and Epigenetics. *Annals of Botany* **101**: 815–823.

Lewis PO. **2001**. A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology* **50**: 913–925.

Li H. **2013**. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. **00**: 1–3.

Lischer HEL, Excoffier L. **2012**. PGDSpider: An automated data conversion tool for connecting population genetics and genomics programs. *Bioinformatics* **28**: 298–299.

Malinsky M, Trucchi E, Lawson DJ, Falush D. **2018**. RADpainter and fineRADstructure: Population Inference from RADseq Data. *Molecular Biology and Evolution* **35**: 1284–1290.

Mallet J, Besansky N, Hahn MW. **2016**. How reticulated are species? *BioEssays* **38**: 140–149.

Marks CE, Ladiges PY, Newbigin E. **2011a**. Karyotypic variation in Nicotiana section Suaveolentes. *Genetic Resources and Crop Evolution* **58**: 797–803.

Marks CE, Newbigin E, Ladiges PY. **2011b**. Comparative morphology and phylogeny of Nicotiana section Suaveolentes (Solanaceae) in Australia and the South Pacific. *Australian Systematic Botany* **24**: 61–86.

Marlin D, Nicolson SW, Sampson JDS, Krüger K. **2016**. Insights into the pollination requirements of the only African wild tobacco, Nicotiana africana (Solanaceae) from the Namib Desert. *Journal of Arid Environments* **125**: 64–67.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, *et al.* 2010. The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research* **20**: 1303.

Pagès H, Aboyoun P, Gentleman R, DebRoy S. **2020**. Biostrings: Efficient manipulation of biological strings. R package version 2.58.0.

Paradis E, Schliep K. **2019**. Ape 5.0: An environment for modern phylogenetics and evolutionary analyses

in R. *Bioinformatics* **35**: 526–528.

Paun O, Turner B, Trucchi E, Munzinger J, Chase MW, Samuel R. **2016**. Processes Driving the Adaptive Radiation of a Tropical Tree (Diospyros, Ebenaceae) in New Caledonia, a Biodiversity Hotspot. *Systematic Biology* **65**: 212–227.

Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE. **2012**. Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE* **7**.

Raguso RA, Levin RA, Foose SE, Holmberg MW, McDade LA. **2003**. Fragrance chemistry, nocturnal rhythms and pollination 'syndromes' in Nicotiana. *Phytochemistry* **63**: 265–284.

Raguso RA, Schlumpberger BO, Kaczorowski RL, Holtsford TP. **2006**. Phylogenetic fragrance patterns in Nicotiana sections Alatae and Suaveolentes. *Phytochemistry* **67**: 1931–1942.

Ratsch A, Steadman KJ, Bogossian F. **2010**. The pituri story: A review of the historical literature surrounding traditional Australian Aboriginal use of nicotine in Central Australia. *Journal of Ethnobiology and Ethnomedicine* **6**: 1–13.

Sang T. **2002**. Utility of Low-Copy Nuclear Gene Sequences in Plant Phylogenetics. *Critical Reviews in Biochemistry and Molecular Biology* **37**: 121–147.

Särkinen T, Bohs L, Olmstead RG, Knapp S. **2013**. A phylogenetic framework for evolutionary study of the nightshades (Solanaceae): A dated 1000-tip tree. *BMC Evolutionary Biology* **13**.

Savolainen V, Chase MW. **2003**. A decade of progress in plant molecular phylogenetics. **19**: 717–724.

Skotte L, Korneliussen TS, Albrechtsen A. **2013**. Estimating individual admixture proportions from next generation sequencing data. *Genetics* **195**: 693–702.

Smith SA, Walker-Hale N, Walker JF, Brown JW. **2020**. Phylogenetic Conflicts, Combinability, and Deep Phylogenomics in Plants. *Systematic Biology* **69**: 579–592.

Soltis DE, Burleigh JG. **2009**. Surviving the K-T mass extinction: New perspectives of polyploidization in angiosperms. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 5455–5456.

Soltis DE, Visger CJ, Blaine Marchant D, Soltis PS. **2016**. Polyploidy: Pitfalls and paths to a paradigm. *American Journal of Botany* **103**: 1146–1166.

Soraggi S, Wiuf C, Albrechtsen A. **2018**. Powerful inference with the D-statistic on low-coverage whole-genome data. *G3: Genes, Genomes, Genetics* **8**: 551–566.

Stamatakis A. **2014**. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.

Symon DE. **2005**. Native Tobaccos (Solanaceae: Nicotiana spp.) in Australia and their Use by Aboriginal Peoples. *The Beagle: Records of the Museums and Art Galleries of the Northern Territory* **21**: 10.

Symon DE, Kenneally KF. **1994**. A new species of Nicotiana (Solanaceae) from near Broome, Western Australia. *Nuytsia* **9**: 421–425.

Wang LG, Lam TTY, Xu S, Dai Z, Zhou L, Feng T, Guo P, Dunn CW, Jones BR, Bradley T, *et al.* 2020. Treeio: An R Package for Phylogenetic Tree Input and Output with Richly Annotated and Associated Data. *Molecular Biology and Evolution* **37**: 599–603.

Wheeler H-M. **1945**. A Contribution to the Cytology of the Australian -South Pacific Species of Nicotiana. *Proceedings of the National Academy of Sciences of the United States of America* **31**: 177–185.

Wickham H. **2016**. *ggplot2: Elegant Graphics for Data Analysis*. Springer Nature.

Williams E. **1975**. A new chromosome number in the australian species nicotiana cavicola L. (Burbidge). *New Zealand Journal of Botany* **13**: 811–812.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. **2009**. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 13875–9.

Wu Y. **2010**. Close lower and upper bounds for the minimum reticulate network of multiple phylogenetic trees. *Bioinformatics* **26**: 140–148.

Yu Y, Ristic N, Nakhleh L. **2013**. Fast algorithms and heuristics for phylogenomics under ILS and hybridization. *BMC bioinformatics* **14 Suppl 1**.

Yu G, Smith DK, Zhu H, Guan Y, Lam TTY. **2017**. Ggtree: an R Package for Visualization and Annotation of Phylogenetic Trees With Their Covariates and Other Associated Data. *Methods in Ecology and Evolution* **8**: 28–36.

Zhou W, Ji X, Obata S, Pais A, Dong Y, Peet R, Xiang QY (Jenny). **2018**. Resolving relationships and phylogeographic history of the Nyssa sylvatica complex using data from RAD-seq and species distribution modeling. *Molecular Phylogenetics and Evolution* **126**: 1–16.

ANNEX 1

species	sample ID	locality	storage conditions	library	repeat library	processing name	NoReads
benthamiana	68171	Silent Gorge	H	RAD 1		ben_68171	2689380
benthamiana	68174	Mount Robinson	H	RAD 1		ben_68174	2064604
benthamiana	68183	Hancock Gorge	H	RAD 1		ben_68183	2799385
benthamiana	68194	Palm Pool	H	RAD 1		ben_68194	3585953
benthamiana	68199	Harding Dam	H	RAD 1		ben_68199	3417632
benthamiana	68218	Whim Creek	H	RAD 1		ben_68218	1701478
benthamiana	68221	McKay Creek	H	RAD 1		ben_68221	3306662
benthamiana	68224	W Pannawonica	H	RAD 1		ben_68224	3028064
benthamiana	68289	Python Pool	H	RAD 1		ben_68289	2070792
goodspeedii	16120	Point Pearce	H	RAD 1		goo_16120	3916605
goodspeedii	16121	Telowie Beach	H	RAD 1		goo_16121	2670474
goodspeedii	16123	Murninni Beach	H	RAD 1		goo_16123	2756628
goodspeedii	16137	Fowler's Bay	H	RAD 1		goo_16137	2786941
goodspeedii	16139	Maralinga Airstrip	H	RAD 1		goo_16139	3226984
goodspeedii	16151	Weebibiddie Cave	H	RAD 1		goo_16151	3437845
goodspeedii	16156	Nuyts NP	H	RAD 1		goo_16156	2573041
hesperis	68240	Exmouth Airport	H	RAD 1		hes_68240	3293118
hesperis	68258	Rocky Pool - type	H	RAD 1		hes_68258	2278202
heterantha	68204	Sherlock River	H	RAD 1		het_68204	2515630
ingulba	68189	Karijini	H	RAD 1		ing_68189	4295519
maritima	16126	Sleaford	H	RAD 1		mar_16126	3786449
maritima	16135	Venus Bay	H	RAD 1		mar_16135	3283555
megalosiphon	17010	Milguy	H	RAD 1		meg_17010	3749173
monoschizocarpa	16001	Daly River Rd Xing	H	RAD 1		mon_16001	3511812
monoschizocarpa	16004	Ooloo Xing	H	RAD 1		mon_16004	1832730
monoschizocarpa	16013	Bitter Springs	H	RAD 1		mon_16013	1373353
obliqua	68157	Kumarina	H	RAD 1		obl_68157	2991228
obliqua	68166	Roy Hill	H	RAD 1		obl_68166	3500420
occidentalis	16038	Mt Benstead	H	RAD 1		occ_16038	3417582
occidentalis	16096	Mulga Park	H	RAD 1		occ_16096	2816358
occidentalis	68165		H	RAD 1		occ_68165	2792531
occidentalis	68167	Roy Hill	H	RAD 1		occ_68167	2414321
occidentalis	68205	Sherlock River	H	RAD 1		occ_68205	4803563
occidentalis	68228	Nanutarra Rd	H	RAD 1		occ_68228	2772097
occidentalis	68231	Burkett Road	H	RAD 1		occ_68231	2979894
occidentalis	68250	Lyndon River	H	RAD 1		occ_68250	3305132
occidentalis obliqua	68279	Murcheson River	H	RAD 1		obl_68279	4742475
occidentalis occidentalis	68216	Port Hedlund	H	RAD 1		occ_68216	3376595
occidentalis occidentalis	68234	Exmouth	H	RAD 1		occ_68234	1932772
rosulata	16132	Minnipa-Yardea	H	RAD 1		ros_16132	2921262
rosulata	16158	Norseman	H	RAD 1		ros_16158	2285394
rosulata	16199	Payne's Find	H	RAD 1		ros_16199	3288165
rosulata	68152	Jibberding	H	RAD 1		ros_68152	5510361
rosulata	68173	Newman	H	RAD 1		ros_68173	3589817
rosulata	68285	Boiada Camp	H	RAD 1		ros_68285	2892531

rosulata	68288	Wubin	H	RAD 1	ros_68288	2818983
sessilifolia	16061	Palm Valley	H	RAD 1	ses_16061	3941051
simulans	16023B	Santa Teresa	H	RAD 1	sim_16023B	2476968
sp. nov.	16027	Lawrence Gorge	H	RAD 1	nov_16027	2929883
sp. nov.	16032	Emily Gap	H	RAD 1	nov_16032	4282845
sp. nov.	16040	Taphina Gorge	H	RAD 1	nov_16040	2896243
sp. nov.	16064	Palm Valley	H	RAD 1	nov_16064	2898268
stenocarpa	16168	Koolkynie Road	H	RAD 1	ste_16168	3600016
suaveolens	17012	Towari	H	RAD 1	sua_17012	2932170
suaveolens	17015	Abercrombie Caves	H	RAD 1	sua_17015	2948772
suaveolens	17022	Buchan	H	RAD 1	sua_17022	4169069
umbratica	68208	Woodstock	H	RAD 1	umb_68208	3507175
umbratica	68214	Shay Gap	H	RAD 1	umb_68214	1794554
velutina	16041	Taphina Gorge	H	RAD 1	vel_16041	3332546
velutina	16059	Palm Valley	H	RAD 1	vel_16059	2455907
africana			H	RAD 2	afr_	2085762
amplexicaulis	14219		KEW	RAD 2	amp_14219	747855
benthamiana	16006	Judbarra-Gregory	H	RAD 2	ben_16006	3241307
benthamiana	68172	Newman	H	RAD 2	ben_68172	1963041
benthamiana	68200	Wittenoom	H	RAD 2	ben_68200	2572116
benthamiana	68209	Marble Bar	H	RAD 2	ben_68209	3118985
benthamiana	68212	Woodstock-Marble Bar Rd	H	RAD 2	ben_68212	2847664
benthamiana	68223	Pannawonica	H	RAD 2	ben_68223	2095693
cavicola	16180	Sandstone	H	RAD 2	cav_16180	5133365
cavicola	68154	Meekathara	H	RAD 2	cav_68154	2696894
cavicola	68261	Congo Creek	H	RAD 2	cav_68261	2078621
sp. Corunna	21019	Sthn Gowler Ranges	KEW	RAD 2	cor_21019	3006703
debneyi	12693		KEW	RAD 2	deb_12693	2376910
fatuhivensis	19752	Marquesas	KEW	RAD 2	fat_19752	2538445
faucicola	3405	Alligator Gorge	H	RAD 2	fau_3405	2218396
faucicola	3621	Brachina Gorge	H	RAD 2	fau_3621	3177738
faucicola	3625	W Brachina Gorge	H	RAD 2	fau_3625	1849518
faucicola	3627		H	RAD 2	fau_3627	2890783
faucicola	3633	Port Germein Gorge	H	RAD 2	fau_3633	2765259
faucicola	3635	Germein Gorge	H	RAD 2	fau_3635	2781239
goodspeedii	16146	Murrawijinnie Cave	H	RAD 2	goo_16146	4650233
goodspeedii	3629		H	RAD 2	goo_3629	1955496
gossei	16066	Re: 68206 - umbratica or cavicola	H	RAD 2	gos_16066	1894936
hesperis	68229	Burkett Road	H	RAD 2	hes_68229	2858837
hesperis	68283	Karara Mine	H	RAD 2	hes_68283	2767748
heterantha	68235	Learmouth Airport	H	RAD 2	het_68235	1925292
maritima?	16125	Carapee Hill	H	RAD 2	mar_16125	2146332
maritima	16130	Coffin Bay	H	RAD 2	mar_16130	3763856

maritima	18005	Dorothee Isld	H	RAD 2	mar_18005	4638465
monoschizocarpa	16010	Waterhouse River	H	RAD 2	mon_16010	1179022
sp. nov.	16049	Simpson's Gap	H	RAD 2	nov_16049	5536026
sp. nov.	16051	Serpentine Gorge	H	RAD 2	nov_16051	3132867
sp. nov. occidentalis	16102	Docker River	H	RAD 2	nov_16102	4455981
obliqua	3615	Corunna	H	RAD 2	obl_3615	3861706
obliqua	68237	Charles Knife	H	RAD 2	obl_68237	3245331
rosulata	16026	Lawrence Gorge	H	RAD 2	ros_16026	2815681
rosulata	16170	E of Leonora	H	RAD 2	ros_16170	2303645
rosulata	68177	Mount Robinson	H	RAD 2	ros_68177	4100812
rosulata	68264	Congo Creek	H	RAD 2	ros_68264	1930395
rosulata	68273	Hamelin	H	RAD 2	ros_68273	3406331
rotundifolia	16157	Dundas Rock	H	RAD 2	rot_16157	2987592
rotundifolia	68277	Nerren Nerren	H	RAD 2	rot_68277	2623527
simulans	16092	Idracowra Station	H	RAD 2	sim_16092	3424730
simulans	3552		H	RAD 2	sim_3552	3477638
simulans	3559		H	RAD 2	sim_3559	3914821
simulans/rosulata?	3564		H	RAD 2	sim_ros_3564	4328601
stenocarpa	16181	E of Sandstone	H	RAD 2	ste_16181	2565029
suaveolens	17017	Whitton Stock Route	H	RAD 2	sua_17017	4022134
symonii	3353		H	RAD 2	sym_3353	1980611
truncata	3599		H	RAD 2	tru_3599	3090858
umbratica	68206	Woodgina Mine	H	RAD 2	umb_68206	2346340
umbratica	68211	Spear Hill	H	RAD 2	umb_68211	3997047
velutina	16017	Native Gap	H	RAD 2	vel_16017	3653834
velutina	16028	Lawrence Gap	H	RAD 2	vel_16028	3306618
velutina	16035	Jessie's Gap	H	RAD 2	vel_16035	3214591
velutina	16069	Glen Helen Gorge	H	RAD 2	vel_16069	5010827
velutina	16086	Watarrka	H	RAD 2	vel_16086	2727400
velutina	16142	Maralinga	H	RAD 2	vel_16142	2976631
velutina	3570	Witjira	H	RAD 2	vel_3570	2964474
velutina	3575		H	RAD 2	vel_3575	4327130
benthamiana	18004	E of Kiwirrkurra	QH	RAD 3	ben_18004	3521935
benthamiana	18007	Little Sandy Desert	QH	RAD 3	ben_18007	2625332
benthamiana	TW16	lab rat form	QH	RAD 3	ben_TW16	4136595
burbidgeae	3571		QH	RAD 3	bur_3571	2973846
burbidgeae	3573		QH	RAD 3	bur_3573	3464577
debneyi	17011	Hat Head NP	QH	RAD 3	deb_17011	2727416
excelsior	16106	Valley of the Winds	QH	RAD 3	exc_16106	2796948
excelsior	17030	Walpa Gorge	QH	RAD 3	exc_17030	2435190
faucicola	17001	Burra Gorge	QH	RAD 3	fau_17001	3190067
faucicola	3619	Bunyeroo	QH	RAD 3	fau_3619	1990114
gascoynica	68253	Gascoyne River Xing	QH	RAD 3	gas_68253	3967757
gascoynica	68257	Rocky Pool	QH	RAD 3	gas_68257	2297128
gascoynica	68260	Mooka Road	QH	RAD 3	gas_68260	3326380

gascoynica	68265	Daurie River Xing	QH	RAD 3	gas_68265	2594324
gascoynica	68268	Wooramel River Xing	QH	RAD 3	gas_68268	2726820
gossei	16083	Watarrka	QH	RAD 3	gos_16083	2116891
gossei	16107	Uluru	QH	RAD 3	gos_16107	2769427
gossei	16217	Simpson's Gap	QH	RAD 3	gos_16217	2489372
gossei	17031	Uluru	QH	RAD 3	gos_17031	1705043
hesperis?	18012	Docker River	QH	RAD 3	hes_18012	2639254
heterantha	16172	Lake Raeside	QH	RAD 3	het_16172	3015064
heterantha	68222	Fortescue	QH	RAD 3	het_68222	2331902
ingulba	17026	Yulara	QH	RAD 3	ing_17026	3452997
ingulba	17027	Docker River	QH	RAD 3	ing_17027	2992946
karijini	18009	Mount Turner	QH	RAD 3	kar_18009	1534688
karijini	68178	Jofre Gorge	QH	RAD 3	kar_68178	3008319
maritima	16118	St. Vincent	QH	RAD 3	mar_16118	3315883
maritima	16129	Yangie Beach	QH	RAD 3	mar_16129	2181971
megalosiphon	17005	Maq's Marshes	QH	RAD 3	meg_17005	3742729
megalosiphon	17009	Mehi River	QH	RAD 3	meg_17009	3203989
minor	16148	Gilgerabbie Hut	QH	RAD 3	min_16148	2504017
non rosulata	68280	Karara Mine	QH	RAD 3	noros_68280	2655779
sp. nov.	16116	Kata-Tjuta	QH	RAD 3	nov_16116	2727025
sp. nov.	16122	Telowi Gorge	QH	RAD 3	nov_16122	3115631
obliqua	68251	Lyndon river	QH	RAD 3	obl_68251	3644320
occidentalis	68202	Point Sampson	QH	RAD 3	occ_68202	4113814
rosulata	16161	Boondi	QH	RAD 3	ros_16161	5146686
rotundifolia	16204	Dalwallinu Village	QH	RAD 3	rot_16204	3137564
rotundifolia	18011	Tom Price	QH	RAD 3	rot_18011	3359791
rotundifolia	68161	Roy Hill	QH	RAD 3	rot_68161	1568545
sessilifolia	16016	Ti-Tree	QH	RAD 3	ses_16016	3438746
sessilifolia	16025	Simpson's Gap	QH	RAD 3	ses_16025	3088035
sessilifolia	16215	Alice Springs	QH	RAD 3	ses_16215	1633398
simulans	3560	Coober Pedy	QH	RAD 3	sim_3560	3756026
stenocarpa	16176	Leonora-Agnew Road	QH	RAD 3	ste_16176	2931425
stenocarpa	16190	E of Yalgoo	QH	RAD 3	ste_16190	2574667
suaveolens	17014	Jenolan Caves	QH	RAD 3	sua_17014	2260742
suaveolens	17016	Whitton Stock Route	QH	RAD 3	sua_17016	3251432
suaveolens	17018	Woolshed Flat	QH	RAD 3	sua_17018	3828962
suaveolens	17019	Pines Picnic Ground	QH	RAD 3	sua_17019	4085406
suaveolens	17020	Wallace Craigie	QH	RAD 3	sua_17020	2707504
suaveolens	17021	Willis Cpgd	QH	RAD 3	sua_17021	2635453
suaveolens	17023	Melbourne	QH	RAD 3	sua_17023	2131216
suaveolens	NGW8382	Morington Peninsula	QH	RAD 3	sua_NGW8382	2453280
symonii	3610	Coruna Station	QH	RAD 3	sym_3610	2813366
aff. velutina	17002	Lake Menindee	QH	RAD 3	vel_17002	2448862
velutina	17003	Louth	QH	RAD 3	vel_17003	2877926
velutina	3575		QH	RAD 3	vel_3575	2154295

velutina	3585		QH	RAD 3		vel_3585	3572347
yandingana	16134	Yandinga	QH	RAD 3		yan_16134	3542819
africana			H	RAD 4	RAD 2	afr_	3269782
amplexicaulis	14219		KEW	RAD 4	RAD 2	amp_14219	1371843
benthamiana	16009	Victoria River Xing	H	RAD 4		ben_16009	2725277
benthamiana	68172	Newman	H	RAD 4	RAD 2	ben_68172	2811523
benthamiana	68183	Hancock Gorge	H	RAD 4	RAD 1	ben_68183	3005703
benthamiana	68185	Weano Gorge	H	RAD 4		ben_68185	3528993
benthamiana	68218	Whim Creek	H	RAD 4	RAD 1	ben_68218	3687626
benthamiana	68289	Python Pool	H	RAD 4	RAD 1	ben_68289	4259332
benthamiana	68174	Mount Robinson	H	RAD 4	RAD 1	ben_68174	2906527
benthamiana	68223	Pannawonica	H	RAD 4	RAD 2	ben_68223	2667718
cavicola	16201	Payne's Find	H	RAD 4		cav_16201	3767489
cavicola	68261	Congo Creek	H	RAD 4	RAD 2	cav_68261	3311508
debneyi	12693		KEW	RAD 4	RAD 2	deb_12693	3216060
excelsior	17030	Walpa Gorge	QH	RAD 4	RAD 3	exc_17030	2625859
excelsior	18003		H	RAD 4		exc_18003	4040805
exigua	18015		H	RAD 4		exi_18015	4116583
fatuhivensis	19752	Marquesas	KEW	RAD 4	RAD 2	fat_19752	2862118
faucicola	3619	Bunyeroo	QH	RAD 4	RAD 3	fau_3619	3474840
faucicola	3625	W Brachina Gorge	H	RAD 4	RAD 2	fau_3625	3121683
faucicola	3405	Alligator Gorge	H	RAD 4	RAD 2	fau_3405	3405557
gascoynica	68257	Rocky Pool	QH	RAD 4	RAD 3	gas_68257	4174542
goodspeedii	3629		H	RAD 4	RAD 2	goo_3629	2619441
gossei	16066	Re: 68206 - umbratica or cavicola	H	RAD 4	RAD 2	gos_16066	3518471
gossei	16083	Watarrka	QH	RAD 4	RAD 3	gos_16083	3340046
gossei	17031	Uluru	QH	RAD 4	RAD 3	gos_17031	2691692
gossei	16217	Simpson's Gap	QH	RAD 4	RAD 3	gos_16217	3080132
hesperis	68274	Hamelin Pool	H	RAD 4		hes_68274	2832585
hesperis	68258	Rocky Pool - type	H	RAD 4	RAD 1	hes_68258	5346174
heterantha	68222	Fortescue	QH	RAD 4	RAD 3	het_68222	4278564
heterantha	68235	Learmouth Airport	H	RAD 4	RAD 2	het_68235	4027307
heterantha	68243	Yardies Creek	H	RAD 4		het_68243	1549899
heterantha	68204	Sherlock River	H	RAD 4	RAD 1	het_68204	3711922
karijini	18002		H	RAD 4		kar_18002	3929761
karijini	18009	Mount Turner	QH	RAD 4	RAD 3	kar_18009	4378237
maritima?	16125	Carapee Hill	H	RAD 4	RAD 2	mar_16125	2603951
maritima	16129	Yangie Beach	QH	RAD 4	RAD 3	mar_16129	4342926
minor	16148	Gilgerabbie Hut	QH	RAD 4	RAD 3	min_16148	3218146
monoschizocarpa	16004	Ooloo Xing	H	RAD 4	RAD 1	mon_16004	2469044
monoschizocarpa	16010	Waterhouse River	H	RAD 4	RAD 2	mon_16010	1050132
monoschizocarpa	16013	Bitter Springs	H	RAD 4	RAD 1	mon_16013	2592052
occidentalis	68250	Lyndon River	H	RAD 4	RAD 1	occ_68250	3706322
occidentalis	68165		H	RAD 4	RAD 1	occ_68165	3168379
rosulata	16124	Carapee Hill	H	RAD 4		ros_16124	2641115
rosulata	16170	E of Leonora	H	RAD 4	RAD 2	ros_16170	4744056

rosulata	16179	Sandstone Sandstone - long tubes	H	RAD 4		ros_16179	4734198
rosulata	16188	Re: 68206 - umbratica or cavicola	H	RAD 4		ros_16188	5036359
rosulata	16207	Wongan Hills	H	RAD 4		ros_16207	3512940
rosulata	18010	Little Sandy Desert	H	RAD 4		ros_18010	3187732
rosulata	68264	Congo Creek	H	RAD 4	RAD 2	ros_68264	3740877
rosulata	68288	Wubin	H	RAD 4	RAD 1	ros_68288	3219727
rosulata	16158	Norseman	H	RAD 4	RAD 1	ros_16158	3532673
rotundifolia	68161	Roy Hill	QH	RAD 4	RAD 3	rot_68161	1955091
sessilifolia	16215	Alice Springs	QH	RAD 4	RAD 3	ses_16215	2840421
simulans	16023B	Santa Teresa	H	RAD 4	RAD 1	sim_ 16023B	3295056
sp. nov.	16027	Lawrence Gorge	H	RAD 4	RAD 1	spn_16027	3691453
sp. nov.	16056	Gosse's Bluff	H	RAD 4		spn_16056	4846783
stenocarpa	16177	Leinster-Wiluna Rd	H	RAD 4		ste_16177	3842407
stenocarpa	16181	E of Sandstone	H	RAD 4	RAD 2	ste_16181	3541041
stenocarpa	16186	Mt Magnet	H	RAD 4		ste_16186	4231650
stenocarpa	16190	E of Yalgoo	QH	RAD 4	RAD 3	ste_16190	3072457
suaveolens	17013	Wollemi NP	H	RAD 4		sua_17013	5253783
suaveolens	17014	Jenolan Caves	QH	RAD 4	RAD 3	sua_17014	3362038
suaveolens	17023	Melbourne	QH	RAD 4	RAD 3	sua_17023	3358999
suaveolens	NGW8382	Morington Peninsula	QH	RAD 4	RAD 3	sua_NGW8382	3231936
symonii	3353		H	RAD 4	RAD 2	sym_3353	2895655
umbratica	68214	Shay Gap	H	RAD 4	RAD 1	umb_68214	3500109
umbratica	68206	Woodgina Mine	H	RAD 4	RAD 2	umb_68206	3865298
aff. velutina	17002	Lake Menindee	QH	RAD 4	RAD 3	vel_17002	3464486
velutina	18018		H	RAD 4		vel_18018	3940395
velutina	16059	Palm Valley	H	RAD 4	RAD 1	vel_16059	3166868
velutina	16142	Maralinga	H	RAD 4	RAD 2	vel_16142	5871375
sp. nov. Wundinna	3853		H	RAD 4		wun_3853	3871165
africana	1			RAD 5		afr_1	3193818
africana	2			RAD 5		afr_2	4700622
amplexicaulis	18151	ArchwayCave	QH	RAD 5		amp_18151	4993366
amplexicaulis	18152	FernCave	QH	RAD 5		amp_18152	4394700
amplexicaulis	18154	MoolayemberGap	QH	RAD 5		amp_18154	3392618
amplexicaulis	18156	GlenleighStation		RAD 5		amp_18156	4954914
amplexicaulis	18157	ChinaWall		RAD 5		amp_18157	4542015
amplexicaulis	18160	Nogoa	QH	RAD 5		amp_18160	4924031
benthamiana	18008	LakeMackay		RAD 5		ben_18008	4246898
benthamiana	18032	CircusRockhole	QH	RAD 5		ben_18032	4638581
benthamiana	18033	Bibarrd	QH	RAD 5		ben_18033	6074371
benthamiana	18038	MtTietkins	QH	RAD 5		ben_18038	3248739
benthamiana	18039	WelliWolli	QH	RAD 5		ben_18039	5864224
benthamiana	18040	DockerRiver	QH	RAD 5		ben_18040	567767
benthamiana	18042	ElizabethCreek	QH	RAD 5		ben_18042	7112516
benthamiana	18061B	Emily Gap	QH	RAD 5		ben_18061B	5738822

benthamiana	18178	Duchess	QH	RAD 5		ben_18178	4642931
benthamina	18183	Pegmatite	QH	RAD 5		ben_18183	6111311
benthamiana	18185	Boulia	QH	RAD 5		ben_18185	4433870
benthamiana	18190	Moondara	QH	RAD 5		ben_18190	7488484
burbidgeae	18034		QH	RAD 5		bur_18034	5808910
debneyi	18030	Woroon	QH	RAD 5		deb_18030	3097824
debneyi	18036	Gallangowan	QH	RAD 5		deb_18036	3266972
debneyi	18159	MtEtna		RAD 5		deb_18159	4314720
excelsior	18046	MimiliW5	QH	RAD 5		exc_18046	3932683
excelsior	18047	Ernabella	QH	RAD 5		exc_18047	3365951
excelsior	18048	MimiliW20	QH	RAD 5		exc_18048	2923012
goodspeedii	3617	BlancheHarbour		RAD 5		goo_3617	4370185
gossei	18045	N?hala	QH	RAD 5		gos_18045	3625274
gossei	18049	Henbury	QH	RAD 5		gos_18049	4413758
ingulba	16085	Watarraka		RAD 5		ing_16085	4356399
ingulba	18056	Barrow Creek		RAD 5		ing_18056	5241296
ingulba	18059	Ethabuka	QH	RAD 5		ing_18059	5872603
ingulba	18064	Tobermorey		RAD 5		ing_18064	5327690
karijini	18029	Paraburdoo	QH	RAD 5		kar_18029	4115760
karijini	18061A	Emily Gap	QH	RAD 5		kar_18061A	3791926
maritima	16119	Wool Bay		RAD 5		mar_16119	5475391
megalosiphon	18161	DouglasPonds		RAD 5		meg_18161	5961101
megalosiphon	18175	Richmond		RAD 5		meg_18175	5795676
obliqua	16141	OakValley	QH	RAD 5		obl_16141	6751521
obliqua	18026	Alcoota	QH	RAD 5		obl_18026	4555842
obliqua	68184	HancockGorge	QH	RAD 5		obl_68184	6196799
obliqua	68250	LakeMacleod		RAD 5		obl_68250	2278293
rotundifolia	18035	Quairading	QH	RAD 5		rot_18035	5213568
rotundifolia	18051	Coolgardie	QH	RAD 5		rot_18051	4263237
rotundifolia	18053	Doodlakine	QH	RAD 5		rot_18053	6479671
rotundifolia	6424A	Kalbari	QH	RAD 5		rot_6424A	3947176
sessilifolia	18176	Cloncurry		RAD 5		ses_18176	4042168
sessilifolia	18191	LeichhardtRiver		RAD 5		ses_18191	2795905
simulans	18058	NewCrown	QH	RAD 5		sim_18058	4585340
sp.nov.	16105	Valleyofthe		RAD 5		spn_16105	4067652
sp.nov.	16143	Maralinga	QH	RAD 5		spn_16143	4668827
sp.nov.	18001	Bates	QH	RAD 5		spn_18001	4074569
suaveolens	17035	Wombeyan	QH	RAD 5		sua_17035	5797041
suaveolens	18060	Yathong		RAD 5		sua_18060	6253546
symonii	3612	CorunnaHills	QH	RAD 5		sym_3612	4860020
truncata	3562	MoonPlain	QH	RAD 5		tru_3562	4311465
velutina	18017	NocolecheNSW	QH	RAD 5		vel_18017	4889238
velutina	18018	VIC		RAD 5	RAD 4	vel_18018	5028228
velutina	18037	MungaThirri	QH	RAD 5		vel_18037	6023690