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The Phylogeny and Biogeography of *Phyla nodiflora* (Verbenaceae) Reveals Native and Invasive Lineages throughout the World

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Academic Editors: Rosa Rao and Giandomenico Corrado
Received: 3 March 2017; Accepted: 4 May 2017; Published: 10 May 2017

Abstract: *Phyla nodiflora* is an herbaceous perennial and an enigmatic species. It is indigenous to the Americas but is considered a natural component of the flora in many areas and a weed in others. Our aim was to circumscribe the native range of *P. nodiflora*, to explore dispersal mechanisms and routes and to test the hypothesis that *P. nodiflora* is native outside of the Americas. Determining whether distributions are natural or human-induced has implications for decisions regarding weed control or conservation. We undertook phylogenetic analyses using sequence variation in nuclear DNA marker ITS (Internal Transcribed Spacer) for a global sample of 160 populations of *P. nodiflora* sourced from Asia, Australia, central America, the Mediterranean, southern North America, South America and Africa. Analyses included maximum likelihood, maximum parsimony, a Bayesian estimation of phylogeny and a parsimony network analysis which provided a genealogical reconstruction of ribotypes. We evaluated phylogenies against extensive historical and biogeographical data. Based on the sequences, 64 ribotypes were identified worldwide within *P. nodiflora* and considerable geographic structure was evident with five clades: one unsupported and the remaining weakly supported (bootstrap support ranging from 52% to 71%). Populations from central and southern North America formed the core area in the indigenous range and we have detected at least three native lineages outside of this range. Within Australia *P. nodiflora* is represented by at least one native lineage and several post-European introductions. *Phyla nodiflora* is one of the few species in the family Verbenaceae to have a pan-tropical native distribution, probably resulting from natural dispersal from America to Africa then to Australasia. However, it has also undergone human-mediated dispersal, which has obscured the native-origin of some ribotypes. These introductions present a risk of diluting the pan-tropical structure evident in this species and therefore they have important conservation implications.

Keywords: biogeography; nuclear DNA ITS variation; native versus alien status; *Phyla nodiflora*; *Phyla canescens*; phylogeny; ribotype analyses

1. Introduction

Cosmopolitan species provide opportunities to test hypotheses on dispersal processes (vicariance versus long distance dispersal) and with modern phylogenetic tools, to clarify the status of species

as native or alien to an area. Native (indigenous) plants have been defined as "Taxa that have originated in a given area without human involvement or that have arrived there without intentional or unintentional intervention of humans from an area in which they are native" [1]. For some species, strong evidence suggests that their wide distribution is due to non-human dispersal mechanisms (e.g., by ocean currents *Cakile edentula*, [2]; ancient tectonic plate movements *Pteridium*, [3]. In other species it is clear that humans have aided the movement of plants (e.g., sweet potato, [4]) and there is a third class in which the distribution of the species is cryptogenic [5]—the basis to the biogeography is puzzling and yet to be resolved.

Phyla nodiflora (L.) Greene (Verbenaceae), a perennial mat-forming herb in neotropical *Phyla* (Verbenaceae), is a cryptogenic species. In the Americas *P. nodiflora* occurs from lower North America (including Florida) to northern South America [6] with a disjunct occurrence in Brazil [6,7]. In Australia, *P. nodiflora* occurs in tropical and sub-tropical regions with several disjunct temperate occurrences [8]. With its distribution on all vegetated continents and many archipelagos [9] occurring over a 9000 km latitudinal range (39.5° N to 44.8° S), it has long intrigued biologists. Depending on their definitions of native status, different authors have considered *P. nodiflora* to be either native to the Americas and dispersed by humans elsewhere [6,8] or with a natural distribution that extends to the old world tropics [10]. The hypothesis that *P. nodiflora* is native outside of the Americas therefore requires testing.

Phyla species radiated within the Americas [11] and *P. nodiflora* is assumed to have originated from there [6]. Recent phylogenetic analyses [11] suggest that the family Verbenaceae has had up to six migration events from the Americas leading to subsequent radiations in Africa (*Lantana*, *Lippia*, *Verbena*, *Chascanum*, *Coelocarpum* and possibly *Stachytarpheta*). A similar natural migration by *Phyla nodiflora* from the Americas to Africa may therefore not be unexpected. Natural migration of species further east to Australia may, however, be exceptional. In fact, Munir [8]) considers that the species was "probably introduced into Australia during the last century and has now become naturalised in most mainland states". However, there is some evidence that it could be native in eastern Australia as the earliest collection was made in 1802 by Robert Brown at Shoalwater Bay (Qld) while on expedition with Captain Matthew Flinders. The Shoalwater Bay area was not occupied by Europeans until 1853 [12] and the only previous European contact in the area was a brief visit by Captain Cook in May 1770.

Our objective was to elucidate the global biogeography of *Phyla nodiflora* and to determine its native/alien status outside of the Americas and particularly for Australasia. This is important because determining whether distributions are natural or human-induced has implications for decisions regarding weed control e.g., providing insights for management [13] and conservation. We constructed a molecular phylogeny of *P. nodiflora* and combined this with phytogeographical and ecological evaluations of the species. We applied these data to a set of criteria we collated from Webb [14] and Bean [15] (Table 1) to determine invasive or native status. Our aim was to circumscribe the native range of *P. nodiflora*, to explore dispersal mechanisms and routes and to test the hypothesis that *P. nodiflora* is native outside of the Americas.

2. Materials and Methods

2.1. Study Species

Phyla nodiflora is an herbaceous perennial from the Americas [16]. It was considered to be a separate species from *P. canescens* (Kunthe) Greene in taxonomic revisions by Kennedy [6] and Munir [8], but most recently O'Leary and Múlgura [7] made a new combination and listed these taxa as varieties of *P. nodiflora* (*P. nodiflora* var. *nodiflora* and *P. nodiflora* var. *minor* (Hook) N. O'Leary & Múlgura). Their distinction was based on leaf shape, leaf size, leaf apex characters and leaf indumentum. In this paper we follow the taxonomy of Kennedy [6] and Munir [8] because (i) the species are morphologically distinctive [6,8,17] (Figure S1a,b); (ii) they appear to have a mostly non-overlapping distribution in their native range [6] and Australia [6,17]; (iii) have distinctive climatic preferences [8,17] and (iv) the species vary in their breeding biology (Fatemi, unpub data, see below; [18]).

Table 1. Criteria and characteristics of alien and native (indigenous) species, particularly for Australia, adapted and modified from Bean [15].

Criterion	Characteristics Likely to be Associated with a Native Species	Characteristics Likely to be Associated with an Invasive Species	Equivocal Information
1	(a) consistently occurs in intact unmodified habitat	(b) species known only from croplands, roadsides and other frequently disturbed sites	
2	(a) is not persistently invasive in its area of occurrence	(b) persistently invades or encroaches upon natural communities	
3	(a) is attended by a range of pests or diseases	(b) species that is pest- and disease-free	(c) Damage to herbarium material may have occurred post collecting
4	(a) displays a range of phenotypic or genetic diversities	(b) phenotypically or genetically uniform populations, probably derived from a single introduction	
5	(a) does not display any post-settlement expansion of geographical range within the region	(b) species that has a known or inferred expansion in its range over the past 100–150 years	
6	(a) any discontinuities of distribution of the species within the region are related to climatic and edaphic factors	(b) a species with a patchy distribution correlated with human settlement patterns is probably alien	
7	(a) a species is probably indigenous if closely related species occur as natives in Australia or nearby (e.g., New Guinea, Timor, Java, New Zealand)	(b) where the closest relatives occur on another continent, the species is likely to be an alien	
8		(b) a species known to be alien in areas outside the region must be under suspicion of being alien within the region	
9		(b) a species is probably not indigenous to the region if the nearest occurrence outside the region represents a major disjunction	
10		(b) the initial herbarium record dates well after the first European settlement of the region	(c) alternatively, if the initial herbarium record precedes or is soon after a European settlement, no useful conclusions can be drawn
11	(a) plant has an established ethnobotanical use by indigenous peoples	(b) a written record exists of the introduction or importation of a species in a journal, nursery catalogue, or botanic gardens listing	(c) alternatively, if no written record exists, no useful conclusions can be drawn

This is further supported by results from this study (see results section). O'Leary and Múlgura [7] do not cite the monograph by Kennedy [6]. However, it would appear that Kennedy [6] and O'Leary and Múlgura [7] also differ in the circumscription of species limits as the same specimens from southern South America are allocated to different species by them (e.g., *MacBride 5880*, *US*, is recognised as *P. canescens* [6] or *P. nodiflora* var *nodiflora* [7]).

2.2. Phylogenetics

2.2.1. Plant Material Collection

Leaf material of *P. nodiflora* was sourced as comprehensively as possible from across its global distribution using herbarium samples, supplemented by additional field surveys conducted in Australia, Iran, eastern Mexico and Florida, USA. In addition, representative *P. canescens* and *Phyla* spp. (specimens that were morphologically similar to either *P. canescens* or *P. nodiflora* but could not be definitively identified) populations were included from a parallel global survey (Fatemi, unpublished data).

For the nuclear DNA assay, we gathered leaf material for *P. nodiflora*, *P. canescens* and *Phyla* spp. from herbarium specimens or living plants from 445 samples (living accessions or herbarium specimens from 20 herbaria, see Acknowledgements) worldwide and from these we successfully extracted DNA from 181 samples, representing 179 populations. These samples were distributed as follows; *P. nodiflora* (160 populations, 161 samples) from the Americas (1 sample from each of $n = 35$ populations), Australasia ($n = 65$), the Mediterranean ($n = 3$), Africa (including the Middle East and Indian oceanic islands, $n = 31$, with two samples from one population in Iran), Asia ($n = 24$) and Hawaii ($n = 2$); *P. canescens* (19 populations, 20 samples) from South America ($n = 6$ populations, with two samples from one population in Bolivia), Hawaii ($n = 1$) and Australia ($n = 6$) and *Phyla* sp. from Australia and South America ($n = 6$). Most of the 181 samples were unequivocally from single plants in discrete populations, i.e., we did not repeat sample at that location, or if we did (Iran and Bolivia) we spaced 20 m between collections. We consider it unlikely that these samples were from the same genet. Effort was directed at sampling areas of taxonomic uncertainty including populations from the Balranald-Mildura region of southern NSW, northern Victoria and south-eastern South Australia ($n = 4$) and northern Argentina ($n = 5$).

2.2.2. DNA Extraction, Amplification and Sequencing

DNA was obtained from small amounts of leaf material (c. 20 mg) taken from herbarium sheets (see above) or from fresh leaves collected during field studies ($n = 22$ populations, Table S1). Our sampling from herbarium sheets included contemporary and very old specimens (*Wilkes s.n.*, 1838, Philippines, NY1239756) and overall we had mixed success with extracting DNA from them. The oldest specimen from which we successfully extracted DNA was from 1899 (*Tracy 6472*, Biloxi, Harrison County, Mississippi, 23 June 1899, US). Many contemporary collections failed to yield useable DNA (e.g., *Okebiro 701*, Kenya, Kiserian-L. Baringo; 19 March 1989, K). Fresh leaf samples were desiccated by placing them in plastic bags containing silica gel and herbarium vouchers for them were deposited with CANB, NE or SI. DNA was readily extracted from all fresh samples. From the 181 samples that yielded DNA of sufficient quality, we examined sequence variation in nuclear ribosomal internal transcribed spacers (nrDNA-ITS, ITS1, 5.8S rDNA and ITS2). Our pilot work with *rbcL* and *trnL-F* and more recent extensive investigation with *trnL-F* and *PetB* using leaf material from 262 populations (Data S1) did not yield variable regions and we abandoned investigations with these markers. Population locations, methods and results for chloroplast investigations are available in Supporting Information (Appendix A, Data S1).

Total genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Chadstone, Vic, Australia) or Wizard SV Genomic DNA Purification System (Catalogue number A2361, Promega, Sydney, NSW, Australia). The entire internal transcribed spacer region (ITS1/5.8S/ITS2) was amplified using primers

ITS4 and ITS5 [19]. Each 25 µL of amplification reaction contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20, 10% DMSO, 4 mM $MgCl_2$, 0.4 mM of each dNTP, 0.5 unit *Taq* Polymerase (GoTaq®Flexi DNA Polymerase, Promega), 0.5 µM each ITS4 and ITS5 primer and 20 ng genomic DNA. Dimethyl sulfoxide (DMSO, 10%) was included in the reaction mix to exclude the amplification of low stability templates and to avoid preferential amplification of paralogous gene copies or nonfunctional pseudogenes [20–22]. Each PCR product was electrophoresed in a 1% agarose gel, stained with SYBR safe (Catalogue number S33102, Invitrogen, Thermo Fisher Scientific Australia, Scoresby, Vic, Australia), then excised and eluted using Wizard SV Gel and PCR Clean-Up System (Catalogue number A2361, Promega). PCR fragments were sequenced using both forward and reverse primers by SUPAMAC (The University of Sydney, Australia).

2.2.3. Sequence Data Preparation

DNA sequences were proofed and edited in FinchTV version 1.4 (available from www.geospiza.com/finchtv). nrDNA-ITS sequences generated in this study have been deposited in the Genbank database and accession numbers are listed in Table S1. Forward and reverse reads were analyzed for all sequences. The guidelines for obtaining reliable nrDNA-ITS sequences in plants [23] were followed to minimise the pitfalls of using ITS sequences to infer phylogenetic patterns. This included the addition of DMSO, repeated PCR reactions, sequencing complementary strands to verify the variable sites and verifying the secondary structure stability of sequences. DNA sequences were aligned using ClustalX [24] with default parameters for gap penalty and extension. One sequence for *Lantana camara* L. (Genbank AF437853) from Taiwan and two sequences for *Lippia alba* (Mill.) N.E.Br. (Genbank EU761076 and EU761078) from Colombia were used as out-groups. The beginning and end of the ITS regions were determined with reference to the *Lantana camara* sequence from Genbank (AF437853). Prior to the phylogenetic analyses, accessions with identical sequences were detected and merged using Jalview 2.4.0.b2 [25]. In total 80 ribotypes were included in the analyses as an ingroup (Table S1).

2.2.4. Consensus Tree Analyses

Consensus tree analyses included maximum likelihood (ML) and maximum parsimony (MP) using PAUP*4.0 beta 10 [26] and MEGA 5.0 [27] and the Bayesian estimation of phylogeny using the MrBayes version 3.1.2 [28]. Maximum parsimony was performed with gap states treated as a fifth character [29] and all character transformations weighted equally. Bootstrap analyses [30] were performed by resampling the data matrix 1000 times. To check for consistency with the results of the parsimony analysis (see below) we first determined the model of nucleotide substitution with ModelTest v. 3.7 [31] and ran two million generations of four simultaneous Markov Chains to approximate the posterior probabilities of trees. A single tree from every 200 generations was retained for the construction of the Bayesian consensus tree. To infer evolutionary divergence between ribotypes within *P. nodiflora*, evolutionary distances were measured in the units of the number of base pair substitutions per site [32] and calculated by using the Maximum Composite Likelihood model [33] incorporated into MEGA 5 [27]. Gaps were treated as complete deletions and standard errors for evolutionary divergence measures were obtained by a bootstrap procedure with 1000 replicates. Values in the distance matrix indicate evolutionary divergence between two ribotypes. A zero indicates that the difference between the two ribotypes is only in a gap or deletion.

2.2.5. Parsimony Network Analyses

We used parsimony networking methods to visualise character incongruence caused by reticulation. Network methods can incorporate population processes in building and refining relationships and also allow a more detailed display of population-level information than bifurcating trees [34]. Parsimony networks were obtained with the software TCS [35], which uses statistical parsimony and genealogical reconstruction algorithms from Templeton et al. [36]. To minimise the

effect of gap coding on the accuracy of the parsimony network and to overcome the problem of order dependent collapsing of sequences into haplotypes, as described by Joly et al. [37], we reshuffled the data matrix and re-analysed the data until all individual sequences were assigned to their respective ribotypes.

2.3. Biogeography—Delimitation of Native-Range Distributions of P. nodiflora Clades

We gathered ecological information on the populations of *P. nodiflora* from journal articles, books, reports, archived newspapers (National Library of Australia, www.//trove.nla.gov.au) and from 336 herbarium vouchers in addition to those sampled for DNA (AD, BRI, CANB, CBG, DNA, HO, MEL, NSW and PERTH). Care was taken with historical searches to exclude *Aloysia citriodora* Ortega ex Pers. (Lemon Verbena) as it has been variously known as 'Lippia' owing to an earlier combination as *Lippia citriodora* H.B. & K. We evaluated our data against Bean's [15] topology, which we broke down into eleven criteria (Table 1), for *Phyla nodiflora* and for each major clade within the species (see results). We gathered information for each criterion where available. For criterion five (post-settlement expansion) and using data from the Australian Virtual Herbarium (sourced 4 April 2012), we compared the number of herbarium vouchers collected over time for *P. nodiflora* ($n = 311$) with (a) endemic *Scutellaria humilis* R.Br (Lamiaceae) ($n = 292$), a small trailing herb found in moist places across five states in Australia and (b) the unequivocally alien shrub in Australia, *Lantana camara* L. (Verbenaceae) ($n = 595$). Our expectation here was that all species would show an increase in the number of voucher records with time since settlement as a consequence of heightened survey effort, but an alien species would show a major range expansion over time compared with the native species, *S. humilis* (and see [15]). Duplicate samples were excluded in the analyses. While herbarium collections can have inherent biases reflecting non-random sampling by botanists (e.g., [38]), we assumed that these biases would be similar across the three species.

3. Results

3.1. Phylogenetics

3.1.1. Characteristics of the ITS Sequences and Ribotype Discovery

The aligned ITS data matrix was 630 bp long. There were 101 variable characters, of which six were indels and 95 were base substitutions. A high number of sites in ITS1 were variable (58 sites), whereas only 10 and 33 variable sites were observed in 5.8S and ITS2 regions respectively (Table S2).

Based on nrDNA-ITS sequences of 181 samples (179 populations), a total of 64 ribotypes were identified worldwide within *P. nodiflora*, 11 for *P. canescens* and five for undetermined *Phyla* species (Table S1). Our global sampling of *P. nodiflora* ($n = 160$ populations) included 35 populations from the Americas where we found 10 ribotypes, a discovery level of 29%; for Australia we sampled 65 populations which yielded 26 ribotypes, a discovery level of c. 40% and from the rest of the world we sampled 61 populations where we found 38 ribotypes at a discovery level of 62%.

3.1.2. Consensus Trees

Five equally maximum parsimonious trees 806 steps long, with a consistency index of 0.75 and retention index of 0.78 were resolved. The strict and semi-strict consensus trees were identical. The Bayesian consensus tree of the 10,000 trees sampled in the analyses was highly congruent with the MP and ML analyses (Figure 1) with slight differences in branching topology among poorly supported nodes. The Bootstrap (BS) and Bayesian analyses provided 100% support for the *Phyla* clade. Within the *Phyla* clade three major *Phyla* lineages were recognized each with at least 97% BS support: a *Phyla* spp. clade, *Phyla canescens* and *Phyla nodiflora* (Figure 1). The *Phyla* spp. clade was recorded in South America, the Gulf of Mexico and southern Australia (Figure 2) and may represent several taxa (analysis not shown). *Phyla canescens* was found in central South America, Hawaii and Australia (Figure 2).

In the Americas *P. nodiflora* was restricted to central and North America and northern South America, but it was the most wide-spread and commonly sampled species elsewhere in the world (Figure 2).

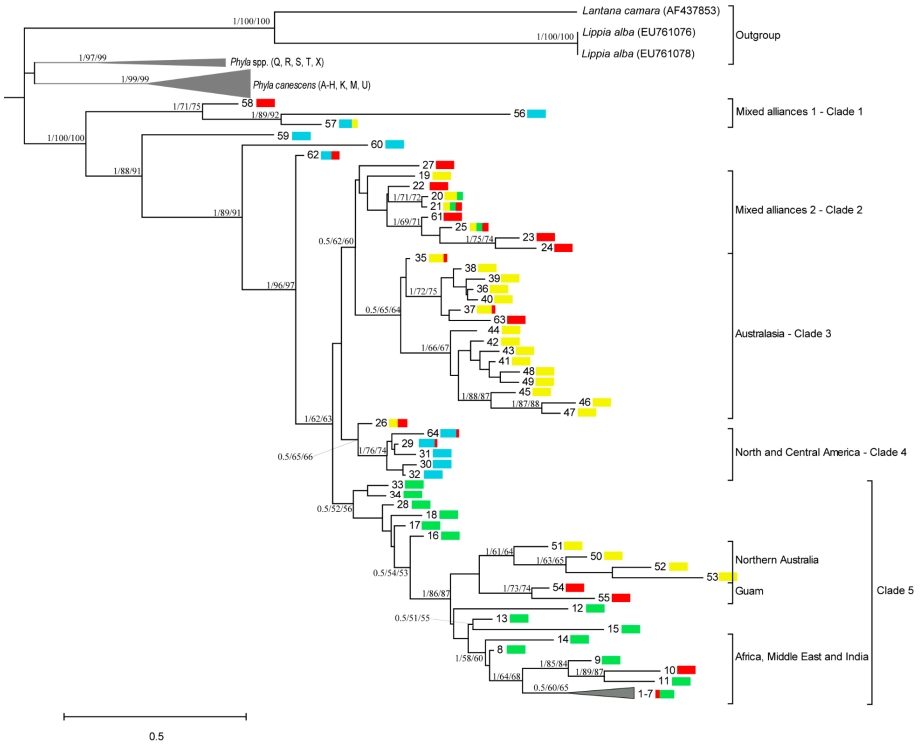

Figure 1. The maximally parsimonious tree based on nrDNA-ITS sequence data. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Numbers on branches indicate posterior probabilities or bootstrap values for Bayesian analyses/parsimony analysis/maximum likelihood. Ribotypes are terminal taxa and details for them are given in Table S1 and Figures 2 and 3. Colour coding matches the global colour scheme in Figure 3 and the bandwidths are not informative. AF437853 = *Lantana camara*. Five clades, 1–5 are recognised.

There was considerable structure evident within the *P. nodiflora* clade. We recognize five clades, one that is unsupported (Clade 2) and the remaining four clades weakly supported with bootstrap support ranging from 52% to 71% (Figure 1). In addition, ribotypes 59 (Utah), 60 (New Mexico) and 62 (Vietnam, Mexico) were not grouped with any major clade within *P. nodiflora* (Figure 1).

Clade 1 contained three distinct ribotypes which together spanned three continents (Asia, North America and Australia) (Figures 1 and 2). It included two ribotypes from Central America, one of which was also recorded in eastern Australia and one only recorded in Asia (China).

Clade 2 was distributed globally, including in Africa, Australia, Asia, Europe and two Oceanic Islands, although it was notably absent from the Americas (Figure 1). This clade contains widely distributed ribotypes 21 and 25, each present across three continents (Figures 1 and 2). Ribotype 21 has the largest latitudinal spread from southwest Western Australia through to China (Figure 2.)

Clade 3 was largely restricted to Australia (and the island of New Guinea and East Timor) (Figure 1). Fifteen of the 16 ribotypes were recorded from Australia. Ribotype 37 was widely distributed across northern Australia, Timor and the island of New Guinea. Only one ribotype, 63 from Palau, was not found within Australasia (Figure 2).

Clade 4 was largely restricted to the Americas, although one ribotype was also present in Taiwan (64) and one in Hawaii (29) (Figure 1). In the Americas clade 4 ribotypes were recorded in south-eastern USA, Mexico, South America (coastal Ecuador and Venezuela) and the Caribbean (Figure 2).

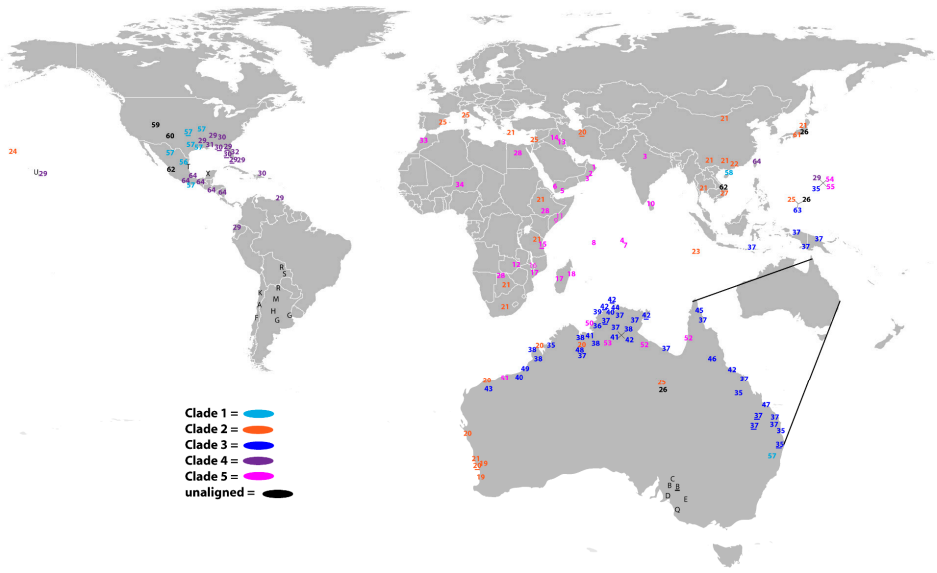

Figure 2. The global distribution of the 64 ribotypes discovered for *Phyla nodiflora* (numbers), the eleven ribotypes discovered for *P. canescens* (A-H, K, M, U) and five ribotypes (unaligned) from an undetermined taxa or taxon within *Phyla* (Q-T, X). Underlined numbers represent multiple collections of that ribotype from that location area. *Phyla nodiflora* ribotypes with clade support are coloured according to clade number on Figure 1 or if unaligned they are in black notation. Further details of ribotypes are given in Table S1.

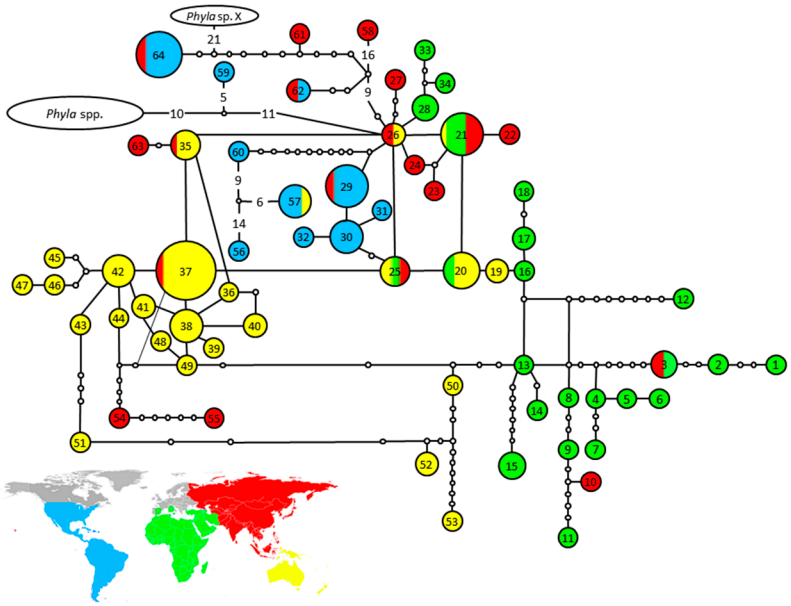

Figure 3. A parsimony network displaying the relationships among the 64 *Phyla nodiflora* nrDNA-ITS ribotypes. Each link between the ribotypes represents one mutational difference. Unlabelled nodes indicate inferred steps not found in sampled populations. Loops in the network are the result of homoplasies. The angle of bifurcation and the length of links between ribotypes have no significance. Each circle is proportional to the ribotype frequency. The colour of each ribotype relates to the biogeographical region in the insert map. Circles with multiple colours are ribotypes present in two or more biogeographic regions. Ribotype numbers and letters are explained in Table S1.

Ribotype 26 was an unsupported sister to clade 4 and was not detected in the Americas but was found on Palau and in Australia.

Considerable genetic and geographic structure was evident in Clade 5 (Figure 1). Most ribotypes were restricted to Africa, the Middle East, the subcontinent and islands in the Indian Ocean (20 ribotypes). However, there were also sub-clades within Clade 5 restricted to Australia (4 ribotypes) and Guam (2 ribotypes).

3.1.3. Parsimony Network

A parsimony network of the ribotypes derived from the ITS region in *P. nodiflora* revealed a complex structure within this species (Figure 3) and is in broad congruence with the phylogenetic analyses. Ribotypes of *P. nodiflora* were connected with 15 or more evolutionary steps to the ribotypes of other species of *Phyla*. Several loops (homoplasies or recombination) were detected within the parsimony network, indicating low resolution in the ITS sequence data or recombination.

Strong biogeographic structure was revealed by the parsimony network analysis (Figures 2 and 3). No ribotype was found on every continent. Only ten of the 64 *P. nodiflora* ribotypes occurred in two or more biogeographical regions (ribotypes 3, 20, 21, 25, 26, 29, 35, 62, 64 and 57). Three of these were present in the Americas (62, 64 and 57). Ribotype 57, a ribotype found extensively in the indigenous American range, was also discovered in eastern Australia in a floodplain disturbed by cattle grazing. Ribotype 25 was the most widely distributed type detected in our study being found in the Middle East, France, Spain, South-East Asia and Australia, but not the Americas. Ribotype 26 was found in Asia (Japan, Palau) and Australia. Ribotype 21 was detected in the Mediterranean, Asia, Africa and Australia. Ribotype 20 was found in Iran and Western Australia. Ribotype 37, the most common ribotype discovered by our sampling in Australia, is shared between Australia (Northern Territory and Queensland), Timor, PNG and West Papua. Ribotype 37 differs by one nucleotide from common ribotypes 25 and 35 and these are the closest links outside of Australia.

Twenty-five of the 64 ribotypes (c. 39%) discovered world-wide were found in Australia and 18 of these (c. 72%) are unique to Australia (ribotypes; 19, 36, 38–53, Figure 3). Ribotype 37, followed by ribotype 42, are the most common endemic types in Australia and they span the tropics of northern Australia (Figure 2). Ribotype 35 is found on the east coast of Australia and in Guam and differs by one nucleotide from the common eastern hemisphere ribotype 26.

Three ribotypes were present on remote islands in the Indian Ocean. Two ribotypes, 4 and 7, found in the Indian Ocean archipelago of Chagos, differed in one deletion and two substitutions respectively at positions 56, 152 and 222 of ITS1. These ribotypes are closely related to ribotypes 5 and 6, found in nearby Yemen, which differ from each other in two substitutions at positions 10 and 182 of ITS1. Ribotype 8, from the Seychelles in the Indian Ocean is closely related to ribotype 9, found in nearby Somalia. They differ in three substitutions at positions 6, 8 and 15 of ITS1 (Table S2).

Ribotypes 54 and 55 in Clade 5 were only found on the island of Guam but differed in five substitutions in their ITS profile (two in ITS1, two in 5.8S and one in ITS2 regions). They were found in disturbed areas near a military base, which would offer opportunities for multiple introductions (e.g., ribotype 35 from Clade 3, ribotype 29 Clade 4 were also found on Guam).

The largest genetic distance observed between two ribotypes was between ribotypes 1 (Oman) and 56 (Mexico) with a divergence value of 0.0826 (Supporting Information, Data S2)—this difference was due to 50 substitutions and three deletions. The differences between ribotype 56 and 11 (Somalia) was also relatively large at 0.0788. Whereas, divergences within regions were generally not pronounced. For example, the distance between ribotypes 29 and 32 was only 0.0016 and ribotype 47 compared with 37, both from Australia, was 0.0065.

3.2. Biogeography—Delimitation of Native-Range Distributions of P. nodiflora Clades

Each of the five clades was associated mostly with one or two biogeographic regions. We attempted to distinguish the likely native-range limits of the clades from human-assisted dispersal, based on our hypothesised phylogeny and additional evidence reviewed against a typology presented in Table 1. In the native range, we would expect a range of genetic diversities to be present with genotypes

structured geographically. Furthermore, a natural distribution would be reflected in the species occurring in intact unmodified habitats, accompanied by a lack of invasive encroachments, a lack of post-European expansion in geographical range and any discontinuities of distribution would be related to climatic, geological and edaphic factors. In an alien species, patterns of genotypic diversity would not be expected to be structured geographically. Human-mediated dispersal would also be indicated by the species being found mainly in disturbed areas, with invasive encroachments on native communities, an expansion of range in the last 200 years and a patchy distribution correlated with human settlement.

We will refer to the criteria in Table 1 as C1 to C11 throughout this section. We found that a constraint to utilising Bean's [15] criterion 1 fully (C1a intact habitat versus C1b frequently disturbed sites) is that *Phyla nodiflora* is a pioneer species in its native range of lower North America to northern South America, where, Kennedy [6] notes that it is weedy. Kennedy [6] also notes that *P. nodiflora* is tolerant to a wide variety of environmental situations and is often associated with water sources. In the context of *P. nodiflora*, we restricted C1a to cover pristine or near pristine habitats and C1b to highly disturbed areas with other signs of gross disturbance including the presence of invasive species.

3.2.1. Clade 1—Mixed Alliances 1: Americas (North, Central, Northern South America)

Ribotypes from this clade occur in the native range of *P. nodiflora* (sensu Kennedy [6]). General ecological information on the species from this region (which could also apply to Clade 4 populations, see below) includes that *Phyla nodiflora* is known to be a pioneer and a weedy species [6]. Plants from ribotypes 56 and 57 grow in river and in irrigation channels, floodplains and alluvial fans and therefore match C1b in Table 1. However, plants have been found in native plant communities (C1a, Table 1) as well as townships (C1b, Table 1) but not as an invasive species (C2a, Table 1). The butterfly *Phyciodes phaon* Edwards (the Phaon crescent), is restricted to the Americas and uses *P. nodiflora* as a food plant for its larva in Florida [39]. This is strong evidence of *P. nodiflora*'s nativity to lower North America (C3a). Furthermore, Genc et al. [39] report that several species of native Lepidoptera use the leaves of *P. nodiflora* as a food plant in this region. Bean [15] argues that a range of phenotypes (C4a) would be indicative of a native species and the corollary that uniform diversity would be suggestive of an alien lineage (C4b). In the Americas however, where *P. nodiflora* is indigenous, Kennedy [6] notes the extensive variation in vegetative characters but that the variation does not have a geographical basis. In Bentham's [40] *Flora Australiensis* and in regard to the species' global diversity, he notes that *P. nodiflora* "is very variable in the breadth of the leaves, the size of the spikes and the flowers, the points and teeth of the bracts, &c". Ribotype 58 was restricted to China and the specimen was found growing on coastal sands. Ribotype 57 was also detected in a single population in eastern Australia (*Nelson, s.n. NE*) in a flood plain disturbed by cattle. This isolated occurrence in Australia (Figure 2), location and clade position (Figure 1) and network position (Figure 3) accords with Bean's [15]) criteria for characteristics associated with an alien species (C1b, C2b, C5b, C6b, C9b, C10b) and we suggest that this ribotype has recently arrived in Australia from the Americas.

3.2.2. Clade 2—Mixed Alliances 2: Asia, Africa, Australia

Ribotypes from this clade are highly mobile with three of them occurring in two or more continents (Figures 1 and 3). We tentatively propose that this clade originates from Asia where it is common. Supporting this and against Criterion 7, the Verbenaceae have been found in fossil pollen records for China (pollen resembling *P. nodiflora*, Miocene Heilongjing Formation, [41]). In Asia, plants have been found along the seashore (Japan) and very disturbed roadsides (China). Further east, on Midway Atoll, the plants were recorded in a drainage ditch and as a new record in 1999 on Sand Island (*BISH 659789*). *Phyla nodiflora* is listed as a food plant for several species of butterfly in Asia including *Junonia almana* L., *Junonia atlites* L., *Junonia lemonias* L. and *Junonia orithya* L. [42] suggesting an evolutionary relationship between these herbivores and host-plant. In Australia and Africa, Clade 2 ribotypes match many of the alien criteria of Bean's (2007 [15]) topology (Table 1; C1b, C2b, C5b, C6b, C9b, C10b) as they grow

in very disturbed areas associated with farming and irrigation and co-occur with invasive species such as *Salvinia molesta* D.Mitch. (Kununurra, W.A., *Thompson s.n. 26 July 2007, CANB*). Location data from specimens of *P. nodiflora* were mapped against region and collection time revealing that Clade 3 is disjunct from the main distribution of *P. nodiflora* in Australia (Figure 4, C9b) with the majority of collections occurring after 1980 (C10b). However, in 1899 E.J. Bickford President of the Mueller Botanical Society of W.A. lists 'Lippia' as a wildflower of the districts and sand plains north of Perth (Western Mail, 9 December 1899). We reject the possibility that this early record was *Phyla canescens* as that species has never been collected from this region in W.A., nor is it found in intact ecosystems. Furthermore the earliest gatherings of *P. nodiflora* in Western Australia were made by Augustus Oldfield (*Oldfield s.n. 1850–1859, MEL583739A*, *MEL583740A*) between 1850–1859 around the Murchison River area near Kalbarri which was being explored for mining and agriculture from 1839 [43], although not well explored until the 1850s (p. 35, [44]). We cannot determine the ribotype of these Oldfield specimens although ribotype 20 from the historic Grey's Well at Kalbarri is a close geographic match. A ribotype from Clade 2 may have existed naturally in the Kalbarri region.

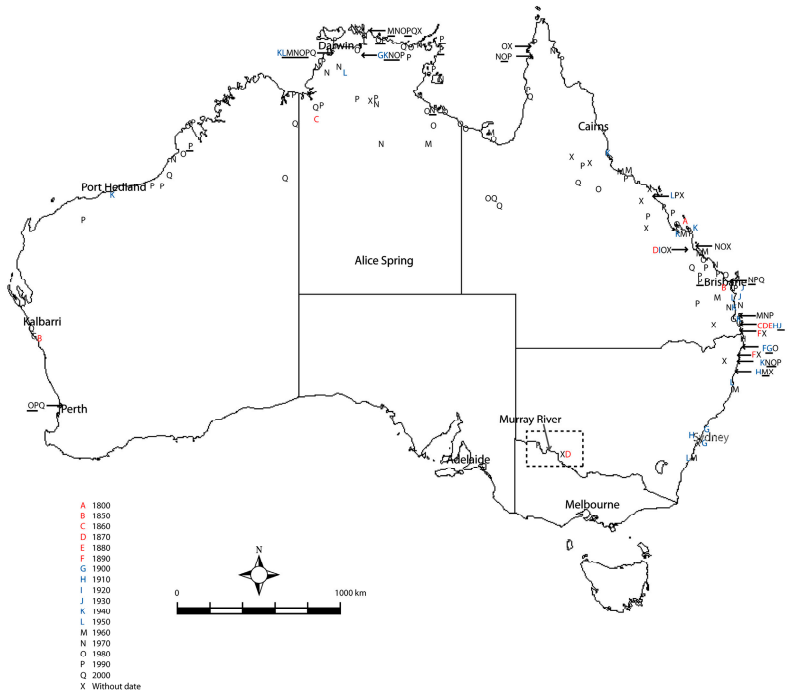

Figure 4. The location and decade of Australian herbarium-collections for *P. nodiflora* in Australia. An underlined letter indicates that multiple collections exist for that location in that decade. Red letters indicate specimens that were collected prior to 1900. The rectangle insert indicates specimens listed in herbaria as *P. nodiflora* but have been determined by us to be *P. canescens*.

3.2.3. Clade 3: Australasia

We propose that this clade is native to Australia. The ribotypes are found in areas that are relatively undisturbed by human activities (C2a, see also C10, Table 1). For example, *Phyla nodiflora* is scantily distributed on the floodplains of Oenpelli, a remote location in the Northern Territory (A. Mitchell, pers. comm. 21 February 2011, *Specht 1181, 13 October 1948, AD*; *Cowie 2119, 13 November 1991, MEL*). On the mainland of Australia we failed to find data (either from herbarium records or from our own field work) where *P. nodiflora* has intense infestations, although plants can be found in disturbed areas co-occurring with invasive species (*Vachellia farnesiana* (L.) Wight & Arn, Bullo River Station, NT, *Kerrigan 1040, 10 March 2006, DNA*). Ribotype 44 was found on mainland Australia and on Croker Island (Arafura Sea, Northern Territory) where *P. nodiflora* has invaded the seasonally

inundated Paludal plains and where the invasion has been facilitated by feral animals overgrazing the area [45]. Clade 3 *Phyla nodiflora* found in both modified and unmodified habitats in Australia (C1a and C1b Table 1) including rubbish tips, drainage channels, roadsides and built recreational areas with lawns (WA, Karratha, R.D. van Klinken s.n. unpub. data). In concert with the behaviour of *P. nodiflora* in its native range, we found habitat preferences suggestive of both a native and alien species. Against Criterion 2, Clade 3 *P. nodiflora* are mostly an occasional or sometimes a common component of natural communities (Ord River, W.A., R.D. van Klinken s.n. 24 July 2007 unpub. data)—but apparently not to the extent that it dominates the community. For Criterion 3 herbarium label data were not informative for the presence or absence of pests and diseases in *P. nodiflora*. Plant collectors may avoid diseased or imperfect specimens when collecting and thus screening herbarium specimens for pests and diseases may not be informative. We have seen an image of the first specimen collected in Australia (*BM, Bennett No. 2335*, image at NE, see Criterion 10) and about half of the leaves are imperfect with holes and other insect damage. This could have happened after the specimen was collected. We found listings for the butterfly *Junonia villida* Fabricius using *P. nodiflora* as a food plant but it also forages on the alien and invasive *P. canescens* (e.g., [46]). We thus do not have unequivocal information of an evolutionary relationship between *P. nodiflora* and native herbivores in Australia.

Bean (2007 [15]) argues that a range of phenotypes would be indicative of a native species and the corollary that uniform diversity would be suggestive of an alien lineage (C4). For Australia, Munir [8]) and Macdonald [17]) note the extensive variability within *P. nodiflora* as well as within the alien and invasive *P. canescens*. Due to the variability expressed in the Americas (see above), we conclude that the variability observed in *P. nodiflora* in Australia is not a useful indicator for its status in Australia. For Criterion 5 the collection dates on herbarium specimens were collated and plotted as a percentage of the collecting effort for each species and against decades for *P. nodiflora*, the native herb *Scutellaria humilis* and the alien *Lantana camara* (Figure 5). As expected *L. camara* has had a rapid expansion of collection records, especially from the 1950s. Overall *S. humilis* was collected more often than *P. nodiflora* up until the 1970s from when *P. nodiflora* was collected more frequently (Figure 4). The data for *P. nodiflora* is somewhat intermediate between the patterns observed for native *S. humilis* and alien *L. camara* and we tentatively suggest a post-settlement expansion in Australia has occurred for *P. nodiflora* especially since 1970.

Against Criterion 6 and Clade 3 in Australia, most of the Australian populations are associated with tropical habitats and freshwater or coastal dune systems. For Criterion 7, *Phyla nodiflora* is most closely related to the South American *P. canescens* [6]. Kennedy [6] also considers the North American species *P. cuneifolia* (Torr.) Greene to be closely related to *P. nodiflora* while O'Leary and Múlgura [7] consider that *P. nodiflora* is the taxon most similar morphologically to *P. lanceolata* (Michx.) Greene. *Phyla canescens* is a highly invasive herb in Australia [18,47–49] whereas *P. cuneifolia*, *P. fructicosa* and *P. lanceolata* are not listed as occurring in Australia (Australia's Virtual Herbarium, accessed 26 February 2017). The taxa outside of Australia with the closest native range to *P. nodiflora* include the Latin American *P. canescens*, *P. betulifolia* (Kunth) Greene and *P. fructicosa* (= *P. nodiflora* var. *reptans* (Kunth) Moldenke, [7]) but none of these taxa is native to Australia's near neighbours of New Guinea, Timor, Java or New Zealand. For Criterion 8 (Table 1) several islands in Asia (e.g., Guam) hold alien populations of *P. nodiflora* and are potential source areas for distribution into Australia. Gupta et al. [10] consider that *P. nodiflora* is native to the whole of Africa, temperate and tropical Asia, Australasia, Europe and tropical America and that it is wide-spread and locally common in "open and wet places near streams, ponds, paddy fields, ditches, backwaters, brackish water". Location data taken from specimens of *P. nodiflora* were mapped against region and collection time revealing that *P. nodiflora* has a disjunct distribution in Australia (Figure 4). Populations are found in the north of Australia, with sparse occurrences along the east coast (C9b, Table 1). For Criterion 10 (Table 1) the earliest collection of *P. nodiflora* from Australia was made in 1802 by Robert Brown at Shoalwater Bay Qld (*BM, Bennett No. 2335*, image at NE) and is within the Clade 3 geographic distribution. At least a further 16 collections from the mid to late 1800s have been lodged in Australian herbaria, including collections

from south-eastern Queensland. None of these areas in southern Queensland was unequivocally pristine at the time these specimens were collected. Captain James Cook for example, had made land in the Shoalwater Bay area in 1770 (late May-early June) where an expedition was made to Pier Headland, in the same vicinity that Robert Brown collected from some 32 years later in 1802. There is no record of a Banks and Solander collection of *P. nodiflora* from 1770 (specimen data from the Herbarium of New South Wales, Royal Botanic Gardens and Domain Trust, 21 February 2011) although botanical gatherings were made in the Shoalwater Bay area by them [50]. Three collections were made in the 1890s along the north coast of New South Wales. One herbarium specimen however, provides strong evidence for a pre-European occurrence of *P. nodiflora* in northern Australia (*Mueller s.n. MEL 583741A*). Mueller was the Botanist on the Gregory Expedition into Northern Australia (1855–1856) and in 1856 he wrote that he collected a 'Lippia' on that expedition [51], Appendix II in [52]. This is a significant finding as the Victoria River area that these explorers traversed at this stage of the Gregory Expedition was not settled and was unchartered territory at the time [53]. We did not find any records of its use as a medicinal plant by the Australian Aboriginal peoples.

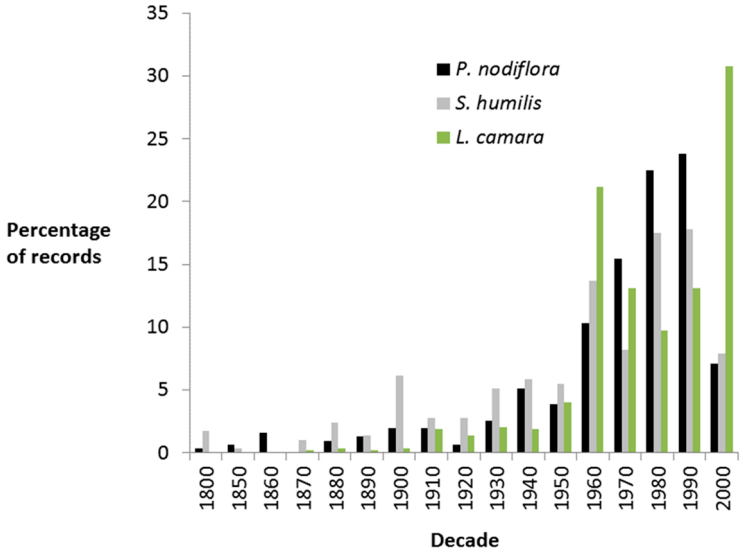

Figure 5. The distribution of Australian collection-records from eight herbaria (AD, BRI, CANB, CBG, HO, MEL, NSW, PERTH) plotted as a percentage of the collecting effort for each species and against time (decadal periods) for *Phyla nodiflora* (n = 311) *Scutellaria humilis* (n = 292) and *Lantana camara* (n = 595).

3.2.4. Clade 4—North and Central America with Asia

We suggest that these ribotypes are native to the Americas with the Asian representatives being exotic to Taiwan and Guam. In the distributional range of Clade 4 in the Caribbean, *Phyla nodiflora* is a host food plant for the butterflies *Junonia genoveva* Cramer and *Junonia evarete* Cramer [42]. In Latin America plants from ribotypes 29 were found along rivers, mangroves, open savannahs, beaches and coastal dunes. In lower North America ribotype 29 plants were found in disturbed areas related to the built environment including airforce bases, boat launches roadsides and empty lots. Ribotype 29 includes a specimen used as a landscaping plant on Guam (*Fosberg 46296 13 July 1965, NY*). We found direct evidence of *P. nodiflora* being accidentally transferred between biogeographic regions. In Hawaii ribotype 29 was grown from soil scraped from a soldier's boot—the soldier was newly arrived from Guam (*BISH 728774*).

3.2.5. Clade 5—Northern Australia, Guam, Africa, Middle East and India

We suggest that this clade is native to Africa but that there has been recent movement from there to Australia and Asia. *Phyla* type pollen fossils have been found in Africa from the Holocene [54] and *P. nodiflora* has a long history as a medicinal plant in Nepal [55], India [56] Taiwan [57] and the Middle East [58]. *Phyla nodiflora* has been recorded from different habitats ranging from pristine dune systems in eastern Africa to black soil flood plains and forests in the Middle East and the Indian subcontinent and from desert oases in Egypt to lake sides in Australia [59–61]. In Africa and the Middle East, ribotypes were found along stream edges, desert oases and natural lakes (C1a) but also in disturbed areas on farms, military camps and air strips (C1b). The ecological data accompanying herbarium specimens for ribotypes 50–53 accords with them being recent arrivals to Australia. All of them were found near coastal settlements and in ruderal habitats such as disturbed areas around developments. In Guam, the samples are also associated with highly disturbed areas (landfill, roadsides on an air base).

4. Discussion

4.1. Molecular Markers

Chloroplast DNA exhibits uni-parental inheritance and a slow rate of mutation [62,63]. We found insufficient sequence variability in three chloroplast regions for resolving relationships within *Phyla nodiflora* or *Phyla canescens*. This is not an unusual finding (but see [64]). For example, in a whole chloroplast genome study of 71 rainforest taxa, Rossetto et al. [65] found 75% of the trees sequenced (up to 18 individuals per species) had less than 25 single nucleotide polymorphisms across an average of 122,373 bp sequenced. Diazgranados & Barber [66], working with 110 species in the subtribe Espeletiinae Cuatrec., Asteraceae, found that most chloroplast regions screened were uninformative or the variability was too low to resolve phylogenetic relationships**.** Low variation in many chloroplast regions highlights that they may be less than ideal for global studies of closely related lineages. ITS regions were more variable and informative with the large clades of *Phyla nodiflora* and *Phyla canescens* strongly supported. Clades within *P. nodiflora* were moderately or weakly supported or unsupported, however the geographic structure of the five clades and the parsimony network of ribotypes discussed below provide a strong argument for the global expansion of *P. nodiflora*.

4.2. The Global Expansion of Phyla nodiflora Involves Native and Invasive Lineages

We set out to determine the native range of *P. nodiflora*, a species with a pantropical distribution. In the Americas, *Phyla nodiflora* is found in unmodified and disturbed habitats and this complicates the determination of its native/alien status in other parts of the world where it also occupies disturbed and undisturbed areas. Our study supports a Neotropical origin for the genus [11] and for *Phyla nodiflora* [6]. Our results also concur with Kennedy's [6] that *P. nodiflora* is indigenous to southern USA, the Caribbean, Mexico and Central America. However, we also found substantial molecular and phytogeographical evidence that *P. nodiflora* has been dispersed widely around the world through both natural and human-assisted spread of propagules. We suggest a North American origin, with subsequent natural dispersal to Africa, Asia and finally Australasia. As such we demonstrate an additional colonisation of Africa to the 5–6 events already determined for Verbenaceae by Marx et al. [11]. In addition, available evidence suggests that human-assisted dispersal has resulted in trans-continental movement of all five clades that we recognized.

Most lineages that we recognized within *P. nodiflora* were present in at least two continents. However, the native range of most could be identified using biogeographic evidence. Two distinct clades were probably native to the Americas, with all five ribotypes from one clade and two of the three ribotypes from another, being recorded from there. The exception was a relatively distinct ribotype that was only recorded from China. Further sampling is required to test its origin, although fossilized pollen evidence from the Miocene Heilongjing Formation (5.3–23 Ma, [41]) does suggest the species, if not necessarily this clade, was already present there at that time.

The status of *P. nodiflora* in Australia is generally considered as naturalised after an introduction to the continent [8], despite it being first recorded there in 1802, soon after European settlement and during first European contact in north-western Australia [51], Appendix II in [52]. However, we identify a lineage that is clearly native to Australia (and possibly Papua New Guinea, West Papua and East Timor). Genetic variation was considerable, although no geographic structure was evident. Only two records of this lineage were found outside Australia, both in Micronesia where all indications are that *P. nodiflora* is alien (see above).

The native-range of the two remaining clades is more ambiguous. The most genetically divergent clade appears to be native throughout Africa, including a subclade that has radiated in and around the Arab Peninsula. A long history of *P. nodiflora* in Africa is also supported by Holocene fossil evidence from Africa [54]. However, a further two sub-clades were only recorded from Australia and Guam respectively, despite available evidence suggesting that they may be alien there and therefore of unknown origin (see below). The final clade, clade 5, was the most ubiquitous, with ribotypes present in Africa (from South Africa to the Mediterranean), Europe, Asia and Australasia and was also the least well supported phylogenetically. Available evidence suggests that it is native to Eurasia, but that it may also have been extensively moved around by humans. For example, it is likely to be alien in Australia where provenances are used as lawns (see below).

Phyla nodiflora grows in surprisingly diverse habitats, in coastal dune systems, along sandy lake shores, shore muds, grasslands, roadsides and salt marshes [59–61,67]. Our sampling included populations growing in seasonally flooded black-soil plains, dune systems and urban environments (such as lawns, roadsides and rubbish tips). However, we found no evidence for ecotypes with differing habitat preferences despite considerable genetic variation within the species.

4.3. Natural Dispersal

All evidence suggests that the genus *Phyla* radiated in the Americas, so *P. nodiflora* most likely dispersed and subsequently radiated from there. Our phylogeny is congruent with movement from the Americas, to Africa and Eurasia and into Australia, although other pathways can't be ruled out. In any case *P. nodiflora* is one of very few non-halophytic and terrestrial plant species known to have achieved such a pantropical distribution (see [3]). However, many taxa have moved naturally across the Atlantic between the Americas and Africa. Renner [68] compiled a list of 110 angiosperm genera in 53 families that contain species from both sides of the tropical Atlantic, with wind and sea currents being identified as the most likely vectors. Dispersal of plant taxa from Africa to Australia has also been documented ([69] and references therein) which Mummenhoff et al. [69] suggest for *Lepidium* L. (Brassicaceae) is congruent with bird migration patterns between Africa and Australia.

We can only speculate on when and how *P. nodiflora* dispersed globally through natural means. Waterbirds are one possibility. *Phyla* seeds have been found in the stomachs of migratory birds in North America [70,71] and seeds are considered to be dispersed by endo and ectozoic means in India [72]. In northern Australia, *Phyla nodiflora* has been found in the stomachs of the Comb Crested Jacana (*Irediparra gallinacea* Temminck) [73], a water bird common to Asia and Australia that is dispersive among water bodies. Sea currents are thought to be responsible for the pantropical distribution of *Hibiscus tiliaceus* L. [74]; and it may also be a pathway for *P. nodiflora* as seeds are tolerant to inundation and saline conditions [75] and *P. nodiflora* grows well in coastal sand dunes in Africa [59] and in Australia (*Carter s.n. 1 July 2007, CANB*).

4.4. Human-Assisted Dispersal

There is evidence that all five clades that we recognize have been moved by humans between biogeographic regions, although some clades appear to have been moved more than others. *Phyla nodiflora*, has long been used and therefore potentially dispersed, by people as a medicinal plant in the tropics of the eastern hemisphere (e.g., [56]), although we failed to find any data on *P. nodiflora* use by indigenous Australians. More recently there are documented cases of *P. nodiflora* being imported

into Australia. For example, 'Lippia' was imported into Tasmania in c. 1902 by the then curator of the Botanical Gardens, Mr Francis Abbot (Western Mail, 16 March 1917). The plant was considered 'an absolute failure' and passed on to Victoria where it was 'condemned as worthless'. Its dismal performance as a lawn substitute in Tasmania and Victoria suggests that the taxon evaluated in 1902 was not *Phyla canescens* and it may have been *P. nodiflora*. It was also imported (as "*Phyla nudiflora*") to Australia in 1948 (Commonwealth Plant Introductions, G. Cook, pers. comm.) from an unknown location and for an unknown purpose. Plant collectors continue to trade "*Phyla nodiflora*" within and among continents (e.g., *Phyla nodiflora* is listed for sale in a Victorian nursery catalogue in 1886, [76]; plants are globally traded (e.g. [77] including as a lawn or ground cover [78]; Cocos (Keeling) Island [79]). However, *P. canescens* plants are commonly mis-labelled and sold as *P. nodiflora*, at least in Australia [17] and so such records should be treated with caution. Nonetheless, we found some *P. nodiflora* ribotypes widely used as ornamentals or as lawns. For example, ribotype 20 was commonly associated with lawns in arid Australian towns (van Klinken, pers. obs) and is one of the ribotypes in that clade that has become naturalized near urban centres in south-western Australia ([80]; Keighery pers. comm. October 2009). On Guam *P. nodiflora* is used as a landscaping plant (*Fosberg 46296 13 July 1965, NY*). There is also evidence of *P. nodiflora* ribotypes being moved unintentionally between continents. For example, the evidence of unintentional movement by humans with the detection of seeds transported from Guam to Hawaii from the soil scraped from a soldier's boot (*BISH 728774, 18 July 2006*).

4.5. Native Lineages Outside of the Americas Have Had Secondary Emigration Events

A grouping of ribotypes (50–53) in Clade 5 from northern Australia is closely aligned with African material and we suggest that these are secondary dispersal events from native African populations to Australia. Each of these ribotypes found in Australia is represented by one specimen. Ribotype 51 was collected on a rubbish tip, ribotype 50 from a swamp and 52 and 53 are from agricultural properties. We tentatively assign these ribotypes as alien to Australia, but further collections from Africa could help resolve their status. This clade also has ribotypes 54 and 55, each with one sample, from disturbed areas on a Military base on Guam. Ribotype 44 from Croker Island, Arafura Sea, Australia, was the accession closest to ribotypes 54 and 55 from Guam and ribotype 37, a common type from northern Australia was also found in Guam and the intermediate step of Papua New Guinea. Croker Island receives Australian and US military vessels from time to time (e.g., [81]). Clade 5 also has 14 ribotypes that are centred on land near the Red Sea, the Persian Gulf and the Arabian Sea. Most of the collections were from disturbed habitats.

4.6. Taxonomic Considerations

Our genetic analyses strongly support the treatment of *P. canescens* and *P. nodiflora* as separate species, a position that is in contrast to the recent morphological work by O'Leary & Múlgura [7] where these taxa are listed as varieties under *P. nodiflora*. We did not sample from or view the type specimen of *Phyla nodiflora*. We accept Munir's [8] position on the nomenclature for the taxon as he viewed microfiche images of syntypes available to Linnaeus and he also accepted Verdcourt's [67] lectotypification of *P. nodiflora* [67] cited in [8]. Verdcourt [67] does not utilise an earlier lectotypification of *P. nodiflora* by Townsend [82] probably due to that specimen being from cultivated material and instead he employed specimens available to Linnaeus. This contrasts with the position taken by O'Leary and Múlgura [7] who note that the lectotype used by Verdcourt may well be the original material for the name but that the earlier lectotypification by Townsend [82] was effectively published.

O'Leary & Múlgura [7] considered varietal status "for taxa when we observed a group of organisms with a gradual variation of characters, which would suggest an incomplete segregation of incipient species sharing the same geographical area". O'Leary & Múlgura [7] did not use a molecular approach but they describe the considerable variation that separates the two taxa in terms of leaf

morphology, indumentum and flower colour and to this we would add the succinct differences in inflorescence structure that the two taxa maintain (Figure S1a,b).

Our work in progress has shown that the two species have different breeding systems; *P. nodiflora* is capable of autogamous seed production (83% of bagged flowers set seed automatically, n = c. 250 flowers, Fatemi, unpub. data, 2006 whereas *P. canescens* is not capable of autogamous seed production, [18]). Our research has found that there is little geographic overlap in the species in either the native or Australian range (Figure 2) which concurs with Kennedy ([6] Figures 8.2 and 8.9) and MacDonald [17] respectively. Our work also revealed additional species that are closely related to *P. canescens* and *P. nodiflora* and these will be discussed elsewhere (M. Fatemi, unpublished data). In most cases our species placements agreed with those of recent taxonomic treatments [6,8], confirming the circumscription of this taxon by these authorities. However, there were some important exceptions. Two specimens from the Caribbean that were determined as *P. nodiflora* by Kennedy [6] were assigned by our analyses to *P. canescens*. The circumscription of *P. nodiflora* in Australia by Munir [8] includes material from the Murray River region (*Lucas 71, Balranald, 1878, MEL583748A*) which would be an outlying population for this species (see Figure 5). Plants from this region show phenotypic variation that warrants further investigation and indeed duplicate specimens of Conn 4043 (*Conn 4043, Kings Billabong, E of Mildura, 16 January 1994, AD*) are variously curated as *P. nodiflora* (AD) or *P. canescens* (BRI, CANB, NSW, MEL). Our ITS studies showed that *Conn 4043* is *P. canescens*. We did not sample from Lucas 71 but we did successfully sample from six herbarium specimens from different populations in the Murray River area and all but one of these was resolved as *P. canescens*. One specimen from Lakala South Australia (Ribotype Q) may be a new taxon for Australia, based on phylogenetic distance, although we are unable to determine which species of *Phyla* it is at present.

4.7. Implications for Conservation

The detection of both native and exotic lineages of *P. nodiflora*, in Africa, Eurasia and Australasia is of conservation significance. First, it confirms that it is native in many parts of the world where its status had previously been considered exotic (e.g., Australia, [8]; South Africa, [83]). Second, exotic lineages could become invasive in their own right, as *P. canescens* has already done in Australia [48,49], although there is currently limited evidence of it doing so. Finally, native and exotic lineages could potentially interbreed, threatening the integrity of native lineages. For example, Saltonstall [84] showed that the American common reed, *Phragmites australis*, had been largely replaced by the cryptic non-native lineages from Europe or Asia over the past 150 years. Cryptic invasion may also contribute to the biological invasion through hybridization with native lineages and introgression [85–87]. Sympatry between *P. nodiflora* and *P. canescens* has not been recorded in Australia although the species are found in the same general regions of the south coast of NSW (*P. canescens Michael s.n. 23-October-1969, CANB*, *Storrie 08/04, 13 February 2004, NSW*; *P. nodiflora Boorman s.n., 28 February 1900, NSW*) and Perth (*P. canescens Reynolds s.n. PERTH*; *Hocking s.n., 2 May 1953 PERTH*; *P. nodiflora Swarbrick 10679, 11 April 1993 BRI*). Further work is required to understand whether or not introgression between native and alien lineages of *P. nodiflora* is occurring and the evolutionary and ecological significance of any such events. *Phyla canescens* is increasingly progressing into regions where *P. nodiflora* occurs in Australia [17] and further work is required to determine the potential for hybridization between the two especially as Kennedy [6] notes that many apparently intermediate forms of *P. nodiflora* and *P. canescens* exist in the Americas.

5. Conclusions

Phyla nodiflora is a cosmopolitan pioneer species of seasonally damp places and sand dunes where it behaves as a weed of minor importance including within its indigenous range of lower North America to northern South America. This is the key to the enigmatic behaviour of *P. nodiflora*—as a pioneer species, it has a set of life history characteristics that promotes dispersal and establishment of propagules to new areas. We have detected at least three native lineages outside of the indigenous

range for *P. nodiflora*, in Australasia, Africa and Eurasia, although this is further confounded by subsequent intercontinental human-movement of several clades. Our results highlight the need for genetic studies, combined with ecological and historical observations, to help elucidate the limits of species' distributions and to identify cases of cryptic invasions facilitated by humans.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/9/2/20/s1. Table S1: Specimens sequenced for nr-DNA and the phylogeography of *Phyla nodiflora*, with voucher information and GenBank accession numbers. Herbarium acronyms stand for: AD = State Herbarium of South Australia; BISH = Herbarium Pacificum; BRI = Queensland Herbarium; CANB = Australian National Herbarium; DNA = Northern Territory Herbarium; FLAS = Florida Museum of Natural History; Gorgan = University of Gorgan; K = Royal Botanic Gardens Kew; MEL = National Herbarium of Victoria; MO = Missouri Botanical Garden Herbarium; MPU = Université Montpellier II; NE = N. C. W. Beadle Herbarium; NSW = Royal Botanic Gardens, Sydney; PERTH = Western Australian Herbarium; PRE = South African National Biodiversity Institute; SI = Museo Botánico; UC = University of California; US = Smithsonian Institution; XAL = Instituto de Ecología, A.C. s.n. = without number. Table S2: The 101 variable sites of the aligned sequences of the ITS1, 5.8S and ITS2 regions yielding 64 ribotypes in *Phyla nodiflora*. A dot (.) indicates that the character states are the same as for ribotype 1 and a dash (-) indicates an insertion in the aligned sequences. Data S1. The mostly univariate sites of the aligned sequences of Petb and trnL-F in *Phyla nodiflora*. A dot (.) indicates that the character states are the same as for ribotype 1, N stands for any base and a dash (-) indicates an insertion in the aligned sequences. Data S2. Estimates of evolutionary divergence between ribotypes (ribotype number is the first column and row). The number of base substitutions per site between sequences is shown. Standard error estimates are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004 [33]) implemented in MEGA5 (Tamura et al., 2011 [27]). The analysis involved 64 DNA sequences and a total of 630 positions in the final dataset. 0 = ribotypes are identical and 1 = ribotypes differ in all positions. Figure S1a: Floral and leaf characters for *Phyla nodiflora* (ribotype 35, GenBank HM194056) from Corindi Beach NSW, Australia (30.0155° S, 153.1855° E) and *Phyla canescens* from Bendigo, Victoria, Australia (36.7570° S, 144.2794° E). Images taken by M. Fatemi 2006. Figure S1b: Habit and spent flower heads of *Phyla nodiflora* (ribotype 26, GenBank HM194113) from Mt Isa, Qld (20.7256° S, 139.4927° E) and *Phyla canescens* from Kialami, New South Wales, Australia (30.4503°S, 151.5325°E). Images taken by A. White (CSIRO) 2007 (lower left) and C.L. Gross 2008 (lower right).

Acknowledgments: This study was supported in part by the Australian Weeds Research Centre (AWRC) and by UNE. We thank A. Haddadchi, M. Macdonald, P. Nelson, C. Xu, T. Woods, S. Smith, S.J. Hadavi, N.A.H. Abbasi, S. Sosa, R. Seguraand M. Martinez for providing leaf samples. Herbarium samples were generously provided by AD, BISH, BRI, CANB, DNA, FLAS, Gorgan, K, MEL, MO, MPU, NE, NSW, NY, PERTH, PRE, SI, UC, US and XAL, and we thank the Directors of these institutions. Louisa Murray is thanked for her assistance with specimens at NSW, B. Conn for informative discussions on *Phyla*, I. Simpson for photographs at NE and J. Bruhl and R. Southwood for facilitating loans to NE from overseas institutions. We thank D. Crayn, K. Schulte, L. Vary, M. Rossetto, J. Bruhl, Wal Whalley, C. Xu, P. Prentice, D. Mackay, A. Bean and M. Harrington for their comments on an earlier version of the manuscript or our approach to analyses. The original idea to investigate the biogeography of *Phyla nodiflora* started with Green (1899) and we were encouraged to embark on the work by John A. Duggin.

Author Contributions: This project was designed by C.L.G., R.V.K., M.F., M.J. The molecular laboratory work was conducted by M.F., the ITS analyses were conducted by M.F., the plastid analysis were conducted by H.M. and C.L.G. with interpretations from all authors. The geography data were collated by C.L.G. with contributions from R.V.K. The paper was written by C.L.G., R.V.K., H.M. with contributions from M.F. & M.J.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Methods for the Chloroplast Regions

Total genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Chadstone, Vic, Australia) or Wizard SV Genomic DNA Purification System (Catalogue number A2361, Promega). Polymerase chain reaction amplification was performed on three intergenic spacer regions from the single-copy portion of the chloroplast genome ([88–90]); these three primers were selected for amplification after testing a set of seven primers (3′rps16/5′trnK UUU, trnL-trnF, trnH-psbA, atpB-rbcL, petB intron, petL-psbE, psbB-psbF). The template specific primers were tailed with universal M13 primers to attach fusion primers (see Table A1).

Our first-round PCR to amplify intergenic regions followed a standard program, adjusted only for annealing temperatures: (1) Initial denaturation was conducted for 2 min at 95 °C. (2) Thirty cycles of denaturation were conducted for 30 s at 94 °C, annealing for 30 s at 56–57 °C temperature depending on the region and elongation for 1 min at 72 °C (3) A final elongation step of 20 min at 72 °C was performed.

Second-round PCR was conducted in 15 µL volume consisting of 7.5 µL 2 × mastermix (GoTaq®G2 Hot Start Colorless Mastermix, Promega), 1 µL PCR product from first-round PCR as template DNA, 5 µM of each fusion primers A and B (Table A1) and PCR grade H2O to make the final volume. The following cycling program was used: (1) Initial denaturation was conducted for 2 min at 95 °C. (2) Twenty cycles of denaturation were conducted for 30 s at 94 °C, annealing for 30 s at 55 °C and elongation for 1 min at 72 °C. (3) A final elongation step of 20 min at 72 °C was performed.

Before sequencing, concentration of the PCR products was determined using QIAxcel multi-capillary electrophoresis system (Qiagen) and then equimolar volumes of each PCR products were pooled into two pools: 144 samples in each (Data S1). The PCR products were then purified using the Wizard®SV Gel and PCR Clean-Up System kit (Promega Corporation, Madison, WI, USA) following manufacturer's instructions. Sequencing of the PCR products was performed on a Roche 454 Genome Sequencer FLX (Roche Diagnostics) with a Titanium XL+ sequencing kit at Macrogen (Seoul, Korea).

Sequences obtained from the 454 Genome Sequencer were assigned to their respective accessions according to MID tags. Fastq files for each of 262 accessions were imported into CLC Genomics Workbench 7.0.4 (CLC). Since reference sequences were not available, a de novo assembly of all samples pooled together was performed with minimum contig length 100, length fraction 0.5 and similarity fraction 0.8. Twenty-five contigs were produced. These were further assembled in Geneious 6.1.7 (Geneious) resulting in 5 contigs. A Blastn search (performed on Genbank) confirmed contig 4 as trnL-trnF, contig 5 as petB intron and 3′rps16/5′trnK UUU was not retrieved. Contigs 4 and 5 were used as reference sequences. Reads for each of 288 accessions were trimmed to a quality value of 0.05 (an error probability calculated in CLC equivalent to a minimum average Phred count of 13 for each sequence) and a minimum length of 50 bp and adaptors were removed. Trimmed sequences were mapped to the reference sequences in CLC and consensus sequences for each accession saved (with low coverage areas, <5×, removed). Consensus sequences were aligned in Geneious in order to compare variation across samples.

Table A1. Primer pairs to study phylogeography based on chloroplast intergenic sequences.

Region	Forward		Reverse		Tm	Length	Source
3′*rps*16/5′*trn*K	(UUU) F	AAAGTGGGTT TTTATGATCC	(UUU) R	TTAAAAGCCG AGTACTCTACC	56	631	[89]
trnL-trnF	UniE	GGTTCAAGTC CCTCTATCCC	UniF	ATTTGAACTGG TGACACGAG	57	273–392	[90]
petB intron	*petB* intron F	AGAGATGGTT CTACTTCGTC	*petB* intron R	ACTTTCATCT CGTACAGCTC	57	552	[88]

[88] Nishizawa T, Watano Y (2000) Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms. *Journal of Phytogeography and Taxonomy* 48:63–64; [89] Shaw J, Lickey E, Schilling E, Small R (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. Am J Bot 94:275–288; [90] Taberlet P, L. Gielly, G. Pautou, J. Bouvet (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol **17**:1105–1109.

References

1. Pyšek, P.; Richardson, D.M.; Rejmánek, M.; Webster, G.L.; Williamson, M.; Kirschner, J. Alien plants in checklists and floras: Towards better communication between taxonomists and ecologists. *Taxon* **2004**, *53*, 131–143. [CrossRef]
2. Gormally, C.; Donovan, J.H.A. Genetic structure of a widely dispersed beach annual, *Cakile edentula* (Brassicaceae). *Am. J. Bot.* **2011**, *98*, 1657–1662. [CrossRef] [PubMed]
3. Der, J.P.; Thomson, J.A.; Stratford, J.K.; Wolf, P.G. Global chloroplast phylogeny and biogeography of bracken (*Pteridium*; Dennstaedtiaceae). *Am. J. Bot.* **2009**, *96*, 1041–1049. [CrossRef] [PubMed]
4. Horrocks, M.; Rechtman, R.B. Sweet potato (*Ipomoea batatas*) and banana (*Musa* sp.) microfossils in deposits from the Kona Field System, Island of Hawaii. *JAS* **2009**, *36*, 1115–1126. [CrossRef]
5. Carlton, J.T. Biological invasions and cryptogenic species. *Ecology* **1996**, *77*, 1653–1655. [CrossRef]

6. Kennedy, K.L. A Systematic Study of the Genus *Phyla* Lour (Verbenaceae: Verbenoideae, Lantanae). Ph.D. Thesis, The University of Texas at Austin, Austin, TX, USA, 1992.
7. O'Leary, N.; Múlgura, M.E. A Taxonomic Revision of the Genus *Phyla* (Verbenaceae). *Ann. Mo. Bot. Gard.* **2012**, *98*, 578–596. [CrossRef]
8. Munir, A.A. A taxonomic revision of the genus *Phyla* Lour.(Verbenaceae) in Australia. *J. Adel. Bot. Gard.* **1993**, *15*, 109–128.
9. Greene, E. Neglected generic types I. *Pittonia* **1899**, *4*, 45–51.
10. Gupta, A.; Sadasivaiah, B.; Bhat, G. *Phyla nodiflora*. Version 2016-3. Available online: www.iucnredlist.org (accessed on 24 February 2017).
11. Marx, H.E.; O'Leary, N.; Yuan, Y.-W.; Lu-Irving, P.; Tank, D.C.; Múlgura, M.E.; Olmstead, R.G. A molecular phylogeny and classification of Verbenaceae. *Am. J. Bot.* **2010**, *97*, 1647–1663. [CrossRef] [PubMed]
12. Cosgrove, B. *Shoalwater Bay: Settlers in a Queensland Wilderness*; Central Queensland University Press: Rockhampton, Australia, 1996.
13. Hawkins, J.; Boutaoui, N.; Cheung, K.; van Klinken, R.; Hughes, C. Intercontinental dispersal prior to human translocation revealed in a cryptogenic invasive tree. *New Phytol.* **2007**, *175*, 575–587. [CrossRef] [PubMed]
14. Webb, D. What are the criteria for presuming native status? *Watsonia* **1985**, *15*, 231–236.
15. Bean, A.R. A new system for determining which plant species are indigenous in Australia. *Aust. Syst. Bot.* **2007**, *20*, 1–43. [CrossRef]
16. Steven, W.; Ulloa, C.; Pool, A.; Montiel, O. Flora de Nicaragua. In *Monographs in Systematic Botany from the Missouri Botanical Garden*; Missouri Botanical Garden: St. Louis, MO, USA, 1978.
17. Macdonald, M.J. Ecology of *Phyla canescens* (Verbenaceae) in Australia. Ph.D. Thesis, University of New England, Armidale, Australia, 2008.
18. Gross, C.; Gorrell, L.; Macdonald, M.; Fatemi, M. Honeybees facilitate the invasion of *Phyla canescens* (Verbenaceae) in Australia–no bees, no seed! *Weed Res.* **2010**, *50*, 364–372. [CrossRef]
19. Roalson, E.; Friar, E. Phylogenetic relationships and biogeographic patterns in North American members of *Carex* section *Acrocystis* (Cyperaceae) using nrDNA ITS and ETS sequence data. *Plant Syst. Evol.* **2004**, *243*, 175–187. [CrossRef]
20. Buckler, E.S.; Ippolito, A.; Holtsford, T.P. The evolution of ribosomal DNA divergent paralogues and phylogenetic implications. *Genetics* **1997**, *145*, 821–832. [PubMed]
21. Consaul, L.L.; Gillespie, L.J.; Waterway, M.J. Evolution and polyploid origins in North American Arctic *Puccinellia* (Poaceae) based on nuclear ribosomal spacer and chloroplast DNA sequences. *Am. J. Bot.* **2010**, *97*, 324–336. [CrossRef] [PubMed]
22. Mäder, G.; Zamberlan, P.M.; Fagundes, N.J.; Magnus, T.; Salzano, F.M.; Bonatto, S.L.; Freitas, L.B. The use and limits of ITS data in the analysis of intraspecific variation in *Passiflora* L. (Passifloraceae). *Genet. Mol. Biol.* **2010**, *33*, 99–108. [CrossRef] [PubMed]
23. Feliner, G.N.; Rosselló, J.A. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Mol. Phylogen. Evol.* **2007**, *44*, 911–919. [CrossRef] [PubMed]
24. Chenna, R.; Sugawara, H.; Koike, T.; Lopez, R.; Gibson, T.J.; Higgins, D.G.; Thompson, J.D. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **2003**, *31*, 3497–3500. [CrossRef] [PubMed]
25. Waterhouse, A.M.; Procter, J.B.; Martin, D.M.; Clamp, M.; Barton, G.J. Jalview Version 2—A multiple sequence alignment editor and analysis workbench. *Bioinformatics* **2009**, *25*, 1189–1191. [CrossRef] [PubMed]
26. Swofford, D.L. *PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods)*, Version 4; Sinauer Associates: Sunderland, MA, USA, 2003.
27. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
28. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **2001**, *17*, 754–755. [CrossRef] [PubMed]
29. Ogden, T.H.; Rosenberg, M.S. How should gaps be treated in parsimony? A comparison of approaches using simulation. *Mol. Phylogen. Evol.* **2007**, *42*, 817–826. [CrossRef] [PubMed]
30. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef]

31. Posada, D.; Crandall, K.A. Modeltest: Testing the model of DNA substitution. *Bioinformatics* **1998**, *14*, 817–818. [CrossRef] [PubMed]
32. Kimura, M. *Population Genetics, Molecular Evolution, and the Neutral Theory: Selected Papers*; University of Chicago Press: Chicago, IL, USA, 1994; p. 704.
33. Tamura, K.; Nei, M.; Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11030–11035. [CrossRef] [PubMed]
34. Posada, D.; Crandall, K.A. Intraspecific gene genealogies: Trees grafting into networks. *Trends Ecol. Evol.* **2001**, *16*, 37–45. [CrossRef]
35. Clement, M.; Posada, D.; Crandall, K.A. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* **2000**, *9*, 1657–1659. [CrossRef] [PubMed]
36. Templeton, A.R.; Crandall, K.A.; Sing, C.F. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **1992**, *132*, 619–633. [PubMed]
37. Joly, S.; Stevens, M.I.; van Vuuren, B.J. Haplotype networks can be misleading in the presence of missing data. *Syst. Biol.* **2007**, *56*, 857–862. [CrossRef] [PubMed]
38. Tobler, M.; Honorio, E.; Janovec, J.; Reynel, C. Implications of collection patterns of botanical specimens on their usefulness for conservation planning: An example of two neotropical plant families (Moraceae and Myristicaceae) in Peru. *Biodivers. Conserv.* **2007**, *16*, 659–677. [CrossRef]
39. Genc, H.; Nation, J.L.; Emmel, T.C. Life history and biology of Phyciodes phaon (Lepidoptera: Nymphalidae). *Fla. Entomol.* **2003**, *86*, 445–449. [CrossRef]
40. Bentham, G. *Flora Australiensis: A Description of the Plants of the Australian Territory*; Reeve & Co.: London, UK, 1870; Volume 5, pp. 31–70.
41. Song, Z.C.; Wang, W.M.; Huang, F. Fossil pollen records of extant angiosperms in China. *Bot. Rev.* **2004**, *70*, 425–458.
42. Robinson, G.S.; Ackery, P.R.; Kitching, I.J.; Beccaloni, G.W.; Hernández, L.M. HOSTS—A Database of the World's Lepidopteran Hostplants. Available online: http://www.nhm.ac.uk/hosts (accessed on 23 February 2017).
43. Grey, G. *Journals of Two Expeditions of Discovery in North-West and Western Australia, during the Years 1837, 38, and 39*; T and W Boone: London, UK, 1841; Volume II.
44. Knibbs, G.H. *Commonwealth Bureau of Census and Statistics Melbourne*; Australian Government: Melbourne, Australia, 1909.
45. Day, K.J.; Forster, B.A. *Report on the land units of Croker Island, N.T.*; Department of Northern Australia, Land Conservation Section: Canberra, Australia, 1975.
46. Edwards, E.D.; Newland, J.; Regan, L. *Lepidoptera: Hesperioidea, Papilionoidea*; CSIRO Publishing: Melbourne, Australia, 2001; Volume 31.6, pp. 545, 615.
47. Leigh, C.; Walton, C.S. *Lippia (Phyla canescens) in Queensland*; Land Protection, Department of Natural Resources, Mines, and Energy: Brisbane, Australia, 2004.
48. Price, J.; Macdonald, M.; Gross, C.; Whalley, R.D.; Simpson, I. Vegetative reproduction facilitates early expansion of *Phyla canescens* in a semi-arid floodplain. *Biol. Invasions* **2011**, *13*, 285–289. [CrossRef]
49. Xu, C.Y.; Julien, M.H.; Fatemi, M.; Girod, C.; Van Klinken, R.D.; Gross, C.L.; Novak, S.J. Phenotypic divergence during the invasion of *Phyla canescens* in Australia and France: Evidence for selection-driven evolution. *Ecol. Lett.* **2010**, *13*, 32–44. [CrossRef] [PubMed]
50. Cilento, R.; Lack, C. *Triumph in the Tropics: An Historical Sketch of Queensland/compiled and edited by Sir Raphael Cilento; with the assistance of Clem Lack; for the Historical Committee of the Centenary Celebrations Council of Queensland*; Smith & Paterson: Brisbane, Australia, 1959; p. 446.
51. Mueller, F. North Australian botany, observations on, by Dr Frederick (sic) Mueller, botanist to the NW Australian Government Expedition, under the command of Mr Surveyor Gregory; in a letter to Sir WJ Hooker (published with the sanction of the Colonial Office). *Hooker's J. Bot. Kew Gard. Misc.* **1856**, *8*, 321–331.
52. Mueller, F. Botanical report on the North Australian exploring expedition, under the command of A.C. Gregory Esq. *J. Proc. Linn. Soc. Lond. Bot.* **1858**, *2*, 137–144. [CrossRef]
53. Knibbs, G.H. *Official Year Book of the Commonwealth of Australia Containing Authoritative Statistics for the Period 1901–1908 and Corrected Statistics for the Period 1788 to 1900. No. 2*; Melbourne, Australia, 1909.

54. Dupont, L.; Behling, H.; Kim, J.-H. Thirty thousand years of vegetation development and climate change in Angola (Ocean Drilling Program Site 1078). *CliPa* **2008**, *4*, 107–124.
55. Dangol, D.; Gurung, S. Ethnobotany of the Tharu tribe of Chitwan district, Nepal. *Int. J. Pharm.* **1991**, *29*, 203–209. [CrossRef]
56. Shukla, S.; Patel, R.; Kukkar, R. Study of phytochemical and diuretic potential of methanol and aqueous extracts of aerial parts of *Phyla nodiflora* Linn. *Int. J. Pharm. Pharm. Sci.* **2009**, *1*, 85–91.
57. Wang, Y.-C.; Huang, T.-L. Screening of anti-*Helicobacter pylori* herbs deriving from Taiwanese folk medicinal plants. *FEMS Immunol. Med. Microbiol.* **2005**, *43*, 295–300. [CrossRef] [PubMed]
58. Ahmed, A.B.A.; Gouthaman, T.; Rao, A.S.; Rao, M.V. Micropropagation of *Phyla nodiflora* (L.) Greene: An important medicinal plant. *Iran. J. Biotechnol.* **2005**, *3*, 186–190.
59. Abuodha, J.; Musila, W.; van der Hagen, H.; van der Meulen, F. Floristic composition and vegetation ecology of the Malindi Bay coastal dune field, Kenya. *J. Coast. Conserv.* **2003**, *9*, 97–112. [CrossRef]
60. Estes, J.R.; Brown, L.S. Entomophilous, intrafloral pollination in *Phyla incisa*. *Am. J. Bot.* **1973**, *60*, 228–230. [CrossRef]
61. Pascual, M.; Slowing, K.; Carretero, E.; Mata, D.S.; Villar, A. Lippia: Traditional uses, chemistry and pharmacology: A review. *J. Ethnopharmacol.* **2001**, *76*, 201–214. [CrossRef]
62. Smith, D.R. Mutation rates in plastid genomes: They are lower than you might think. *Gen. Biol. Evol.* **2015**, *7*, 1227–1234. [CrossRef] [PubMed]
63. Wolfe, K.H.; Li, W.-H.; Sharp, P.M. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 9054–9058. [CrossRef] [PubMed]
64. Call, A.; Sun, Y.X.; Yu, Y.; Pearman, P.B.; Thomas, D.T.; Trigiano, R.N.; Carbone, I.; Xiang, Q.Y.J. Genetic structure and post-glacial expansion of *Cornus florida* L. (Cornaceae): integrative evidence from phylogeography, population demographic history, and species distribution modeling. *J. Syst. Evol.* **2015**, *54*, 136–151. [CrossRef]
65. Rossetto, M.; McPherson, H.; Siow, J.; Kooyman, R.; Merwe, M.; Wilson, P.D. Where did all the trees come from? A novel multispecies approach reveals the impacts of biogeographical history and functional diversity on rain forest assembly. *J. Biogeogr.* **2015**, *42*, 2172–2186. [CrossRef]
66. Diazgranados, M.; Barber, J.C. Geography shapes the phylogeny of frailejones (Espeletiinae Cuatrec., Asteraceae): A remarkable example of recent rapid radiation in sky islands. *PeerJ* **2017**, *5*, e2968. [CrossRef] [PubMed]
67. Verdcourt, B. Flora of Tropical East Africa. In *Verbenaceae*; CRC Press: London, UK, 1992.
68. Renner, S. Plant dispersal across the tropical Atlantic by wind and sea currents. *Int. J. Plant Sci.* **2004**, *165*, S23–S33. [CrossRef]
69. Mummenhoff, K.; Linder, P.; Friesen, N.; Bowman, J.L.; Lee, J.-Y.; Franzke, A. Molecular evidence for bicontinental hybridogenous genomic constitution in *Lepidium sensu stricto* (Brassicaceae) species from Australia and New Zealand. *Am. J. Bot.* **2004**, *91*, 254–261. [CrossRef] [PubMed]
70. Pettingill, O.S. Additional information on the food of the American woodcock. *Wilson Bull.* **1939**, *51*, 78–82.
71. Rundle, W.D.; Sayre, M.W. Feeding ecology of migrant Soras in southeastern Missouri. *J. Wildl. Manag.* **1983**, *47*, 1153–1159. [CrossRef]
72. Razi, B.A. A contribution towards the study of the dispersal mechanisms in flowering plants of Mysore (south India). *Ecology* **1950**, *31*, 282–286. [CrossRef]
73. Dostine, P.; Morton, S.R. Seasonal abundance and diet of the comb-crested Jacana *Irediparra gallinacea* in the tropical Northern Territory. *Emu-Austral Ornithol.* **2000**, *100*, 299–311. [CrossRef]
74. Takayama, K.; Kajita, T.; Murata, J.; Tateishi, Y. Phylogeography and genetic structure of *Hibiscus tiliaceus*—Speciation of a pantropical plant with sea-drifted seeds. *Mol. Ecol.* **2006**, *15*, 2871–2881. [CrossRef] [PubMed]
75. Baldwin, A.H.; McKee, K.L.; Mendelssohn, I.A. The influence of vegetation, salinity, and inundation on seed banks of oligohaline coastal marshes. *Am. J. Bot.* **1996**, *83*, 470–479. [CrossRef]
76. Brookes, M.; Barley, R. *Plants Listed in Nursery Catalogues in Victoria 1855–1889*; Published for the Ornamental Plant Collections Association Inc., Royal Botanic Gardens: Melbourne, Australia, 1992.
77. Lorenz's OK Seeds. Available online: http://www.lorenzsokseedsllc.com/?s=lippia (accessed on 26 February 2017).
78. Moldenke, H. *Verbenaceae*; Amerind Publishing Co. Pvt. Ltd.: New Delhi, India, 1983.

79. Barker, R.; Telford, I. Oceanic Islands 2. In *Flora of Australia*; Australian Biological Resources Study and the Australian Government Publishing Service: Canberra, Australia, 1993; Volume 50, p. 415.
80. Hussey, B.; Keighery, G.; Dodd, J.; Lloyd, S.; Cousens, R. *Western Weeds: A Guide to the Weeds of Western Australia*; The Plant Protection Society of Western Australia: Victoria Park, Australia, 2007.
81. HMAS Inverell. Available online: http://www.navy.gov.au/hmas-inverell (accessed on 26 February 2017).
82. Townsend, C. Verbenaceae. In *Flora of Turkey and the East Aegean Islands*; Davis, P., Ed.; Edinburgh University Press: Edinburgh, UK, 1982; pp. 31–35.
83. Germishuizen, G.; Meyer, N. *Plants of Southern Africa: An Annotated Checklist*; National Botanical Institute Pretoria: Pretoria, South Africa, 2003.
84. Saltonstall, K. Cryptic invasion by a non-native genotype of the common reed, *Phragmites australis*, into North America. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2445–2449. [CrossRef] [PubMed]
85. Ellstrand, N.C.; Schierenbeck, K.A. Hybridization as a stimulus for the evolution of invasiveness in plants? *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7043–7050. [CrossRef] [PubMed]
86. Mason-Gamer, R.J. Reticulate evolution, introgression, and intertribal gene capture in an allohexaploid grass. *Syst. Biol.* **2004**, *53*, 25–37. [CrossRef] [PubMed]
87. Miura, O. Molecular genetic approaches to elucidate the ecological and evolutionary issues associated with biological invasions. *Ecol. Res.* **2007**, *22*, 876–883. [CrossRef]
88. Nishizawa, T. Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms. *J. Phytogeogr. Taxon* **2000**, *48*, 63–66.
89. Shaw, J.; Lickey, E.B.; Schilling, E.E.; Small, R.L. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *Am. J. Bot.* **2007**, *94*, 275–288. [CrossRef] [PubMed]
90. Taberlet, P.; Gielly, L.; Pautou, G.; Bouvet, J. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* **1991**, *17*, 1105–1109. [CrossRef] [PubMed]