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The application of high-throughput sequencing for taxonomy: The case of *Plantago* subg. *Plantago* (Plantaginaceae)



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ABSTRACT

Plantago is a cosmopolitan genus including over 250 species, concentrated in temperate and high-elevation tropical regions. The taxonomy of *Plantago* is very difficult, mainly because of its reduced morphology, which features relatively few characters for species classification. Consequently, the infrageneric classification of the genus remains controversial and inadequate. In this study we applied high-throughput plastid genome skimming to provide powerful phylogenetic resolution to clarify the relationships within subg. *Plantago*, which is the largest, most broadly distributed and poorest understood subgenus of *Plantago*. Ninety-four samples covering ~56% of all species and representing all sections of subg. *Plantago* as well as an outgroup were successfully sequenced. The resulting phylogenetic topology was used, complemented by field and herbarium studies, to revise the sectional classification of subg. *Plantago* and present a complete listing of the accepted species in the subgenus. Our phylogenetic results were also tested for their usefulness in clarifying the taxonomic placement of some taxonomically complicated species in the subgenus. We conclude that a combination of morphological studies and state-of-the art high-throughput DNA data provide a useful toolbox for resolving outstanding taxonomic puzzles exemplified by the genus *Plantago*.

1. Introduction

Determining the genealogy of each great Kingdom of Nature was Charles Darwin's dream (Darwin and Darwin, 1887). Since the emergence of Sanger sequencing techniques in the 1970s (Sanger et al., 1977), DNA sequencing has greatly advanced our understanding of the tree of life and shed new light on previous classifications based on taxonomic studies of primarily morphological characters (Savolainen and Chase, 2003; Morey et al., 2013; van Dijk et al., 2014; Heather and Chain, 2015). Starting in 2004, the so called next-generation sequencing, or high-throughput sequencing (HTS) became available, greatly increasing the speed and the amount of generated data, and hugely decreasing the sequencing cost per base (Morey et al., 2013; van Dijk et al., 2014; Heather and Chain, 2015). The massive amount of data generated by HTS techniques has powerful applications in potentially all fields of the biological sciences (Delseny et al., 2010; Koboldt et al., 2013; Buermans and den Dunnen, 2014), including taxonomy (Harrison and Kidner, 2011; Straub et al., 2012; Soltis et al., 2013). However, the associated costs and necessary infrastructure are still the main restrictors of its use (Delseny et al., 2010), especially in developing countries and low-funding research environments such as taxonomy. For this reason, and also due to the lack of plant taxonomists in most high-funding institutions (see e.g. Agnarsson and Kuntner, 2007; Ebach et al., 2011; Wägele et al., 2011; Sluys, 2013), the application of HTS to resolve taxonomic problems is still in its infancy (e.g. Gardner et al., 2016; Hou et al., 2016; Uribe-Convers et al., 2017). In order to take full benefit from this new potential, a working connection between phylogeny, morphology and nomenclature is necessary, and without which phylogeny is not translated into advanced systematics.

In this study we demonstrate the utility of applying state-of-the art high-throughput DNA data to test current taxonomic understanding based on morphology and help resolve outstanding taxonomic problems exemplified by the plant genus *Plantago* L. (Plantaginaceae).

The Plantaginaceae had its circumscription radically altered with

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Fig. 1. Photographs of species in Plantago subg. Plantago. A. P. eriopoda. B. P. elongata. C. P. alpestris. D. P. euryphylla. E. P. glacialis. F. P. major. G. P. asiatica.

recent molecular phylogenetic studies (Olmstead et al., 2001; Albach et al., 2005), having been greatly expanded with the inclusion of a large number of species from the former Scrophulariaceae (sensu lato) plus Callitrichaceae, Globulariaceae and Hippuridaceae (Albach et al., 2005). Within the family, tribe Plantagineae (Albach et al., 2005) comprises *Plantago, Littorella* P.J.Bergius (Hoggard et al., 2003; Hassemer et al., 2018) and *Aragoa* Kunth (Bello et al., 2002). Molecular phylogenetic analyses have also been used to review the circumscriptions, biogeography and phylogenetic relationships of taxa within Plantagineae (Bello et al., 2002; Rønsted et al., 2002; Hoggard et al., 2003; Cho et al., 2004; Dunbar-Co et al., 2008; Meyers and Liston, 2008; Ishikawa et al., 2009; Tay et al., 2010a; Iwanycki Ahlstrand et al., 2019).

Plantago is a cosmopolitan genus which has diversified into over 250 species which are usually anemophilous herbs or rarely subshrubs, perennial or annual, and concentrated in temperate and high-elevation

tropical regions (Figs. 1, 2 and 3; Pilger, 1937; Rahn, 1996; Li et al., 2011). Although some species have wide geographic distributions, a few such as *P. major* and *P. lanceolata* L. being cosmopolitan ruderals, many others have restricted geographic distributions, occurring in more specialised environments, and several of these are endemic to oceanic islands (Dunbar-Co et al., 2008; Meudt, 2012; Hassemer et al., 2016; Iwanycki Ahlstrand et al., 2019). A number of *Plantago* species are well-known for their medicinal properties, and also for other traditional uses (Samuelsen, 2000; Weryszko-Chmielewska et al., 2012; Gonçalves and Romano, 2016).

In many areas, *Plantago* species have successfully colonised new habitats and then have undergone consequent rapid and recent diversification, including an extremely high level of mitochondrial DNA evolution often contrasting with low morphological variation (Rønsted et al., 2002; Cho et al., 2004; Meyers and Liston, 2008; Tay et al., 2010a; Ishikawa et al., 2009). Although the genus is one of the most



Fig. 2. Photographs of species in Plantago subg. Plantago. A. P. macrocarpa. B. P. tehuelcha. C. P. bradei. D. P. napiformis. E. P. rahniana. F. P. commersoniana.

well-studied plant genera from a taxonomic viewpoint, this low morphological variation, reduced morphology, and lack of useful taxonomic characters have precluded a full understanding of the evolution and classification of the genus and its species (Rahn, 1996; Rønsted et al., 2002; Ishikawa et al., 2009; Tay et al., 2010b; Meudt, 2011). Trichomes and seeds are considered the most informative morphological characters (Rahn, 1992, 1996), and both trichomes (e.g. Andrzejewska-Golec, 1991; Rahn, 1992; Andrzejewska-Golec and Świętosławski, 1993) and seeds (e.g. Liu et al., 1992; Shipunov, 1998; Klimko et al., 2004; Shehata and Loutfy, 2006) have been investigated. A series of chemotaxonomic studies have also been conducted (e.g. Andrzejewska-Golec et al., 1993; Jensen et al., 1996; Rønsted et al., 2000, 2003; Taskova et al., 2002). However, none of these characters have enabled a satisfying infrageneric classification of the genus. To complicate things further, there is evidence of polyploidy (Murray et al., 2010; Wong and Murray, 2012, 2014), hybridisation (Rahn,

1974; Wong and Murray, 2014) and reticulate evolution (Ishikawa et al., 2009) in *Plantago*.

According to the classification of Rahn (1996) based on a cladistic analysis of morphological characters, with updates by Rønsted et al. (2002) and Hoggard et al. (2003) using plastid *trn*L–F and nuclear encoded ITS sequence data, the genus *Plantago* is subdivided into four subgenera: *Bougueria* (Decne.) Rahn (once considered to be its own monotypic genus; Rahn, 1996; Rønsted et al., 2002), *Coronopus* (Lam. & DC.) Rahn, *Plantago* and *Psyllium* (Mill.) Harms & Reiche. Also according to this classification, subgenus *Plantago*, the focus of the current study, is cosmopolitan, includes 143 species, and is in turn subdivided into five sections: *Mesembrynia, Micropsyllium, Plantago, Oliganthos* and *Virginica* (see Table 1). Rahn (1996) deemed his sects. *Mesembrynia* and *Plantago* to be not monophyletic, whereas his sects. *Micropsyllium, Oliganthos* and *Virginica* were monophyletic according to his analyses.

Previous molecular phylogenetic studies by Rønsted et al. (2002),



Fig. 3. Photographs of species in Plantago subg. Plantago. A. P. cordata. B. P. triandra. C. P. spathulata. D. P. myosuros. E. P. udicola. Photo credits: Mei Lin Tay (B, C, E) and Luís Adriano Funez (D).

Table 1

Summary of the sections accepted by Rahn (1996) in *Plantago* subg. *Plantago*, the distribution and the number of species in each section, and the monophyletic status according to his analyses.

Section	Native distribution	No. of species	Monophyletic?
Mesembrynia	Australasia and Eurasia	32	No
Micropsyllium	North America and Eurasia	6	Yes
Oliganthos	Australasia and South America	24	Yes
Plantago	Worldwide except mainland South America and Australasia	53	No
Virginica	The Americas	28	Yes

Hoggard et al. (2003), Ishikawa et al. (2009) and Tay et al. (2010a) based on Sanger sequencing of a limited number of DNA regions from a combined number of ca. 40 species (\sim 28%) of subg. *Plantago* indicated that, although there is strong evidence pointing to the monophyly of subg. *Plantago*, and for sect. *Micropsyllium* being sister to the remainder of the subgenus, most sections are not monophyletic nor well resolved, demonstrating the need for further investigations to understand the phylogenetic relationships within this group. A recent phylogenetic study (Iwanycki Ahlstrand et al., 2019) based on five DNA regions (*nr*ITS and four plastid regions) focusing on the biogeography of the oceanic island endemic species in subg. *Plantago* (30 species included),

confirmed the polyphyly of all sections except *Micropsyllium* and *Virginica*. Thus, a revised molecular phylogeny of subg. *Plantago* with increased sampling and more informative markers is therefore needed to better understand the taxonomy, phylogenetic relationships, biogeography and evolutionary history of this group.

The objective of this study is to apply HTS genome skimming techniques to reconstruct phylogenetic relationships within *Plantago* subg. *Plantago* and to propose a new sectional classification of the subgenus.

2. Materials and methods

2.1. Herbarium specimens revision

Due to the difficult taxonomy and identification of *Plantago*, which leads to a considerable proportion of herbarium specimens being misidentified, we found it necessary to conduct an extensive revision of herbarium specimens to ensure that we associate the correct names to the sampled specimens. Special attention was given to type specimens, which fix the application of names and are critical for the correct taxonomic classification. Collections from the following herbaria were studied: AK, ASE, BHCB, C, CEN, CGMS, CHR, CIIDIR, DDMS, EAC, EFC, FI, FLOR, FT, FURB, GB, GH, HAS, HBR, HO, HRB, HURB, IAC, ICN, K, LD, MA, MBM, MVFA, MVJB, MVM, OTA, P, PI, RB, SGO, TANG, TEPB, TUB, UB, UESC, UFMT, UPCB, UPS and WELT (herbarium

codes follow Thiers, 2018). Furthermore, images of specimens kept at the following herbaria were studied: A, B, BBF, BM, BR, COI, CONC, CORD, CTES, DD, E, ESA, F, G, GOET, HFLA, IRAI, L, LD, LE, LINN, M, MO, MPU, PH, PRC, R, RO, S, SP, UC, UEC, US and W. The nomenclature presented here follows the *Shenzhen Code* (Turland et al., 2018). Author names of species and sections included in the taxonomic treatment or in Table 3 are not repeated elsewhere in the text. The terminology and interpretation of morphological characters for *Plantago* follow Rahn (1992, 1996). Unless otherwise informed, all field photographs were taken by the authors.

2.2. Sampling strategy

The samples used in this study were obtained through field work, from cultivated individuals at the Botanical Garden of the Natural History Museum of Denmark, University of Copenhagen, and also from herbarium specimens. Furthermore, nineteen DNA extracts from the Kew DNA Bank (http://apps.kew.org/dnabank/homepage.html) were used. In addition to these, two published reference plastomes were also included in our phylogenies: *P. maritima* L. (GenBank acc. nr. KR297244) and *P. media* (GenBank acc. nr. KR297245) (Zhu et al., 2015).

For this study we successfully sequenced 94 DNA accessions corresponding to 87 *Plantago* species and one *Littorella*. Details of voucher materials are listed in Table 2; these sequences will be submitted to TreeBase during the review process. The five included outgroups were *L. uniflora* (L.) Asch. and two samples each of *Plantago* subgenera *Coronopus* and *Psyllium*. Eleven additional samples (10 *Plantago* and one *Aragoa* species) were sequenced but could not be used for the phylogenies due to highly degraded DNA or evident contamination. Construction of libraries failed twice with samples of *P. nubicola* (Decne.) Rahn, the sole representative of *Plantago* subg. *Bougueria*.

The sampling strategy focused on covering all sections recognised by Rahn (1996) for Plantago subg. Plantago. We also included species whose phylogenetic placement had already been considered problematic in the literature, especially the six Eurasian species of Rahn's (1996) sect. Mesembrynia (P. arachnoidea, P. camtschatica, P. depressa, P. komarovii, P. perssonii and P. schwarzenbergiana), and as many species as possible of sect. Plantago, which Rahn himself deemed to be paraphyletic (Rahn, 1996). Furthermore, we included several species from South America, Australia and New Zealand, as these species-rich areas have been under-sampled in previous molecular studies. Additional samples of some species were included with the purpose of testing the phylogenetic placement of taxonomically problematic subspecies or populations of P. australis and P. lanigera.

2.3. High-Throughput DNA sequencing

Whole genomic DNA was extracted from fresh, silica gel dried or herbarium specimens using the Qiagen DNeasy Minikit (Qiagen, Germany) following the manufacturer's protocol with the three following modifications to increase yield: (1) 50–60 mg dried pulverised tissue was used for each extraction; (2) 50 μ l proteinase K was added and incubated for 1 h at 45 °C following the second step in the manufacturers protocol (i.e. add 400 μ l of AP1 buffer and 4 μ l of RNase A, mix, and incubate for 10 min at 65 °C); and (3) the final elution step was done thrice using 120 μ l AE buffer, but re-pipetting the flow-through onto the spin column each time (instead of adding new AE buffer). DNA was quantified using a Qubit 2.0 fluorometer (Life Technologies, USA) following the manufacturer's instructions for high sensitivity.

Prior to preparing the libraries for sequencing, DNA was fragmented to ca. 300 basepairs (bp) using a Bioruptor (Diagenode, Belgium), running four cycles, with 15 s ON/90 s OFF. Illumina-compatible 100 bp paired-end libraries from DNA extracts were prepared using NEBNext Library building kits (New England Biolabs, USA, catalogue nr. E6070L) following the manufacturer's protocol. The libraries were

amplified using AmpliTaq Gold (Life Technologies, USA), and had their quality checked using a 2200 TapeStation (Agilent Technologies, USA). Subsequently, the libraries were multiplexed and sequenced on three lanes with 32, 40 and 42 samples respectively using an Illumina HiSeq2000 platform at the Danish National High-Throughput DNA Sequencing Centre. For this study we used the Illumina platform because of the large amount of data that it generates, but also because the error-rate in base calling of this method is the lowest, making it advantageous for the purposes of this study compared to other highthroughput sequencing methods available (Bruun-Lund et al., 2017).

2.4. Data analyses

2.4.1. Reference-based plastome assembly

The sequencing resulted in 180.27 gigabytes of reads for 105 samples. Following the analysis pipeline of Bruun-Lund et al. (2017), the sequences were filtered to remove adaptors and low quality reads using AdaptorRemoval v. 2 (Schubert et al., 2016) running with the default settings and a minimum read length set to 30 bp. The data were then tested for quality using FastQC (http://www.bioinformatics.babraham. ac.uk/projects/fastqc). Reads were then imported into Geneious v. 9.1.8 (Biomatters Ltd., New Zealand). The resulting high-quality reads were then paired and subsequently reference-based assembled to a published plastome of P. media (GenBank acc. nr. KR297245; Zhu et al., 2015) using the Bowtie2 v. 2.3.2 (Langmead and Salzberg, 2012) plugin in Geneious, choosing end to end, high-medium sensitivity. This reference was chosen because it was the only published plastome of a species in subg. Plantago. Then the consensus sequence of the result was extracted using a 50% (strict) threshold, calling "?" if no coverage or coverage is less than 10 reads. For each of the samples, between 949,457 and 1,049,210 reads were mapped to P. media, with coverage ranging from 16 to $1346 \times$ (with most samples around $100-300 \times$), when used as a reference to the mapping process.

Next, successive alignments of the 105 samples sequenced, in addition to the two reference plastomes of *P. media* and *P. maritima*, were made using the MAFFT v. 7.309 (Katoh and Standley, 2013) plugin for Geneious, choosing the default settings. At this point the need for removing eleven of the sequenced samples was verified, either because of lack of high levels of endogenous DNA (two samples) or because of the negative impacts that highly degraded DNA caused to the alignments (nine samples), and thus the final alignment was reduced to 96 sequences including the two reference plastomes. In order to avoid artificially increasing the phylogenetic signal from the inverted repeated region in chloroplast genomes, one of these repeated regions was removed prior to analysis (Bruun-Lund et al., 2017). The final aligned matrix of the 96 sequences included 215,259 bp before removing one of the inverted repeated regions and 164,720 bp after.

2.4.2. Phylogenetic analyses

The final plastome alignment of 96 taxa and 164,720 bp was used for two separate sets of analyses:

In the first analysis (I) we considered the plastome as a single heritable unit where the most appropriate model of evolution was tested for using jModelTest2 v. 2.1.6) (Darriba et al., 2012) using the default settings. According to the Akaike information criterion, as recommended by Posada and Buckley (2004), the model was inferred to be GTR + G.

In the second set of analyses (II) we used *P. media* again as a reference for annotations using the 'transfer' option in Geneious. Using PartitionFinder (Lanfear et al., 2012), we determined the partitioning strategy and models of evolution that fitted the data best when using all genes and regions between genes as input. The alignment and partition strategy was uploaded to CIPRES (www.phylo.org).

Next, a maximum likelihood (ML) analysis was conducted on both (I) and (II) to search for the best tree using RAxML-HPC v. 8.2.9 (Stamatakis, 2014) in CIPRES, with the following changes from the

Table 2

List of 94 DNA samples successfully sequenced used for the final phylogenetic analysis. Herbarium codes are listed in parenthesis.

Taxon	Voucher	Sample provenance
L. uniflora	Chase 2798 (K); Kew DNA Bank 2798	England
P. alismatifolia	Mosquin et al., 6813 (GH)	Northwestern Mexico
P. alpestris	Briggs 10181 (NSW-884676)	Southeastern Australia
P. arachnoidea	Gubanov and Kamelin 2662a (MW)	Mongolia
P. arborescens	Hassemer 918 (C)	Madeira Island, Portugal: cultivated in Copenhagen
P. asiatica	Liu 15395 (C)	Central China
P. aucklandica	Wright s.n. (WELT-SP090482)	Auckland Islands, New Zealand
P australis subsp australis	Hassemer 738 (FLOR)	Grão Pará, southern Brazil
P australis subsp. cumingiana	Hassemer 917 (C)	Central Chile: cultivated in Copenhagen
P australis subsp. birtella	Hassemer 768 (C)	Joinville southern Brazil
P australis subsp. leioloma	Arsène 5422 (US-00137259)	Central Mexico
P bradei	Hassemer 826 (C)	Alto Caparaó, eactern Brazil
P. camtschatica	Rahn 684 (C): Kew DNA Bank 9402	Origin unknown: cultivated in Copenhagen
D canescens	Pospelov s.n. (MW)	Northern Russia
P catharinea 1	Hassemer 706 (FLOR)	Florianópolis, southern Brazil
P. catharing 2	Hassemer 210 (C)	Contos, southern Prezil
P. caularinea Z	Gine Brit our Conceller 025 (V): Kow DNA Berl 21022	Salitos, southern Brazil
P. cavaleriel	Shio-Brit, exp. Galigshall 935 (K); Kew DIVA Balik 31933	Yunnan, China
P. commersoniana	Hassemer 832 (C)	Montevideo, Uruguay
P. cordata	Wagner and Fritsch 90012 (NY)	Michigan, USA
P. cornutii	Rønsted 31 (C); Kew DNA Bank 11180	Origin unknown; cultivated in Copenhagen
P. corvensis	Hassemer 737 (FLOR)	Grão Pará, southern Brazil
P. daltonii	Briggs 9782 (NSW-743874)	Tasmania, Australia
P. debilis	Briggs 10184 (NSW-899215)	Eastern Australia
P. depressa	Yongsok 6295 (F-1535438)	Ulleung Island, South Korea
P. elongata	Bare 1113 (NY)	North Dakota, USA
P. eriopoda	Anonymous s.n. (OKL); Kew DNA Bank 30432	USA
P. euana	Sykes 879/T (US-3121974)	Tonga Islands
P. euryphylla	Briggs 10175 (NSW-884716)	Southeastern Australia
P. fernandezia	Solbrig et al. 3907 (GH)	Juan Fernández Islands, Chile
P. floccosa	Spellman et al. 990 (MO-2898184)	Central Mexico
P. gaudichaudii	Hosking 3286 (NSW-841427)	Eastern Australia
P. gentianoides	Buja et al. s.n. (NY)	Romania
P glacialis	Briggs 10180 (NSW-884675)	Southeastern Australia
P guilleminiana	Hassemer 884 (C)	Urubici southern Brazil
P hatschlachiana	No voucher: photo: Fig. 3 in Hassemer (2016)	Ponta Grossa southern Brazil
D hawaiansis	Dunbar Co 2002	Hawaii Island USA
P. hadlari	Sood 21 (NSW 797700)	Lord Howa Island, Australia
P. healey	Seeu 31 (NSW-767790)	Loru Howe Island, Australia
P. humatata	Stewart 218/1 (N1)	Kasininir Commé constitues Busell
P. numbolatiana	Hassemer 766 (C)	Corupa, southern Brazil
P. incisa	Filip H5/8184-52 (K); Kew DNA Bank 11191	Java, Indonesia
P. komarovii	Petelin 99-546 (MW)	Mongolia
P. lanceolata	Hassemer 364 (FLOR)	Florianopolis, southern Brazil
P. lanigera 1	Meudt 268 (WELT-SP090353)	Rock and Pillar Range, New Zealand
P. lanigera 2	Heenan s.n. (CHR-688758)	Sewell Peak, New Zealand
P. longissima	Glen 1928 (US-3438221)	Northern South Africa
P. macrocarpa	Volkova et al. s.n. (MW-0156805)	Bering Island, northeastern Russia
P. major	Hassemer 760 (C)	Florianópolis, southern Brazil
P. maxima	Rønsted 28 (C); Kew DNA Bank 11181	Origin unknown; cultivated in Copenhagen
P. moorei	Moore 729 (GH)	West Falkland, UK
P. muelleri	Briggs 10179 (NSW-884674)	Southeastern Australia
P. myosuros 1	Hassemer 834 (FURB)	Montevideo, Uruguay
P. myosuros 2	Hassemer 837 (C)	Lavalleja, Uruguay
P. napiformis	Hassemer 809 (C)	Ponta Porã, western Brazil
P. novae-zelandiae	Tay 52 (WELT-SP090356)	Ruahine Range, New Zealand
P. pachyneura	Hassemer 805 (C)	Central Chile; cultivated in Copenhagen
P. pachyphylla	Dunbar-Co 2155 (PTBG)	Oahu Island, USA
P. palmata	Rønsted 9 (C)	Rwanda
P. palustris	Hosking 2486 (NSW-693662)	Eastern Australia
P. paradoxa	Briggs 9781 (NSW-743924)	Tasmania, Australia
P. personii	Oinghai-Xizang exp. 870947 (PE): Kew DNA Bank 20552	Xinjiang, China
P. picta	Atkins s.n. (WELT-SP086772)	Tolaga Bay, New Zealand
P polysperma	Tsvelev et al. 995 (LF)	Kazakhstan
D princens	Dunbar-Co 2341 (DTRG)	Ophy Jeland USA
D pusilla	Cusick and Cardner 260E4 (NV)	Indiana USA
r. pusuu D. ashaisan	Usecomer 706 (C)	Illulalla, USA Dom Jandim do Como
r. runniana	nassemer / 80 (C)	Bom Jardim da Serra, southern Brazil
P. raoutu	Meudt 281 (WELT-SP086777)	Puketapu, New Zealand
P. rapensis	Motley 2740 (K); Kew DNA Bank 20557	Rapa Iti Island, France
P. reniformis	Rønsted 42 (C); Kew DNA Bank 9446	Origin unknown; cultivated in Copenhagen
P. rhodosperma	No voucher; photo: Fig. S1	Texas, USA
P. rigida	Chase 2767.B (K); Kew DNA Bank 2767.1	Peru
P. rugelii	Rønsted 37 (C); Kew DNA Bank 9447	Ontario, Canada
P. rupicola	Dunbar-Co 2268 (PTBG)	Rapa Iti Island, France
P. schwarzenbergiana	Boros s.n. (GH)	Hungary
P. sparsiflora	LeBlond 5305 (CSU); Kew DNA Bank 30433	Origin unknown

(continued on next page)

Table 2 (continued)

Taxon	Voucher	Sample provenance
P. spathulata	Garnock-Jones 2629 (WELT-SP090461)	Marfells Beach, New Zealand
P. spathulata × raoulii 1	Tay 49 (WELT-SP090387)	Sugarloaf Pass, New Zealand
P. spathulata × raoulii 2	Barkla s.n. (WELT-SP087211)	Old Man Range, New Zealand
P. stauntonii	Rahn 706 (C)	St. Paul and New Amsterdam Islands, France
P. subnuda	McClintock and Wheeler s.n. (UC-530075)	California, USA
P. subspathulata	Hassemer 808 (C)	Madeira Island, Portugal; cultivated in Copenhagen
P. subulata	Hassemer 916 (C)	Origin unknown; cultivated in Copenhagen
P. tanalensis	Deroin 260 (MO-5970257)	Madagascar
P. tasmanica	Briggs 9791 (NSW-743928)	Tasmania, Australia
P. tehuelcha	Eyerdam et al. 24025 (GH)	Southern Argentina
P. tomentosa	Hassemer 793 (C)	Santo Antônio das Missões, southern Brazil
P. triandra	Tay 55 (WELT-SP090357)	Manaia, New Zealand
P. trinitatis	Port s.n. (FLOR-49242)	Trindade Island, Brazil
P. tubulosa	Webster 67 (K); Kew DNA Bank 19210	Puno, Peru
P. turficola	Hassemer 621 (FLOR)	Urubici, southern Brazil
P. tweedyi	Hoggard 518 (CSU); Kew DNA Bank 30436	Origin unknown
P. udicola	Sneddon s.n. (WELT-SP090378)	Tablelands, New Zealand
P. unibracteata	Meudt 273 (WELT-SP090464)	Rock and Pillar Range, New Zealand
P. varia	Briggs 10177 (NSW-884666)	Eastern Australia
P. weddelliana	Hjerting et al. 180 (F-1607387)	Northwestern Argentina

default settings: maximum hours to run: 100; model for bootstrapping phase: GTRGAMMA; analysis type: rapid bootstrap analysis / search for best-scoring ML tree; bootstrapping type: rapid bootstrapping; boot-strap iterations: 1000 (the maximum value allowed).

To verify the results of the ML analyses, a Bayesian inference analysis was conducted with MrBayes v. 3.2.6 (Ronquist et al., 2012), also in CIPRES on both (I) and (II), using two independent runs and four chains, sampling every 500 generations for up to 50 million generations, and capped at 100 h of analysis resulting in 10,110,000 generations. Chain convergence and effective sample size parameters were inspected with Tracer v. 1.6 (http://tree.bio.ed.ac.uk/software/tracer) and the first 25% of the trees sampled from the posterior were discarded as burn-in. Using the program sumtrees.py from DendroPy v. 4.0.3 (https://github.com/jeetsukumaran/dendropy) (Sukumaran and Holder, 2010) we produced a maximum credibility clade tree. The best tree obtained from both maximum likelihood and Bayesian inference was viewed and annotated using FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

3. Results

3.1. Plastome phylogeny

The final alignment of the plastome dataset included 96 samples encompassing 88 species, and allowed the highest coverage to date (83 species; ~56%) of *Plantago* subg. *Plantago*, which is here estimated to include 147 described species. The completeness of the plastomes was variable due to the set 50% threshold, calling "?" if no coverage or coverage is less than 10 reads, to ensure only high quality assemblies was included in the subsequent analysis. For all samples, the proportion of missing data was limited. No structural variation other than length variation was found. This alignment was submitted to Treebase (www. treebase.org), submission no. 24429.

The two sets of analyses considering either the plastome data as one inheritable unit (I) or using a partitioning approach (II) resulted in largely similar topologies. Support values differed slightly but did not follow a consistent pattern. Consequently, we present only the results of analysis I.

The topology of the trees obtained using the two different analyses (maximum likelihood with RAxML and Bayesian inference with MrBayes; see above) was identical (Fig. 4; see also Figs. S2 and S3), confirming the robustness of our data. The support for clades at higher levels was generally high (most often 100% bootstrap support and posterior probability = 1.00). However, the support for some more

terminal clades, especially in the more species-rich clades (sects. *Mesembrynia* and *Virginica*, see below) was generally much lower.

The infrageneric taxa mentioned here refer to the classification of Rahn (1996), updated by Rønsted et al. (2002) and Hoggard et al. (2003). The RaxML consensus tree is presented in Fig. 4 with clades with low posterior probabilities (PP < 1.00 or BS < 100%) indicated on the branches. ML and Bayesian phylograms showing branch lengths are included in the Supplementary Material, Figs. S2 and S3. The resulting plastome tree topology (Fig. 4) is in accordance with the topologies from previous phylogenetic studies based on Sanger sequencing of both plastid and nuclear data (Rønsted et al., 2002; Hoggard et al., 2003; Ishikawa et al., 2009; Tay et al., 2010a; and Iwanycki Ahlstrand et al., 2019), but providing significantly improved resolution of *Plantago* subg. *Plantago*.

The plastome topology obtained here shows subg. *Plantago* to be monophyletic with strong support (BS = 100%; PP = 1.00). *Plantago* sect. *Micropsyllium* (clade A; BS = 100%; PP = 1.00) is sister to the remainder of subg. *Plantago* (BS = 100%; PP = 1.00). The next dichotomy is between a clade of two species of sect. *Plantago* from southeastern Europe and Asia Minor (clade B; *P. gentianoides* and *P. reniformis*; BS = 97; PP = 1.00) and the remaining clade, which has lower support (BS = 52%; PP = 0.95). Within this clade, a well-supported clade (clade C; BS = 100%; PP = 1.00) consisting of three species from sect. *Mesembrynia* (*P. arachnoidea*, *P. perssonii* and *P. schwarzenbergiana*) and three from sect. *Plantago* (*P. canescens*, *P. maxima* and *P. media*) intermixed, is sister to a clade of the remaining species (BS = 100%; PP = 1.00).

Within this clade a well-supported clade (clade D; BS = 100%; PP = 1.00) with three Sub-Saharan African species of sect. Plantago (P. longissima, P. palmata and P. tanalensis), is sister to the remaining species (BS = 100%; PP = 1.00). The next dichotomy consists of a larger well-supported clade (clade E; BS = 100%; PP = 1.00) with species from Australia, New Zealand and St. Paul and New Amsterdam Islands, coming from sects. Mesembrynia, Oliganthos and Plantago, and a clade of the remaining species (BS = 100%; PP = 1.00). Within this remaining clade we obtain a clade (BS = 100%; PP = 1.00) with six species, which splits into two clades: one clade (Clade F; BS = 100%; PP = 1.00) consisting of three species from sect. *Plantago* (including *P*. major), and another clade (BS = 55%; PP = 0.73) consisting of P. cordata (from sect. Plantago), and a clade (Clade G; BS = 100%; PP = 1.00) with two species from sect. Oliganthos (P. rigida and P. tu*bulosa*). Subsequently, a well-supported clade (clade H; BS = 100%; PP = 1.00) including 12 species from Asia, North America and oceanic Pacific islands, nine from sect. Plantago and three from sect.



Fig. 4. Phylogenetic hypothesis of *Plantago* subg. *Plantago*. Best tree obtained from the RaxML analyses based on plastome data. Clades with low support (PP < 0.95 or BS < 100%) are indicated. The updated classification of *Plantago* subg. *Plantago* is shown on the right.

Mesembrynia, is sister to a clade of the remaining species (BS = 100%; PP = 1.00). The last major dichotomy shows *P. macrocarpa* (sect. *Plantago*), from northwestern North America and northeastern Asia, as sister to a clade (clade I; BS = 87%; PP = 1.00) of American species

from sects. Oliganthos, Plantago (P. fernandezia) and Virginica.

Table 3

Accepted species in *Plantago* subg. *Plantago* and their native distributions. Their former sectional placement and chromosome numbers are according to Rahn (1996). The corresponding clades highlighted in Fig. 4 are indicated after the section names. Species not included in the present phylogeny, but included in the phylogeny of Iwanycki Ahlstrand et al. (2019) are marked with an exclamation mark (!); species not included in either phylogenies, but whose position we inferred based on the accumulated knowledge (see Rosenberg and Kumar, 2001) are marked with an asterisk (*).

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* P. polita Craven New Guinea Oliganthos ? P. raoulii Deene. New Zealand Mesembrynia 48 P. spathulata Hook.f. New Zealand Mesembrynia 48 P. stauntonii Reichardt St. Paul and New Amsterdam Islands (France) Mesembrynia 24 * P. stenophylla Merr. & L.M.Perry New Guinea Oliganthos ? P. tasmanica Hook.f. Tasmania (Australia) Mesembrynia 12 P. trandra Berggr. New Zealand New Zealand Oliganthos 12 P. triandra Berggr. New Zealand New Zealand Oliganthos 12 * P. triandra Berggr. New Zealand Oliganthos 12 * P. triandra Berggr. New Guinea Oliganthos 12 * P. trinchophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 <		P. picta Colenso	New Zealand	Mesembrynia	- 48
P. raoulii Decne. New Zealand Mesembrynia 48 P. spathulata Hook.f. New Zealand Mesembrynia 48 P. stauntonii Reichardt St. Paul and New Amsterdam Islands (France) Mesembrynia 24 * P. stenophylla Merr. & L.M.Perry New Guinea Oliganthos 2 P. tasmanica Hook.f. Tasmania (Australia) Mesembrynia 12 P. traindra Berggr. New Zealand New Zealand Oliganthos 12 * P. triandra Berggr. New Zealand (New Zealand) and Tasmania (Australia) Oliganthos 12 * P. triandra Berggr. New Guinea Oliganthos 12 * P. triandra Berggr. New Guinea Mesembrynia 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 96	*	P. polita Craven	New Guinea	Oliganthos	?
P. spathulata Hook.f.New ZealandMesembrynia48P. stauntonii ReichardtSt. Paul and New Amsterdam Islands (France)Mesembrynia24*P. stenophylla Merr. & L.M.PerryNew GuineaOliganthos?P. tasmanica Hook.f.Tasmania (Australia)Mesembrynia12P. traindra Berggr.New ZealandOliganthos48*P. triandra Berggr.New ZealandOliganthos12*P. triandra Berggr.Auckland Islands (New Zealand) and Tasmania (Australia)Oliganthos12*P. trichophora Merr. & L.M.PerryNew GuineaMesembrynia?*P. turiffera B.G.Briggs et al.AustraliaMesembrynia12P. uticiola Meudt & GarnJonesNew ZealandMesembrynia?		P. raoulii Decne.	New Zealand	Mesembrynia	48
P. stauntonii Reichardt St. Paul and New Amsterdam Islands (France) Mesembrynia 24 * P. stenophylla Merr. & L.M.Perry New Guinea Oliganthos ? P. tasmanica Hook.f. Tasmania (Australia) Mesembrynia 12 P. triandra Berggr. New Zealand New Zealand Oliganthos 48 * P. triandra Bergegr. Auckland Islands (New Zealand) and Tasmania (Australia) Oliganthos 48 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. turifera B.G.Briggs et al. New Zealand Mesembrynia 12 * P. turifera B.G.Briggs et al. New Zealand Mesembrynia 12		P. spathulata Hook.f.	New Zealand	Mesembrynia	48
* P. stenophylla Merr. & L.M.Perry New Guinea Oliganthos ? P. tasmanica Hook.f. Tasmania (Australia) Mesembrynia 12 P. triandra Berggr. New Zealand Oliganthos 48 * P. triandra Spreng. Auckland Islands (New Zealand) and Tasmania (Australia) Oliganthos 12 * P. trichophora Merr. & L.M.Perry New Guinea Oliganthos 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia ? * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. utrifera B.G.Briggs et al. New Zealand mone (new species) 96		P. stauntonii Reichardt	St. Paul and New Amsterdam Islands (France)	Mesembrynia	24
P. tasmanca Hook.f. Tasmania (Australia) Mesembrynia 12 P. triandra Berggr. New Zealand Oliganthos 48 * P. triandra Spreng. Auckland Islands (New Zealand) and Tasmania (Australia) Oliganthos 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. utrichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. udicola Meudt & GarnJones New Zealand mone (new species) 96	*	P. stenophylla Merr. & L.M.Perry	New Guinea	Oliganthos	?
* P. triantha Spreng. New Zealand Oliganthos 48 * P. triantha Spreng. Auckland Islands (New Zealand) and Tasmania (Australia) Oliganthos 12 * P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia ? * P. turifera B.G.Briggs et al. Australia Mesembrynia 12 * P. uticiola Meudt & GarnJones New Zealand none (new species) 96		P. tasmanica Hook.t.	Tasmania (Australia)	Mesembrynia	12
* P. trichophora Merr. & L.M.Perry New Guinea Mesembrynia 12 * P. turrifera B.G.Briggs et al. Australia Mesembrynia 12 P. udicola Meudt & GarnJones New Zealand New Zealand none (new species) 96	*	r. u ulluru berggr. D triantha Spreng	www zealallu Auchland Islands (New Zealand) and Taemania (Auctralia)	Oliganthos	48 19
* <i>P. turifera</i> B.G.Briggs et al. Australia <i>Mesembrynia</i> 12 <i>P. udicola</i> Meudt & GarnJones New Zealand none (new species) 96	*	P. trichophora Merr. & I. M Perrv	New Guinea	Mesembrynia	14
P. udicola Meudt & GarnJones New Zealand none (new species) 96	*	P. turrifera B.G.Briggs et al.	Australia	Mesembrynia	12
		P. udicola Meudt & GarnJones	New Zealand	none (new species)	96

(continued on next page)

Table 3 (continued)

Species		Native distribution	Previously in sect.	2n =
	P. unibracteata Rahn	New Zealand	Oliganthos	60, 72
	P. varia R.Br.	E Australia	Mesembrynia	12
Sect. Mici	ropsyllium Decne. — clade A — 6 species			
!	P. bigelovii A.Gray	W North America	Micropsyllium	20
	P. elongata Pursh	W North America	Micropsyllium	12, 36
*	P. heterophylla Nutt.	North America	Micropsyllium	12
	P. polysperma Kar. & Kir.	C Asia	Micropsyllium	?
	P. pusilla Nutt.	North America	Micropsyllium	12
!	P. tenuflora Waldst. & Kit.	W Eurasia	Micropsyllium	24
Sect. Paci	fica Hassemer — clade H — 26 species			
*	P. alata Nakai	Jeju (Korea)	Plantago	?
	P. asiatica L.	E and SE Asia	Plantago	24
	P. camtschatica Link	NE Asia	Mesembrynia	12
*	P. curuleriel H.Lev.	S Chilla Jeiu (Korea)	Plantago	? 2
	P. depressa Willd.	Asia	Mesembrynia	12
	P. eriopoda Torr.	North America	Plantago	24
*	P. glabrifolia (Rock) Pilg.	Hawaiian Archipelago (USA)	Plantago	
*	P. grayana Pilg.	Hawaiian Archipelago (USA)	Plantago	
*	P. hakusanensis Koidz.	Japan	Plantago	?
*	P. hasskarlii Decne.	Java (Indonesia)	Plantago	?
	P. hawaiensis (A.Gray) Pilg.	Hawaiian Archipelago (USA)	Plantago	?
×	P. hillebrandii Pilg.	Hawaiian Archipelago (USA)	Plantago	2
	P. Incisu Hassk. P. komarovii Pavlov	G Asia	Planlago Masambrunia	? 2
*	P krajinaj Pilo	Hawaijan Archinelago (IISA)	Plantago	•
*	P. melanochrous Pilg.	Hawaiian Archipelago (USA)	Plantago	
*	P. muscicola Pilg.	Hawaiian Archipelago (USA)	Plantago	
	P. pachyphylla A.Gray	Hawaiian Archipelago (USA)	Plantago	24
	P. princeps Cham. & Schltdl.	Hawaiian Archipelago (USA)	Plantago	12
	P. rapensis Pilg.	Rapa Iti Island (France)	Plantago	?
	P. rugelii Decne.	E North America	Plantago	24
	P. rupicola Pilg.	Rapa Iti Island (France)	Plantago	?
	P. sparsiflora Michx.	SE North America	Plantago	24
!	P. taquetti H.Lev. P. tugadhi A. Gray	Jeju (Korea) W North America	Plantago Plantago	? 24
	r. tweeuyt A.Glay	W North Allerica	Fiantago	24
Sect. Plan	ttago — clade F — 5 species			
	P. cornutii Gouan	S Europe	Plantago	12
!	P. grijitnii Deche.	S Asia	Plantago (as a synonym)	? 2
*	P. tatarica Decne	S Asia	Plantago (as a synonym)	: ?
	P. major L.	Eurasia	Plantago	12
Cost Vine	inia Dama 9 Stainh an Damáand alada I 46		0	
sect. virg	<i>P. alismatifolia</i> Dila	C Mexico	Virginica	24
*	P argentina Pilo	NW Argentina	Virginica	24 48
	P. australis Lam.	South America and S North America	Virginica	24, 48
!	P. barbata G.Forst.	S Argentina and S and C Chile	Oliganthos	48, 72
*	P. berroi Pilg.	Uruguay and E Argentina	Virginica	24
	P. bradei Pilg.	E Brazil	Virginica (as a synonym)	?
*	P. buchtienii Pilg.	W Bolivia and NW Argentina	Virginica	48
	P. catharinea Decne.	S Brazil	Virginica	24
	P. commersoniana Decne. & Barneoud	Uru, S Brazil and SE Par.	Virginica	?
~	P. correae Rann D. correae Rann	S Argentina and S Chile	Oligantnos	96
	P. corvensis Hasseliner P. cumingiang Fisch & C.A. Mey	S ord C Chile and W Argentina	Virginica (as a suponym)	? 2
*	P. dielsiana Pilg.	E Argentina and S Uruguay	Virginica	: 24
	P. fernandezia Bertero ex Barnéoud	Juan Fernández Islands (Chile)	Plantago	?
*	P. firma Kunze ex Walp.	C Chile	Virginica	24
	P. floccosa Decne.	NE Mexico	Virginica	24
*	P. galapagensis Rahn	Galápagos Islands (Ecuador)	Virginica	?
	P. guilleminiana Decne.	S Brazil	Virginica	?
	P. hatschbachiana Hassemer	S Brazil	none (new species)	?
*	P. numbolatiana Hassemer	S Brazil	none (new species)	?
0	r. jujuyensis kann P. moorei Pahn	NW Argentina Weet Folkland (UK)	virginica Oliganthos	24
	r. moorel Kalli P. myosuros I am	west Faikland (UK) S and W South America	Uugununos Virainica	د 24
	P naniformis (Rahn) Hassemer	NE Arg Par and S Brazil	Virginica (as a subspecies)	2⊐ ?
*	<i>P. orbignyana</i> Steinh. ex Decne.	Ecu., Peru, Bol. and NW Arg.	Virginica	24. 48
	P. pachyneura Steud.	N and C Chile	Virginica	24
*	P. penantha Griseb.	Uru., NE Arg. and S Brazil	Virginica	24
*	P. pretoana (Rahn) Hassemer	SE Brazil	Virginica (as a subspecies)	?
*	P. pulvinata Speg.	S Argentina and S Chile	Oliganthos	24
*	P. pyrophila Villarroel & J.R.I.Wood	E Bolivia	none (new species)	?

(continued on next page)

Table 3 (continued)

Species		Native distribution	Previously in sect.	2n =
	P. rahniana Hassemer & R.Trevis.	S Brazil	none (new species)	?
	P. rhodosperma Decne.	SW USA and NE Mexico	Virginica	48
*	P. sempervivoides Dusén	S Argentina and S Chile	Oliganthos	?
	P. subnuda Pilg.	W USA	Virginica	48
	P. tehuelcha Speg.	S Argentina and S Chile	Oliganthos	24
*	P. tenuipala (Rahn) Rahn	C Colombia	Virginica	?
	P. tomentosa Lam.	Arg., Bol., Par., Uru. and S Brazil	Virginica	24
	P. trinitatis Rahn	Trindade Island (Brazil)	Virginica	?
*	P. truncata Cham. & Schltdl.	C Chile	Virginica	?
	P. turficola Rahn	S Brazil	Virginica	?
*	P. uniglumis Wallr. ex Walp.	S Argentina and S and C Chile	Oliganthos	48, 72
*	P. veadeirensis Hassemer	C Brazil	none (new species)	?
*	P. ventanensis Pilg.	E Argentina	Virginica	24
*	P. venturii Pilg.	W Argentina	Virginica	24
!	P. virginica L.	North America	Virginica	24
	P. weddelliana Decne.	S Bolivia and NW Argentina	Virginica	24
Incertae	sedis — 1 species			
*	P. robusta Roxb.	Saint Helena Island (UK)	Plantago	?

4. Discussion

4.1. Revised sectional classification

Supported by our plastome phylogeny (Fig. 4), in addition to the revision of herbarium collections, and a comprehensive revision of the taxonomic and phylogenetic literature on *Plantago*, we propose here a revised sectional classification of *Plantago* subg. *Plantago* (Table 3).

There is evidence of polyploidy, hybridisation and reticulate evolution in *Plantago* as discussed in the introduction. The plastome is maternally inherited and would therefore not provide evidence of conflicting evolutionary history between organelles. However, we observe good alignment of the plastome topology with other data including morphology and biogeography as well as topologies obtained in a recent study including both plastid and nuclear Sanger sequencing data (e.g. Iwanycki Ahlstrand et al., 2019). The hereby proposed classification is a valuable update to existing classifications, but a full revision of *Plantago* awaits additional HTS data covering also the nuclear genome, which could for example be obtained by gene capture methods.

The new sectional classification proposed here follows the following principles:

(1) All accepted sections must be monophyletic;

- (2) The recognised sections must be morphologically (especially regarding fruit and flower, the evolutionarily most conservative characters) and secondarily geographically coherent;
- (3) The new classification should take into consideration, as much as possible, aspects from the latest classification (Rahn, 1996), but also elements of previous classifications (Barnéoud, 1844, 1845; Decaisne, 1852; Pilger, 1937) when these were proved correct in light of the revised phylogeny.

Plantago subg. Plantago

Lectotype (designated by Britton and Brown 1913: 245): *P. major* L. Our results corroborate, with a strong support (BS = 100%; PP = 1.00), the already well-established perception (Rønsted et al., 2002; Hoggard et al., 2003; Ishikawa et al., 2009) that subg. *Plantago* is monophyletic. We estimate that there are 147 species in the subgenus (Table 3), although this number certainly will change in the future, with new species being described, species being re-established and names being synonymised as taxonomic knowledge of the group advances.

Plantago sect. Micropsyllium Decne. in A.DC., Prodr. 13(1): 696. 1852

Lectotype (designated by Dietrich 1980: 563): P. tenuiflora Waldst. & Kit.

= *Plantago* sect. *Diandra* H.Dietr., Wiss. Z. Friedrich-Schiller-Univ. Jena, Math.-Naturwiss. Reihe 29(4): 563. 1980

Holotype: P. elongata Pursh

Plantago sect. *Micropsyllium* comprises six species from North America and Eurasia (Table 3), is morphologically well defined (Bassett 1966; Rahn 1996) and is the only section within subg. *Plantago* that did not undergo any changes in this study. The three sampled species form a well-supported clade (BS = 100%; PP = 1.00; Fig. 4, clade A).

This section appears to have originated in Eurasia and subsequently colonised North America. In terms of morphology, sect. *Micropsyllium* is characterised by the following apomorphic characters: diminutive annual plants; annual root; hairs on scapes antrorse; corolla lobes shorter than 1 mm; anthers less than 1 mm long; seeds shorter than 2 mm. One important plesiomorphic character shared by all species is the linear leaves. Reported chromosome numbers are variable, including 2n = 12, 24, 36 and 20 (this last one, reported by Bassett (1966) for *P. bigelovii*, is the only record of $\times = 5$ in the subg. *Plantago*, and needs to be confirmed).

Selected taxonomic references: Pilger (1937), Bassett (1966) and Moore et al. (1976).

Plantago sect. Eremopsyllium Pilg., Pflanzenr. 102: 283. 1937

Holotype: P. reniformis Beck

= *Plantago* sect. *Gentianoides* Pilg., Pflanzenr. 102: 306. 1937, *syn. nov.*

Holotype: P. gentianoides Sibth. & Sm.

The two species included in the hereby re-established sect. *Eremopsyllium* (Table 3) were both sampled here and form a well-supported clade (BS = 97%; PP = 1.00; Fig. 4, clade B) that is sister to all the rest of subg. *Plantago* except for sect. *Micropsyllium*. These species are distributed in southeastern Europe and Asia Minor. Here, we do not accept *P. griffithii* as a subspecies of *P. gentianoides* (see Hassemer 2018). In terms of morphology, sect. *Eremopsyllium* is characterised by the following apomorphic characters: adventitious roots; spike less than 1/3 the length of the scape; anthers white both when fresh and when dried. Reported chromosome numbers are 2n = 12.

Selected taxonomic references: Pilger (1937), Moore et al. (1976) and Tutel (1982).

Plantago sect. Lamprosantha Decne. in A.DC., Prodr. 13(1): 697. 1852

Lectotype (designated by Rahn 1996: 196): P. media L.

The hereby re-established sect. *Lamprosantha* (Table 3) includes six species, all of which were sampled here: three species from Rahn's (1996) sect. *Plantago* (*P. canescens, P. maxima* and *P. media*) and three

from sect. *Mesembrynia* (*P. arachnoidea*, *P. perssonii* and *P. schwarzenbergiana*). This section is distributed in temperate Eurasia, with the exception of *P. arachnoidea* which occurs in northern Asia and northwestern North America. This section forms a well-supported clade (BS = 100%; PP = 1.00; Fig. 4, clade C) which includes the rare and threatened *P. maxima* as sister to a clade (BS = 100%; PP = 1.00) which includes the remainder of the species included in the section.

It should be noted that the support for the clade that includes sect. Lamprosantha plus all subsequent groups within subg. Plantago is relatively low (BS = 52%; PP = 0.95). It is possible that further investigations could indicate that sects. Eremopsyllium and Lamprosantha are sisters, in which case their merger into an enlarged sect. Lamprosantha would be desirable due to morphology and biogeography. However, our plastome phylogenies do not support this (Fig. 4), and therefore we recognise these two sections as distinct.

This section apparently has a temperate Eurasian origin. In terms of morphology, sect. *Lamprosantha* is characterised by the following apomorphic characters: spike less than 1/3 the length of the scape; seeds shorter than 2 mm. Reported chromosome numbers are 2n = 12, with the exception of a few populations of *P. media*, which have 2n = 24.

Selected taxonomic references: Pilger (1937), Grigoriev (1958), Moore et al. (1976) and Li et al. (2011).

Plantago sect. Leptostachys Decne. in A.DC., Prodr. 13(1): 720. 1852

Holotype: P. leptostachys E.Mey. ex Decne., nom. illeg., non Hook.f. (1847), nec Ledeb. (1849) — = P. laxiflora Decne.

= Plantago sect. Palaeopsyllium Pilg., Pflanzenr. 102: 75. 1937, syn. nov.

Lectotype (designated here): P. palmata Hook.f.

The seven species recognised in the hereby re-established sect. *Leptostachys* (Table 3) occur in sub-Saharan Africa and Madagascar, comprise the most tropical among the species within subg. *Plantago*, and comprise species included in Rahn's (1996) sect. *Plantago*. The three sampled species (*P. longissima*, *P. palmata* and *P. tanalensis*) form a clade which has a high support (BS = 100%; PP = 1.00; Fig. 4, clade D). Taxonomic knowledge of this section is the poorest among subg. *Plantago* and a taxonomic revision is critically needed, especially to clarify questions regarding *P. leptostachys* E.Mey. ex Decne. *nom. illeg.*, which is the type species of the section, and its supposedly accepted name, *P. laxiflora* (fide Pilger, 1937), which was not sampled here.

This section appears to have originated in continental sub-Saharan Africa and subsequently expanded to Madagascar. In terms of morphology, sect. *Leptostachys* is characterised by the following apomorphic characters: adventitious roots; corolla lobes shorter than 1.5 mm. One important plesiomorphic character shared by all species is the spikes normally equalling the length of the scape. Chromosome numbers are unknown for all species except for *P. palmata*, which has 2n = 24.

Selected taxonomic references: Pilger (1937) and Verdcourt (1971). *Plantago* sect. *Mesembrynia* Decne. in A.DC., Prodr. 13(1): 701. 1852

Lectotype (designated by Rahn 1996: 196): P. debilis R.Br.

= Plantago sect. Microcalyx Pilg., Pflanzenr. 102: 122. 1937, syn. nov.

Lectotype (designated by Rahn 1996: 196): P. triandra Berggr.

Plantago sect. *Mesembrynia* is hereby accepted as including 44 species (Table 3), although this number will probably increase in the future with the discovery of new species. Of these 44 species, 23 were sampled here which form a well-supported clade (BS = 100%; PP = 1.00; Fig. 4, clade E). This section encompasses all *Plantago* species native to Australia, New Zealand and New Guinea, and also species from some neighbouring islands (Auckland Islands, Lord Howe Island and Tonga), in addition to two species from St. Paul and New Amsterdam Islands in the southern Indian Ocean. Our expanded sect. *Mesembrynia* includes all species in Rahn's (1996) homonymous section except for the six Eurasian species (*P. arachnoidea, P. camtschatica, P. depressa, P. komarovii*,

P. perssonii and *P. schwarzenbergiana*), 15 species from his sect. *Oliganthos*, plus two island endemics (*P. aucklandica* and *P. hedleyi*) from his sect. *Plantago*.

Our cpDNA tree topology is similar but not identical to a previous phylogenetic study of the Australasian species, which analysed ITS, cpDNA and mtDNA sequences (Tay et al., 2010a). Our results indicate that at least four long-distance dispersal events occurred for the New Zealand *Plantago* species, likely originating from Australian ancestors, thus corroborating the conclusions of Tay et al. (2010a) who indicated three such events. One of these dispersal events comprises the clade of *P. lanigera* and *P. novae-zelandiae*, the second dispersal event comprises *P. unibracteata*, the third comprises *P. triandra*, and the fourth comprises the clade of *P. picta*, *P. raoulii*, *P. spathulata* and *P. udicola*. The small oceanic island species *P. euana*, *P. hedleyi* and *P. stauntonii* are spread across sect. *Mesembrynia*, again likely originating from Australian ancestors.

Although we attempted sequencing of samples of two species from New Guinea, *P. aundensis* and *P. papuana*, these were not able to be included here due to highly degraded DNA. In accordance with the accumulated biogeographic and morphological knowledge of these species (Craven, 1976; van Royen, 1983; Rahn, 1996), the seven *Plantago* species from New Guinea are here grouped together with the other Australasian species. However, we advocate that future phylogenetic works should focus on sampling New Guinean species, in order to confirm their phylogenetic placement and fully understand the biogeography of this Southern Hemisphere section.

Our results show that the two sampled individuals of *P. lanigera*, although in the same lineage, are paraphyletic relative to the closely related species, *P. novae-zelandiae* (BS = 100%; PP = 1.00). Because our study used cpDNA only on very few (1-2) individuals per species, it is not possible to speculate further on the taxonomic implications of this finding due to the complex polyploid evolutionary history of the New Zealand species (Meudt, 2011, 2012; Ishikawa et al., 2009). Nevertheless, the plants from Sewell Peak, from which the sample "*P. lanigera* 2" was collected, will be further investigated in regards to their morphology and will be compared with the types of other New Zealand *Plantago*, as it is possible that this sample could correspond to a still undescribed species.

This section appears to have originated in Australia and subsequently spread to New Zealand and other neighbouring islands in several separate dispersal events (Tay et al., 2010a). However, it should be noted that the lack of sampling of New Guinean species precludes the inference of the centre of origin of this section. In terms of morphology, apparently there is not a single apomorphic character that is shared by all species in sect. Mesembrynia. This is certainly the reason why the species included in the section have never before been all placed under the same section-there are considerable morphological differences between the species formerly placed in sects. Mesembrynia and Oliganthos, which is not reflected in the phylogeny. Some plesiomorphic characters seem to be shared by all the species in the section, such as: lamina with attenuate base or not distinguishable from the petiole; apex of the leaves without a colourless acumen; pedicel absent; corolla lobes always patent; anthers never white; ovary with more than 4 ovules; carpophore absent. Reported chromosome numbers are 2n = 12, 24,36, 48, 60, 72 and 96, with 12 being the most common and apparently the ancestral condition.

Selected taxonomic references: Briggs et al. (1973, 1977), Craven (1976), Briggs (1980), van Royen (1983) and Meudt (2012).

Plantago sect. Plantago

Type species: P. major L.

= *Plantago* sect. *Major* Barnéoud, Rech. Plantagin. Plumbagin.: 17. 1844

Holotype: P. major L.

= Plantago sect. Polyneuron Decne. in A.DC., Prodr. 13(1): 694. 1852

Lectotype (designated by Rahn 1996: 196): P. major L.

The hereby much reduced sect. *Plantago* (Table 3), with only five species, is without doubt the most unexpected and drastic change departing from Rahn's (1996) classification, who recognised 53 species (although with much doubt) in his admittedly paraphyletic sect. *Plantago*. The three species sampled here (*P. cornutii*, *P. himalaica* and *P. major*) form a clade, which is well supported (BS = 100%; PP = 1.00; Fig. 4, clade F). The five species in this section are all originally distributed in temperate Eurasia. The type species of the genus, subg. and sect. *Plantago*, *P. major*, is now a cosmopolitan species with a Eurasian origin. The morphologically similar *P. cornutii*, from southern Europe, and *P. griffithii*, *P. himalaica* and *P. tatarica*, from southern Asia (see Hassemer, 2018), are also included in this section.

This section appears to have its origin in southwestern Eurasia. In terms of morphology, sect. *Plantago* is characterised by the following apomorphic characters: adventitious roots; lamina more than four times as wide as the petiole; leaves remaining green on drying; hairs on scape antrorse; spur-like elongation on lowermost cell of non-glandular hairs on the scape; corolla lobes shorter than 2 mm; pyxidia globose, not conspicuously elongated. Chromosome numbers are known for two species (*P. cornutii* and *P. major*), 2n = 12.

Selected taxonomic references: Pilger (1937), Moore et al. (1976) and Hassemer (2018).

Plantago sect. Carpophorae Rahn, Nordic J. Bot. 5: 144. 1985 Holotype: P. rigida Kunth

The hereby re-established sect. *Carpophorae* (Table 3) comprises two species from mountains in Central America and western South America (Rahn, 1985). Both species sampled here form a clade that is well supported (BS = 100%; PP = 1.00; Fig. 4, clade G) and is sister to *P. cordata* (sect. *Heptaneuron*)—although with rather low support (BS = 55%; PP = 0.73). *Plantago* sect. *Carpophorae* is notable among *Plantago* for producing a carpophore (Rahn, 1985). The section was created with this exact circumscription by Rahn (1985), who later (Rahn 1996) changed this taxon to the series level, *Plantago* ser. *Carpophorae* (Rahn) Rahn, under *Plantago* sect. *Oliganthos*, because of the numerical results of his morphological phylogeny.

From a biogeographic perspective, it would be expected that the species in sect. *Carpophorae* would be closely related to the predominantly South American sect. *Virginica* (see below). From a morphological perspective, however, the two species in sect. *Carpophorae* could well be placed in a separate subgenus, given their unique, very distinct fruit morphology (Rahn, 1985). The inflorescences with very few flowers (normally 1, rarely 2–3) led to the two species in sect. *Carpophorae* having been included in sect. *Oliganthos* in most classification systems (e.g. Pilger, 1937; Rahn, 1996).

It would be extremely undesirable to unite the species in sects. *Carpophorae (P. rigida* and *P. tubulosa)* and *Heptaneuron (P. cordata)* in a single section, due to the very distinctive morphology of the two species in sect. *Carpophorae*, which is unique in *Plantago* and not coherent with that of *P. cordata*. Of less importance is the fact that *P. cordata* is a semi-aquatic plant endemic to eastern North America, whereas the species in sect. *Carpophorae* are endemic to mountains in western South America and southern North America (southern Mexico southwards). Although our phylogeny would allow the merger of these two sections, it would also allow their recognition as distinct, and we believe that classification systems based on molecular phylogenies should nevertheless be coherent from a morphological point of view.

It would have been possible, based on our phylogeny, to include the clade that encompasses sects. *Heptaneuron* and *Carpophorae* in a more broadly defined sect. *Plantago*—this clade of seven species is strongly supported (BS = 100%; PP = 1.00). Nevertheless, as explained above, this enlarged circumscription would be extremely undesirable because of considerable morphological differences of the two species in sect. *Carpophorae*. Therefore, we opted to recognise sect. *Carpophorae*, and as a consequence it was also necessary to recognise the monotypic sect. *Heptaneuron* (see below). However, due to the low support (BS = 55%; PP = 0.7306) of the clade containing sects.

Carpophorae + *Heptaneuron*, further phylogenetic investigation is critically needed to elucidate this group of sections. If improved phylogenies would in the future show that sects. *Heptaneuron* and *Plantago* are sister, they should probably be merged. In any case, because of morphology, the species in these two sections should not be merged with sect. *Carpophorae*.

This section appears to have originated in the Andes and subsequently colonised southern North America. In terms of morphology, sect. *Carpophorae* is well-characterised by the following apomorphic characters: adventitious roots; scape very short, less than a quarter of the supporting leaf; hairs on scape antrorse; small, three-celled, glandular hairs placed in cavities; flower solitary, only one bract present; anthers longer than 2 mm long; carpophore present. Reported chromosome numbers are 2n = 24, 48 and 72.

Selected taxonomic reference: Rahn (1985).

Plantago sect. Heptaneuron Decne. in A.DC., Prodr. 13(1): 698. 1852

Lectotype (designated by Rahn 1996: 196): P. cordata Lam.

The hereby re-established sect. Heptaneuron (Table 3) is monotypic, including only the semi-aquatic eastern North American P. cordata, previously in Rahn's (1996) sect. Plantago. Plantago sect. Heptaneuron is sister to sect. Carpophorae (BS = 55%; PP = 0.73) and, due to considerable morphological differences, should not be united with the latter (see explanation above). Plantago cordata is unique in the genus in that the fruits are still green and alive at the time of dehiscence, when the lid of the pyxidia readily falls off, and the seeds with the entire fleshy placenta fall out as a unit; this structure is buoyant and may represent an adaptation to dispersal by water (Tessene, 1969; Rosatti, 1984). In terms of morphology, sect. Heptaneuron is characterised by the following apomorphic characters: lamina less than 1.9 times as long as wide; lamina more than 4 times as wide as the petiole; base of lamina truncate; corolla lobes shorter than 1.5 mm; anthers longer than 2 mm long; ovary with four ovules. The chromosome number of P. cordata is 2n = 24.

Selected taxonomic reference: Pilger (1937) and Bassett (1973). *Plantago* sect. *Pacifica* Hassemer, *sect. nov.*

Diagnosis: plants perennial; apex of the leaves without a colourless acumen; scape length at least more than a quarter of the supporting leaf; scape not elongating conspicuously after anthesis; trichomes on leaves up to 2 mm long; trichomes on leaves more than 0.04 mm wide; absence of small, three-celled, glandular hairs placed in cavities; normal spike with 12 flowers or more; sepals glabrous on the back; corolla lobes always patent; corolla lobes up to 3 mm long; stamens 4; anthers never white; anthers longer than 0.5 mm; carpophore absent; ovary with more than 4 ovules; mature pyxidia pyriform, elongated; seeds shorter than 3 mm.

Holotype: P. princeps Cham. & Schltdl.

This new section, which we estimate to include 26 species, 15 of which were sampled here (Table 3), corresponds to a well-supported clade (BS = 100%; PP = 1.00; Fig. 4, clade H) sister to sects. *Holopsyllium* + *Virginica*. All species were formerly placed in Rahn's (1996) sect. *Plantago*, except for three species from his sect. *Mesembrynia* (*P. camtschatica*, *P. depressa* and *P. komarovii*). Because this lineage does not include *P. major*, the type species of sect. *Plantago*, it required a new name at section level. A thorough sampling, especially of the Eurasian species in subg. *Plantago*, is needed to confirm the circumscriptions of sects. *Pacifica* and *Plantago*. The name of the new section is a reference to the distribution of its species in Asia and North America, i.e. at both sides of the Pacific Ocean, and also in some Pacific oceanic islands (Hawaiian Archipelago and Rapa Iti Island).

The central Asian *P. komarovii* is sister to the remainder of the species in the section (BS = 100%; PP = 1.00). This clade then splits into an Asian clade (BS = 100%; PP = 1.00) including *P. asiatica*, *P. camtschatica*, *P. cavaleriei*, *P. depressa* and *P. incisa*, and another clade (BS = 100%; PP = 1.00) comprising North American and the oceanic species mentioned above. Our phylogeny indicates that the Hawaiian

(*P. glabrifolia*, *P. grayana*, *P. hawaiensis*, *P. hillebrandii*, *P. krajinai*, *P. melanochrous*, *P. muscicola*, *P. pachyphylla* and *P. princeps*) and Rapa Iti Island (*P. rapensis* and *P. rupicola*) species in this section originated from North American ancestors, as all these island species are included in the clade that also includes the North American *P. eriopoda*, *P. rugelii*, *P. sparsiflora* and *P. tweedyi*. The closest living relatives of the Hawaiian and Rapa Iti *Plantago* were indicated, in a strongly-supported clade (BS = 100%; PP = 1.00), to be *P. rugelii* and *P. sparsiflora*.

Based on morphology and biogeography, it would appear that P. alata, P. coreana and P. taquetii, all described from Jeju Island, could be synonyms of *P. asiatica*—however, a comprehensive taxonomic revision of the Korean *Plantago* is needed to confirm this. A comprehensive taxonomic treatment of the Hawaiian *Plantago* is also urgently needed. as the synonymisation of P. glabrifolia, P. grayana, P. hillebrandii, P. krajinai, P. melanochrous and P. muscicola under P. pachyphylla done by Wagner et al. (1990) appears weakly supported from a morphological point of view (G. Hassemer, pers. obs.), which may have been why Rahn (1996) decided to keep these six species in his phylogenetic study. Furthermore, there is phylogenetic and morphological evidence (Dunbar-Co et al., 2008, 2009) that there are more species in the Hawaiian Archipelago than currently recognised. We are here following the treatment of Pilger (1937) regarding the Hawaiian Plantago, because it seems to better reflect the specific diversity in this group than the treatment of Wagner et al. (1990). Such as occurred with some other plant groups such as Asteraceae (Baldwin and Sanderson, 1998; Knope et al., 2012) and Campanulaceae (Givnish et al., 2009), we believe it possible that a great diversification occurred upon the arrival of Plantago from North America to Hawaii, due to the abundance of unoccupied niches. The taxonomic resolution of Hawaiian Plantago is critical because of implications it would have for the conservation of narrowly endemic species, but also for allowing a better understanding of sect. Pacifica.

This section appears to have its origin in central and eastern Eurasia, and subsequently colonised North America, and from there it spread to the Hawaiian archipelago and Rapa Iti Island. In terms of morphology, apparently there is not a single apomorphic character that is shared by all species in sect. Pacifica. Some plesiomorphic characters seem to be shared by all the species in the section, such as: plants perennial; apex of the leaves without a colourless acumen; scape length at least more than a quarter of the supporting leaf; scape not elongating conspicuously after anthesis; trichomes on leaves up to 2 mm long; trichomes on leaves more than 0.04 mm wide; absence of small, threecelled, glandular hairs placed in cavities; normal spike with 12 flowers or more; sepals glabrous on the back; corolla lobes always patent; corolla lobes up to 3 mm long; stamens 4; anthers never white; anthers longer than 0.5 mm; carpophore absent; ovary with more than 4 ovules; mature pyxidia pyriform, elongated; seeds shorter than 3 mm. Reported chromosome numbers are 2n = 12 and 24.

Selected taxonomic references: Pilger (1937), Grigoriev (1958), Bassett (1973), Wagner et al. (1990), Yamazaki (1993) and Li et al. (2011).

Plantago sect. Holopsyllium Pilg., Pflanzenr. 102: 101. 1937 Holotype: P. macrocarpa Cham. & Schltdl.

This monotypic section (Table 3), hereby re-established, is sister to the predominantly South American sect. *Virginica* (BS = 100%; PP = 1.00). Its only species, *P. macrocarpa*, occurs on the coast of northwestern North America, the Aleutian archipelago and the Commander Islands (Russia). The uniqueness of several morphological characters of *P. macrocarpa* has already been evidenced by Pilger (1937), of which the most prominent are the indehiscent pyxidia. Because of pronounced morphological differences, it would be undesirable to merge sect. *Holopsyllium* with its sister, sect. *Virginica*, and for this reason both sections are accepted. In terms of distribution and phylogeny, sect. *Holopsyllium* could perhaps be a testimony of the crossing of subg. *Plantago* from Eurasia to the Americas. In terms of morphology, sect. *Holopsyllium* is characterised by the following apomorphic characters: anterior sepals distinctly narrower than the posterior, and differently shaped; corolla lobes shorter than 2 mm; ovary with two ovules, and no rudiment of an upper compartment; fruit an indehiscent pyxidium; seeds longer than 3 mm. The chromosome number of *P. macrocarpa* is 2n = 24.

Selected taxonomic references: Pilger (1937), Grigoriev (1958) and Bassett (1973).

Plantago sect. Virginica Decne. & Steinh. ex Barnéoud, Rech. Plantagin. Plumbagin.: 17. 1844

Holotype: P. virginica L.

= Plantago sect. Cleiosantha Decne. in A.DC., Prodr. 13(1): 721. 1852

Lectotype (designated by Rahn 1996: 196): *P. veratrifolia* Decne. — *P. australis* subsp. *hirtella* (Kunth) Rahn

= Plantago sect. Dendriopsyllium Decne. in A.DC., Prodr. 13(1): 704. 1852, syn. nov.

Lectotype (designated by Rahn 1996: 196): P. fernandezia Bertero ex Barnéoud

= Plantago sect. *Fernandezia* Barnéoud, Rech. Plantagin. Plumbagin.: 19. 1844, *syn. nov.*

Holotype: P. fernandezia Bertero ex Barnéoud

= *Plantago* sect. *Novorbis* Decne. in A.DC., Prodr. 13(1): 724. 1852 Lectotype (designated by Rahn 1996: 196): *P. tomentosa* Lam.

Plantago sect. Oliganthos Barnéoud, Rech. Plantagin. Plumbagin.:
 17. 1844, syn. nov.

Lectotype (designated by Rahn 1984: 609): *P. pauciflora* Lam. — = *P. barbata* G.Forst.

= Plantago sect. Oreophytum Decne. in A.DC., Prodr. 13(1): 704. 1852

Holotype: P. orbignyana Steinh. ex Decne.

= Plantago sect. Plantaginella Decne. in A.DC., Prodr. 13(1): 727. 1852, syn. nov.

Lectotype (designated by Rahn 1984: 609): P. barbata G.Forst.

With 46 recognised species, one of which (*P. cumingiana*) hereby reestablished (see below), our enlarged sect. *Virginica* (Table 3) is sister to the monotypic sect. *Holopsyllium*, which has distinct fruit morphology and distribution (see above). The clade of sect. *Virginica* is well-supported in our phylogeny (BS = 87%; PP = 1.00; Fig. 4, clade I), and includes all species in Rahn's (1996) homonymous section, in addition to the seven American species in series *Oliganthos*, and *P. fernandezia*, which was previously placed in sect. *Plantago*. This predominantly South American clade has two centres of diversity: one in high-elevation grasslands and open coastal vegetation of central-eastern South America, including Uruguay, southern Brazil and eastern Argentina, and another in moist rocky environments of southern South America, which includes the American species in Rahn's (1996) series *Oliganthos*.

In *Plantago* sect. *Virginica*, our phylogeny indicated an early split between a clade (BS = 100%; PP = 1.00) including the southern South American *P. fernandezia* and *P. tehuelcha*, and another clade (BS = 100%; PP = 1.00) including the remainder of species sampled, including the West Falkland endemic *P. moorei*. Based on our phylogeny it is impossible to infer the position of the five unsampled American species in Rahn's (1996) series *Oliganthos (P. barbata, P. correae, P. pulvinata, P. sempervivoides* and *P. uniglumis*) between these two possible early branches within sect. *Virginica*. Similarly to the case of another large section, i.e. sect. *Mesembrynia*, we consider that sect. *Virginica* constitutes a phylogenetically and biogeographically coherent unit whose splitting would be undesirable because the species previously recognised in sect. *Oliganthos* (due to distinct morphology) are spread in multiple branches through the phylogeny of the section.

This section appears to have its origin in southern South America, and subsequently expanded to the rest of South America and also to North America. In terms of morphology, apparently there is not a single apomorphic character that is shared by all species in sect. *Virginica*. This is certainly the reason why the species included in the section have never before been all placed under the same section—there are considerable morphological differences between the species formerly placed in sects. *Virginica* and *Oliganthos*, which is not reflected in the phylogeny. Some plesiomorphic characters seem to be shared by all the species in the section, such as: nerves of dead leaf never remaining on the plant as long bristles; lamina with attenuate base or not distinguishable from the petiole; scape not elongating conspicuously after anthesis; spike open and cylindrical, the rachis visible between the flowers; pedicel absent; corolla lobes longer than 1 mm; stamens 4; anthers never white; carpophore absent; mature pyxidia pyriform, elongated. Reported chromosome numbers are 2n = 24, 48, 72 and 96, with 24 being the most common and apparently the ancestral condition.

Our phylogenetic results indicated that the current concept of P. catharinea is polyphyletic; this taxonomic problem, which is caused by some populations of P. napiformis being misidentified as P. catharinea, was discussed in detail in Hassemer (2019). We also highlight that our sampling of P. australis, albeit limited considering its continental distribution with eight subspecies currently recognised, clearly indicated that one of its subspecies, i.e. P. australis subsp. cumingiana, is polyphyletic in relation to the rest of the sampled subspecies, which formed a monophyletic clade. The monophyly of the remainder of the sampled subspecies of P. australis does not disagree with the current taxonomic treatment of the species (Rahn, 1974; Hassemer et al., 2015), but also does not necessarily agree with the recognition of these taxa at the subspecies rank. The resolution of the P. australis complex will require an extensive sampling of populations encompassing all subspecies and preferably all environmentally distinct regions where it occurs, coupled with comprehensive morphological and nomenclatural knowledge of the group.

Selected taxonomic references: Rahn (1974, 1984), complemented with novelties in Villarroel and Wood (2011), Hassemer and Baumann (2014), Hassemer et al. (2014, 2015); Hassemer (2016, 2017, 2019) and Hassemer and Rønsted (2016).

Species incertae sedis:

P. robusta Roxb.

Based on our results, it is not possible to ascertain the phylogenetic position of *P. robusta*—unfortunately, the sample of this species that we sequenced was contaminated with a species belonging to *Plantago* subg. *Coronopus.* This species is endemic to Saint Helena, a small (122 km²) South Atlantic oceanic island more than 2000 km from the nearest major landmass (Africa). This species has aerial woody stems like other oceanic island endemics such as *P. fernandezia* and *P. trinitatis.* However, based on morphology we cannot infer its phylogenetic placement. Therefore, new sampling of this species, preferably from living specimens, is necessary.

Revalidation of Plantago cumingiana

Plantago cumingiana Fisch. & C.A.Mey., Index Seminum [St. Petersburg] 3: 44–45. 1837

= Plantago australis subsp. cumingiana (Fisch. & C.A.Mey.) Rahn, Bot. Tidsskr. 60: 48–49. 1964

Lectotype (or maybe neotype, designated by Rahn 1964: 48–49): CHILE. S.d., *H. Cuming s.n.* (LE-00016458! [Fig. S4]).

In all plastome trees *P. australis* subsp. *cumingiana* did not form a clade with the three other sampled subspecies of *P. australis* (*P. australis* subsp. *australis* subsp. *australis* subsp. *hirtella* and *P. australis* subsp. *leioloma*). In the plastome tree, *P. australis* subsp. *cumingiana* is sister (BS = 52%, PP = 0.98) to a clade (BS = 49%, PP = 0.77) which includes, among other species, *P. bradei* and *P. tomentosa* in addition to the other *P. australis* samples. Although the inclusion of *P. bradei* is not strongly supported, the next clade excluding it is very well supported (BS = 100%, PP = 1.00). This phylogenetic evidence, in addition to the study of several hundred specimens of *P. australis* from all over its distribution, has convinced us of the need for re-establishing this species, whose geographic dispersion does not overlap with the huge extent of occurrence of *P. australis*, the most common and widespread species in *Plantago* sect. *Virginica* (see Rahn, 1974).

Plantago cumingiana occurs in central (Valparaíso) to southern

(Tierra del Fuego) Chile, and also in southwestern Argentina, in the western parts of the provinces of Chubut, Neuquén and Río Negro (see Rahn, 1974; Murillo, 2012). Some morphological differences from the other subspecies of *P. australis* have been observed during the revisions of herbarium collections, and also with cultivation experiments: a taproot is often present among cord-like secondary roots, the leaves have a slightly thicker consistency, the corollas are slightly longer, and the length/breadth ratio of the seeds is slightly less (slightly more globose-like than ellipsoid). However, P. australis is a morphologically very variable species, and outlier specimens exist for most of its subspecies, what makes us conclude that morphology alone is not enough to resolve the P. australis species complex. This could explain why Rahn (1964, 1974) decided to lump together over a dozen previously-accepted species in his enlarged concept of P. australis. Our results show that the three sampled subspecies (P. australis subsp. australis, P. australis subsp. hirtella and P. australis subsp. leioloma) cluster together in a single clade, indicating that it is possible that they are conspecific, whereas P. cumingiana clearly constitutes a separate phylogenetic branch. Our results suggest that P. australis does not occur in Chile-all records of this species in this country are P. cumingiana instead. Inclusion of multiple accessions and the other synonymised previously recognised species, as well as nuclear molecular markers, is necessary to resolve the P. australis complex in the future.

Notes on the new classification

The hereby-proposed classification system for subg. *Plantago*, with 11 accepted sections, recognises considerably more sections than that of Rahn (1996), which accepted five sections (Table 1), but slightly less sections than Pilger (1937), who accepted 13 sections (i.e. sects. *Eremopsyllium, Gentianoides, Holopsyllium, Lamprosantha, Mesembrynia, Microcalyx, Micropsyllium, Novorbis, Oliganthos, Oreophytum, Palaeopsyllium* and *Polyneuron*) for the subgenus as we understand it. Compared to Rahn's (1996) classification, the most important changes were the transfer of the majority of the species of his admittedly nonmonophyletic sect. *Plantago* to six other sections (i.e. sects. *Eremopsyllium, Heptaneuron, Holopsyllium, Lamprosantha, Leptostachys* and *Pacifica*) and the disintegration of his sect. *Oliganthos,* the species of which were transferred to sects. *Mesembrynia* and *Virginica* following a geographically coherent pattern.

From our revised classification it is evident that some morphological characters that have been used for classifying the species in subg. Plantago are not appropriate to this end, as they overlap across different clades. Examples of such are number of flowers in the inflorescences, and trichome and seed morphology. Other characters are more conserved across the phylogeny and therefore significant for infrageneric classification, namely flower (flower symmetry, number of stamens, and the flowers being hermaphroditic or not) and fruit (fruit shape and dehiscence, and number of seeds) characters. Based on our results, we argue that morphology remains the most adequate tool for the discovery of new species of flowering plants, as molecular phylogenetic techniques are still very far from being universally available, and are not helpful when exploring the biodiversity out in the field or during herbarium revisions. Furthermore, it should be highlighted that the correct identification of specimens relies on morphology, and phylogenies based on misidentified specimens are very detrimental to science. Regarding chromosome numbers, some sections are relatively homogenous, while others present wider variation (e.g. sects. Carpophorae, Mesembrynia, Micropsyllium and Virginica).

The considerably reduced morphology within subg. *Plantago*, and the fact that molecular phylogeny evidenced that most characters formerly used to distinguish sections are variable within and overlap between different sections, has convinced us that an attempt to produce an identification key to the sections of subg. *Plantago* would most probably result in an impractical and unusable key, thus thwarting the purpose of an identification key which is to facilitate the identification of specimens by non-specialists. For this reason, we do not provide such a key here. The identification of specimens of *Plantago* requires the consultation to specialised taxonomic works and regional floras, which have paramount importance for the advancement of the taxonomic knowledge.

4.2. The application of molecular phylogeny to classification

The final alignment of the plastome dataset included 96 samples encompassing 88 species providing a significant improvement compared to previous studies (from 40 species, $\sim 28\%$ of the subgenus previous, to 83 species, $\sim 56\%$ here) of *Plantago* subg. *Plantago*. Ten additional samples were sequenced, but could not be included due to too much degradation of DNA or contamination. Although HTS approaches have proven very efficient in obtaining DNA from even highly degraded species in general compared to Sanger sequencing, difficulties in obtaining samples of sufficient DNA quality of rare and rarely collected species remains a problem. However, overall our phylogenetic results, combined with insights from the extensive herbarium and literature revision, has evidenced that HTS is a very promising tool to support the resolution of taxonomic problems and we have here been able to propose a new sectional classification of the taxonomically difficult subg. *Plantago*.

Our newly proposed classification departs considerably, in many aspects, from all previous major classification systems for *Plantago*, all based on morphology: Barnéoud (1844, 1845), Decaisne (1852), Pilger (1937) and Rahn (1978, 1996). Some of the proposed changes to the most recent and currently accepted system, Rahn (1996), re-established aspects from previous classifications, including Rahn's previous ideas, as in the case of sect. *Carpophorae*. This had already happened before, when Rønsted et al. (2002) indicated that *Plantago* subg. *Albicans* Rahn was paraphyletic to subg. *Psyllium* and argued for its merging with subg. *Psyllium* as discussed above. *Plantago* subg. *Albicans* was described by Rahn (1996) as result of his phylogeny based on morphology, and was a departure from his previous proposal, based on his taxonomic experience and insight, to unite several of Pilger's (1937) sections into a much enlarged subg. *Psyllium* (Rahn 1978).

Despite the great value of morphology for the classification and identification of species, the ineffectiveness of morphological phylogeny (Rahn 1996) to infer relationships within Plantago becomes evident with the results of our molecular phylogeny. This is probably aggravated by the general morphological reduction of most reproductive structures in Plantago, and possibly also the parallel evolution of similar characteristics in similar habitats. One illustrative example is the trichomes on scapes, which have repeatedly been reported as one of the most important taxonomic characters for Plantago (Rahn, 1974, 1992, 1996; Andrzejewska-Golec, 1991; Andrzejewska-Golec and Świętosławski, 1993; Hassemer et al., 2014, 2015; Hassemer, 2016, 2017). Our phylogenetic results indicate that, although this character is very useful to classify and identify species, even closely-related species can differ considerably.

Even so, the morphology-based phylogeny of Rahn (1996) is more similar to our findings than molecular phylogeny based on the nuclear marker SUC1 (Ishikawa et al. 2009), emphasising the need for inclusion of multiple markers and interpretation in the light of current taxonomic understanding based on morphology, biogeography, and other evidence. A molecular phylogeny is only reliable when the samples used are correctly identified, which requires morphological knowledge of the taxa studied, and also nomenclatural knowledge, otherwise it is impossible to link morphologies to names. Furthermore, errors committed during the laboratory work, especially contamination, can also compromise the reliability of the resulting phylogenies and lead to erroneous conclusions in the worst case.

5. Future perspectives

Future phylogenetic research on *Plantago* should include the species indicated here as *incertae sedis* (*P. robusta*), as well as *P. nubicola* and the

New Guinean species, which unfortunately could not be included in this study. Taxonomic revisions are critically necessary for the African, southern Asian and Hawaiian species of subg. *Plantago*. Furthermore, intensified taxonomic work is necessary to discover and present to science the still undescribed narrowly endemic species in sects. *Mesembrynia* and *Virginica*, whose species numbers are certainly underestimated.

A species level phylogeny including multiple accessions of all species and using nuclear as well as chloroplast DNA markers would greatly contribute towards the necessary knowledge for the appropriate development and application of conservation efforts and strategies for the narrowly endemic, endangered *Plantago* species (e.g. Hassemer and Baumann, 2014; Hassemer, 2016, 2017; Hassemer and Rønsted, 2016), including the still little-understood cryptic species (Rahn, 1974; Hassemer et al., 2015). Conservation biologists should rely on the most reliable information available on the species, i.e. the most updated taxonomic treatments, and consider the combined knowledge accumulated by taxonomists and the results of new tools and techniques.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.05.013.

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