Nucleotide diversity and genetic relationships of orchardgrass (*Dactylis glomerata*) inferred from two non-coding regions of the chloroplast.

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A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Bachelor of Science – Honours Biology

April 2016, Halifax, Nova Scotia

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Date: April 13, 2016

Abstract

Nucleotide diversity and genetic relationships of orchardgrass (*Dactylis glomerata*) inferred from two regions of the chloroplast.

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Orchardgrass (*Dactylis glomerata* L.), an important member of the Poaceae family, is a cool-season perennial forage grass, and is widely used for hay production throughout temperate and subtropical regions of the world. Despite its economic importance, nucleotide diversity of orchardgrass species remains largely unexplored. In this study, we characterized nucleotide diversity of orchardgrass species using the chloroplast *rps*16 intron and Asp(GUC) – Thr(GGU) intergenic region. Genetic relationships among 40 orchardgrass accessions of eight *Dactylis glomerata* subspecies were examined. The estimates of nucleotide diversity (π) for *trn*D – *trn*tW and *rps*16 varied from 0.00073 for *Dactylis glomerata* subsp. *glomerata* to 0.00484 for *Dactylis glomerata* subsp. *smithii*, 0.00011 for *Dactylis glomerata* subsp. *glomerata* to 0.00393 for *Dactylis glomerata* subsp. *lusitanica* and *Dactylis glomerata* subsp. *woronowii* respectively. A dendrogram based on genetic distance of the Asp(GUC) – Thr(GGU) intergenic region revealed that four accessions, two from *Dactylis glomerata* subsp. *hispanica*, one from *Dactylis glomerata* subsp. *glomerata*, and one from *Dactylis glomerata* subsp. *woronowii* were more closely related to one another than the rest of the *Dactylis glomerata* subspecies/accessions. A dendrogram based on genetic distance of the *rps*16 intron revealed that four accessions, two from *Dactylis glomerata* subsp. *glomerata*, one from *Dactylis glomerata* subsp. *lusitanica* and one from *Dactylis glomerata* subsp. *woronowii* were more closely related to one another than to the rest of the *Dactylis glomerata* subspecies/accessions.

April 13, 2016

Acknowledgments

Foremost, I want to express my sincerest gratitude to my supervisor Dr. Genlou Sun for giving me this opportunity and providing me with his patience, insight, reassurance, and financial support. I would like to thank the members of the Sun Lab: Kai Zhou and Afnan Khalid, whose help with laboratory techniques and guidance was always greatly appreciated. Moreover, I would like to thank Dr. Zhongmin Dong, and the faculty and staff of Saint Mary's University Biology Department for their advice and assistance throughout this process. Finally, I would like to thank my family and friends for everything they have provided me with, their never ending support and encouragement helped make this possible.

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Introduction

Genetic Diversity

Phylogeny is the study and reconstruction of evolutionary relationships of organisms (Aluru, 2006). DNA sequences incur variations through evolutionary time that can be used to evaluate species relationships and produce a phylogenetic tree. Although there are multiple methods for constructing a phylogenetic tree, the most popular method is a maximum parsimony analysis, which systematizes the species into a tree requiring the minimum amount of evolutionary change to explain pragmatic differences (Ye, 2008). The integrity of the generated tree can be assessed using bootstrap analyses (Aluru, 2006; Ye, 2008).

Taxonomy is defined as the description, identification, nomenclature and classification of an organism. Plant systematics is a method that combines phylogenetic analyses and molecular data to provide a background and tools to understand the relationship at all levels of the taxonomic hierarchy (Small et al., 2004). The taxonomy for some species, as those present in this study, can be exceedingly multifaceted due to the morphological variation within and between species. Therefore, to further describe and ultimately classify species, multiple taxonomic and phylogenetic parameters need to be analyzed to truly reveal relationships between and within species.

Genetic diversity is universal in nature and is the basis for survival,

adaption and provides raw material for evolution of a species by natural selection (Fisher, 1930). Central questions have always been: "How much genetic variation occurs within and between species?," and "What is the structure of this variation among populations?" (Nevo and Beiles, 1989). The magnitude of genetic variability within a population is a fundamental source of biodiversity. In order to successfully plan, implement, and manage conservation programs, knowledge of genetic diversity and population structure is necessary (Sun et al., 2001). Higher genetic variation is linked to survival, adaptation, and is important for approximating populations constraints, such as evolutionary studies of mating systems and relatedness (Sun et al., 2001). Previously, the detection and analysis of genetic diversity was based on Mendelian methods concerned with morphological and cytological variants. Later methods have progressed to statistical analysis of quantitative variation to biochemical assays and, currently, molecular genetic markers (Sun et al., 2002).

DNA molecular markers

In the past decade, numerous molecular markers have been utilized for genetic diversity, genetic mapping and quantitative loci analysis in orchardgrass (Jiang et al., 2014). DNA molecular techniques have gained popularity, principally those based on polymerase chain reaction (PCR), such as random amplified

polymorphic DNA (RAPD) (Kölliker et al., 1999; Tuna et al., 2004), amplified fragment length polymorphism (AFLP) (Peng et al., 2008; Reeves et al., 1998), inter-simple sequence repeats (ISSR) (Madesis et al., 2014; Zeng et al., 2006), sequence-related amplified polymorphism (SRAP)(Xie et al., 2011; Zeng et al., 2008), simple sequence repeats (SSRs) (Hirata et al., 2011; Xie et al., 2011, 2012), expressed sequence tags (EST)-SSRs (Bushman et al., 2011; W. G. Xie et al., 2010), and start codon-targeted (SCoT) polymorphism (Jiang et al., 2014; Zeng et al., 2014). These markers have become the norm in part as they provide a large number of potentially polymorphic loci (Heun et al., 1994). Each molecular marker unveils unique properties that are useful depending on the desired outcome. DNA molecular markers are routinely used to detect individual genotypes, study population structures, and create genome maps (Sun et al., 1998).

Ever since RAPD technology was first introduced (Williams et al., 1990), it has become widely used for assessing genetic variability between closely related cultivars (Bhat et al., 1995; Hilu and Stalker, 1995; Hu and Quiros, 1991; Liu et al., 1994; Rieseberg, 1996; Wilde et al., 1992; Williams and Clair, 1993). RAPD works by amplifying unstipulated DNA segments from one parent but not the other with single primers of arbitrary nucleotide sequence. These polymorphisms are inherited in a Mendelian fashion and can be used to construct genetic maps in a variety of species (Williams et al., 1990). The RAPD technique has numerous

advantages over its predecessors: it requires only small amounts of DNA, is technically simple, and is proven to be a fast method of polymorphic site detection. RAPD analysis does not come without its limitations. RAPDs are sensitive to reaction conditions, lack band reproducibility, and potentially co-migration of different amplification products. The major disadvantage to RAPD analysis is the dominant marker allelic segregation that occurs (i.e. heterozygosity is not detectable).

AFLP markers have been widely used for population genetic studies in a wide variety of taxa. The origination of AFLP fragments is a multi step process, commencing with endonuclease digestion of DNA. Universal linkers are then ligated to the restriction fragments, which are amplified by PCR primers complementary to both linkers and the restriction sites. Several types of polymorphisms are, theoretically, detectable by analysis of AFLPs (Wong et al., 2001).

Microsatellites (simple sequence repeats SSRs) are short DNA sequences with tandem repeated motifs (2-6 bp) (Litt and Luty, 1989), are highly polymorphic, abundant and are accessible to other research laboratories via published primers sequences. They have become the marker of choice for genetic mapping, gene tagging, genetic diversity study, genomic and DNA fingerprinting

(W. G. Xie et al., 2010). Inter-simple sequence repeats (ISSRs) are the genome region between two microsatellite loci. The complementary regions flanking the loci are used as PCR primers, and the variable region between get amplified. The ISSR-PCR technique is not without limitations as there is a possibility as with RAPD that fragments with the same mobility originate from non-homologous regions, which can contribute to some distortion in the estimates of genetic similarities (Reddy et al., 2002; Sanchez de la Hoz et al., 1996). SSRs in general have limitations, the fact that the PCR primers used to amplify SSRs are frequently species-specific, meaning markers developed for one taxa cannot be readily transferred to another. This lack of transferability means that interspecific comparisons are often based on disparate sets markers, confounding species differences with possible locus-specific effects (Ellis and Burke, 2007). The rapid and inexpensive development of SSRs from expressed sequence tags (EST) database has been shown to be a more practical option for obtaining high quality nuclear markers (Bhat et al., 2005; Ellis and Burke, 2007; Gupta et al., 2003). EST-SSRs have been found to be significantly more transferable across taxonomic boundaries than traditional SSRs (Chagné et al., 2004; Ellis and Burke, 2007; Gutierrez et al., 2005; Liewlaksaneeyanawin et al., 2004; Pashley et al., 2006). Since the cDNA from which ESTs are derived lack introns, one possible concern with EST-SSRs is that unrecognized intron splice sites could disrupt priming sites, resulting in failed amplification (Ellis and Burke, 2007).

Sequence related amplified polymorphisms (SRAPs) markers are the first marker to amplify a targeted sequence of choice. Previous polymorphic markers amplified anonymous regions (Li and Quiros, 2001). SRAPs have several advantages over other techniques: simplicity, reasonable throughput rates, discloses numerous co-dominant markers, allows for easy isolation of bands for sequencing and, most importantly, it targets ORFs (Li and Quiros, 2001).

Sequence codon targeted (SCoT) markers (Collard and Mackill, 2009), are quickly becoming the marker of choice in the past half decade for generating gene-targeted markers in plants. SCoT polymorphisms are based on the short conserved regions in plant's genes surrounding the ATG translation start codon, PCR primers designed from the short conserved regions flank the ATG start codon that is conserved for all genes (Collard and Mackill, 2009). Therefore, the technique is similar to RAPD or ISSR. The main applications of SCoT markers are QTL mapping by increasing marker density to a specific chromosomal region, bulk segregant analysis, and genetic diversity studies (Collard and Mackill, 2009).

Single nucleotide polymorphism (SNP) is a single nucleotide variation in a genome sequence of individuals of a population. They constitute the most abundant molecular markers in the genome and are widely distributed throughout genomes and distribution varies among species. SNPs are usually more abundant

in non-coding regions of the genome.

Chloroplast DNA

The chloroplast is an organelle required for protein synthesis, production, and metabolism. The genome of chloroplast is circular, follows uniparental inheritance and is haploid in nature; therefore intraspecific variation is reduced and this can be used to identify maternal lineages and genome donors (Xu and Ban, 2004). Moreover, chloroplast exhibits a non-Mendelian inheritance and consists of highly conserved coding regions. The non-coding regions are variable in their content and arrangement. This is especially true for related species that do not display recombination, thus the regions are useful in studying the phylogeny, evolution and genetic diversity of a species (Aluru, 2006; Bateman et al., 2003; Sun et al., 2002). Non-coding regions can provide valuable information in phylogenetic studies at the species level because of their high variability, simple amplification and abundance of developed universal primers. Alternatively, nuclear DNA may explain other characteristics of phylogenetic and/or evolutionary relationships as a result of bi-parental inheritance.

There is a growing interest in comparative analysis of non-coding chloroplast sequences for plant systematics studies at low taxonomic levels (Kelchner, 2000). Non-coding regions experience limited or no selective pressure

and are likely to evolve at rates far surpassing those of genic regions (Böhle et al., 1994; Curtis and Clegg, 1984; Olmstead and Palmer, 1994; Palmer, 1991, 1987; Wolfe et al., 1987). There are also assumptions that non-coding regions should experience random and independent mutations, both mode and distribution (Kelchner, 2000).

For these reasons, a considerable amount of work has been published demonstrating the phylogenetic utility of discrete non-coding regions in the chloroplast: the *trn*L-*trn*F spacer (Bayer and Starr, 1998; Cros et al., 1998; Gielly and Taberlet, 1994; Mes and Hart, 1994; van Ham et al., 1994), the *trn*T-*trn*L spacer (Böhle et al., 1994; Bohle et al., 1996; Small et al., 1998), the *trn*L intron (Bayer and Starr, 1998; Bayer et al., 2000; Kajita et al., 1998; Sang et al., 1997), and the *rps*16 intron (Liden et al., 1997; Oxelman et al., 1999). In this study, we used the *trn*D – *trn*tW spacer and *rps*16 intron of the chloroplast, this spacer as previously mentioned is a non-coding regions and functions similar as those mentioned above.

Dactylis

The genus *Dactylis* consists of a single perennial forage species, *Dactylis glomerata* L. known as orchardgrass or cocksfoot (Stewart and Ellison, 2011). Orchardgrass is the fourth most widely used forage grasses used with 3.3% of the

world's temperate seed (Bondensen, 2007; Stewart and Ellison, 2011). The genus *Dactylis glomerata L.* consists of fourteen subspecies with diploid (2n=2x=14), and three species and subspecies with tetraploid (2n=4x=28) (Amirouche and Misset, 2007; Borrill, 1977; Domin, 1943; Stebbins and Zohary, 1959).

The taxa in the *Dactylis glomerata* complex are subjected to different interpretations in different regions of its natural range (Stewart and Ellison, 2011). There are no modern taxonomic treatments which interpret all forms on the same basis (Stewart and Ellison, 2011). Jogan (2002) interpreted *D. glomerata* to be monotypic, and consists of one diverse species complex. Borrill (1977) stated that the genus is clearly on the verge of speciation with many diploid, tetraploid and a hexaploid subspecies, and reduced fertility when diploid forms are hybridized.

Populations of diploid forms of *D. glomerata* are usually described as subspecies, or even separate species, and publications vary in their interpretation (Stewart and Ellison, 2011). Diploid forms of *D. glomerata* occur from China to Portugal and North Africa as well as the Canary islands and Cape Verde (Stewart and Ellison, 2011). Using nuclear internal transcribed spacers (ITS) and *trn*L intron chloroplast sequences, Stewart and Ellison (2011) supported the hypothesis that the diploid progenitor originated in, and migrated out from Central Asia.

Tetraploid forms of *Dactylis glomerata* virtually occupy all areas the species is located, consisting of both autotetraploids and interecotypic hybrids where one

or more diploid subspecies is/are involved (Stewart and Ellison, 2011). In addition, the interecotypic hybrids often gain extensive evolutionary success due to their vigor and enhanced genetic variations (Stebbins, 1971). The process of tetraploidization seems to be initiated early in the development of the *Dactylis* genus, unreduced gametes frequently occur in diploid populations, and gene flow is from diploid to tetraploid (Lumaret and Barrientos, 1990; Lumaret et al., 1992). It is frequently reported that nearly all the diploid entities have a sympatric association of autotetraploids or at minimum tetraploids derived chiefly from a single diploid (Stewart and Ellison, 2011).

Objectives

The objective of this study is to use the non-coding region of the *trn*D-*trn*tW intergenic spacer and the *rps16* intron in the chloroplast, to detect and analyze nucleotide diversity within and between 43 accessions of the *Dactylis* genus originating from Asia, Australia and Oceania, the Middle east, North Africa, Europe and North America, and to elaborate on the most likely phylogenetic relation amidst the species.

Materials and Methods

Plant materials

The species studied include: *Dactylis glomerata* subspecies (subsp.) *glomerata*, *D. glomerata* subsp. *hispanica*, *D. glomerata* subsp. *juncinella*, *D. glomerata* subsp. *lobata*, *D. glomerata* subsp. *lusitanica*, *D. glomerata* subsp. *santai*, *D. glomerata* subsp. *smithii*, *D. glomerata* subsp. *woronowii*, and *D. marina*. Table 1 provides a list of species, accession numbers, and origins used in this study. Seeds were provided by the United States Department of Agriculture (USDA), and were germinated and transplanted to a common potting soil mixture, then maintained in a greenhouse located at Saint Mary's University in Halifax, Nova Scotia.

DNA Extraction

Leaves of young plants were harvested for DNA extraction as per the manufacturer's instructions using a GeneJet™ Plant Genomic DNA Purification Mini Kit (#K0792 from Thermo Scientific). One hundred milligrams of fresh plant tissue was crushed into a fine powder in liquid nitrogen using a mortar and pestle, and transferred to a 1.5 mL centrifuge tube with 350 μL of Lysis Buffer A. Fifty microliters of Lysis Buffer B and 20 μL of RNase A were added, and the tube was incubated at 65°C for 10 minutes. The tube was incubated on ice for 5 minutes, then, 130 μL of Precipitation Solution was combined with the mixture. The

supernatant was collected and placed in a new 1.5 mL centrifuge tube with 400 μL of Plant gDNA Binding Solution and 400 μL of 95% Ethanol and emulsified. The solution was centrifuged for 1 minute at 6,000 x g, 500 μL of Wash Buffer 1 was added to the column after discarding the flow through. The column was then centrifuged for 1 min at 3,000 x g, 500 μL of Wash Buffer 2 was added to the column to be centrifuged again at ≥ 20,000 x g for 3 minutes. One hundred microliters of Elution Buffer containing 10 mM Tris-HCl, 0.5 mM EDTA at a pH of 9.0, was added and incubated at room temperature for 5 minutes, thereafter centrifuged for 1 minute at 8,000 x g. A second elution step of 100 μL Elution Buffer was performed. DNA was then stored at -20°C.

Polymerase Chain Reaction (PCR)

Primers *trn*-D (5'-ACCAATTGAACTACAATCCC-3') and *trn*-tW (5'-CCCTTTTAACTCAGTGGTAG-3') (Sun, 2002) were used to amplify non-coding chloroplast DNA between genes, which consists of 1,200 base pairs. Meanwhile, Primers *rps*16F (5'-GTGGTAGAAAGCAACGTGCGACTT-3'), and reverse *rps*16R (5'-TCGGGATCGAACATCAATTGCAAC-3') (Popp and Oxelman, 2007) were used to amplify the *rps*16 gene within the chloroplast DNA.

A master mix for both sets of primers consisted of 3.8 μL of autoclaved water, 2 μL of BioBasic Inc. 10 X reaction buffer, 2 μL of BioBasic Inc. 20 μM $MgSO_4$, 4 μL

of 1mM deoxynucleotides (dNTP), 3 μL of 2 μM forward primer, 3 μL of 2 μM reverse primer, 0.2 μL of BioBasic *Taq* DNA Polymerase and 3.0 μL of template DNA in a 20 μL thin wall centrifuge tube. For each accession, two 20 μL replicates of PCR amplification products were obtained and later combined to reduce the chance of PCR selection or drift due to *Taq* Polymerase errors during the process (Yan and Sun, 2011; Zeng et al., 2010).

The polymerase chain reaction (PCR) consisted of three phases: denaturation, annealing, and extension. A Bio-Rad T100 Thermal Cycler was used for DNA amplification. For the *trn*D/tW primers, the PCR series was: one cycle at 95°C for 3 minutes, 40 cycles of 30 seconds at 95°C, 30 seconds at 54°C, 1 minute 45 seconds at 72°C, one cycle at 72°C for 10 minutes, and an infinite hold temperature of 8°C. For the *rps*16 primers, the PCR reaction cycle consisted of: one cycle at 95°C for 4 minutes, 40 cycles of 40 seconds at 95°C, 40 seconds at 63°C, 1 minute 30 seconds at 72°C, followed by one cycle at 72°C for 10 minutes, and an infinite hold temperature of 4°C.

Electrophoresis and Visualization

PCR product was separated using agarose gel electrophoresis. The PCR products were mixed with 5 μL of loading dye and separated on a 1% agarose gel (1% agarose,1X TBE buffer, EDTA, Tris base, boric acid and water). The gel was

run at approximately 140 volts for 35 minutes using a Fisher Biotech electrophoresis system (FB-SBR-1316). After electrophoresis, the gel was stained with a 0.1% ethidium bromide (EtBr) and the bands were visualized under UV light using a SynGene bioimaging system. Figure 1 shows an example of successful PCR products.

Sequencing & Data analysis

PCR products successfully amplified were purified and sequenced by the Taihe Biotechnology Company in Beijing, China. Sequences were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/) to ensure the sequences from the gene desired were amplified. Multiple sequences were aligned using the MAFFT v7 (Katoh and Standley, 2013). The Phylogenetic Analysis Using Parsimony (PAUP) program was used to analyze the phylogenetic relationship of the subspecies. *Festuca arundinacea*, was used as an outgroup, based on previous studies of phylogenetic analysis of Poaceae (Charmet et al., 1997; Quintanar et al., 2007). Gaps were treated as missing data while all characters were considered unweighted and ungrouped. A distance dendrogram was created by unweighted pair group mean algorithm (UPGMA) using the Tamura – Nei substitution model (Tamura and Nei, 1993) in the PAUP 4.0 program (Swofford, 2003). One thousand bootstrap replications were conducted to determine the clade consistency

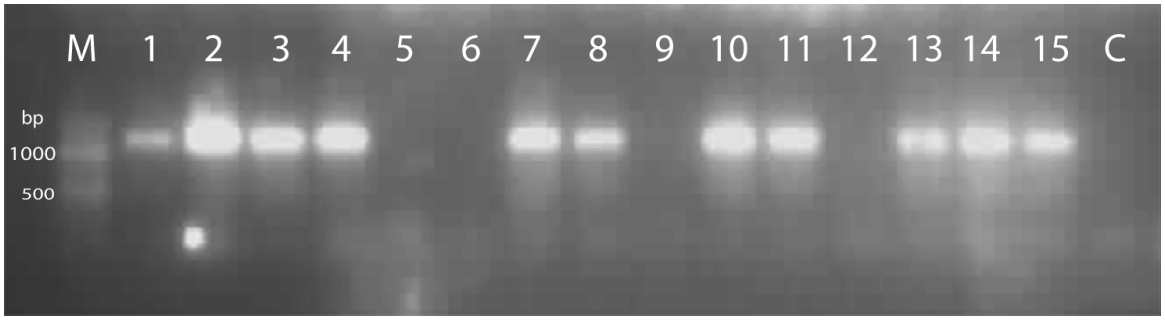
(Felsenstein, 1985), and a 50% majority rule consensus tree was created. Waterson's (Watterson, 1975) θ, Fu and Li's F and D statistics (Fu and Li, 1993), and Tajima's (Tajima, 1989) π statistics were used to estimate nucleotide diversity. Additionally, the number of haplotypes and the number of polymorphic sites were examined. The DnaSP v5.0 program (Librado and Rozas, 2009) was used for the analyses of DNA polymorphism data and tests of neutral evolution described by Fu and Li (1993) and Tajima (1989). Bayesian analyses were performed using MrBayes v3.2 (Ronquist et al., 2012). Default uniform priors were used for all model parameters. One cold chain and three incrementally heated Markov Chain Monte Carlo (MCMC) chains were run simultaneously, 500,000 generations for *trn*D/tW data and 750,000 generations for *rps*16 data with default heat temperature of 0.2. For *trn*D/tW, samples were taken every 1000 generations under the GTR model with equal rate variation across sites and a proportion of variable site. For *rps16*, samples were taken every 1000 generations under the F81 model with equal rate variation across sites and a proportion of variable site. The first 25% of the samples from each run were discarded as a burn in to ensure the convergence of the chains. Bayesian posterior probability (PP) values were obtained from a 95% majority rule consensus tree generated from the remaining sample trees.

Table 1: Species names, accession numbers and origin of *Dactylis* species used in this study

Taxon	Accession	Origin
Dactylis glomerata subsp. *glomerata*	PI 174774	Sweden
	PI 175131	India
	PI 186308	Australia
	PI 189388	New Zealand
	PI 199245	Greece
	PI 201887	Egypt
	PI 201942	Israel
	PI 202281	Chile
	PI 202697	Uruguay
	PI 209885	Australia
	PI 222761	Argentina
	PI 235124	Netherlands
	PI 260244	Germany
	PI 272114	Poland
	PI 273738	Lithuania
	PI 288998	Hungary
	PI 308542	Colombia
	PI 371711	USA, Alaska
	PI 418667	Norway
	PI 435996	Iran
	PI 440283	Kazakhstan
	PI 442475	Belgium
	PI 499409	China
	PI 502340	Estonia
	PI 542738	Croatia
	PI 577055	Spain
	PI 578603	Cyprus
	PI 578661	Jamaica
	PI 594977	France
	PI 598843	Morocco
	PI 634258	Albania
	PI 636535	Tunisia
	PI 659865	USA, Oakland
	W6 10026	Romania

D. glomerata subsp. *hispanica*	PI 221085	Israel
	PI 230115	Iran
	PI 231517	Morocco
	PI 231544	Greece
	PI 632497	Tunisia
D. glomerata subsp. *ibizensis*	PI 237609	Spain
D. glomerata subsp. *juncinella*	PI 237601	Spain
	PI 418678	Spain
D. glomerata subsp. *lobata*	PI 283241	Germany
	PI 316209	Bulgaria
	PI 420744	Australia
	PI 420746	Australia
D. glomerata subsp. *lusitanica*	PI 237603	Portugal
	PI 634265	Spain
Dactylis marina	PI 229472	Algeria
D. glomerata subsp. *santai*	PI 237605	Algeria
	PI 237606	Morocco
	PI 368880	Algeria
D. glomerata subsp. *smithii*	PI 237607	Spain
	PI 441032	United Kingdom
	PI 441034	United Kingdom
D. glomerata subsp. *woronowii*	PI 237610	Iran
	PI 283243	Former Soviet Union

Figure 1: Example of agarose gel electrophoresis after staining with ethidium bromide and visualization under UV light for *D. glomerata* subspecies amplified with *trn*D/*trn*tW primers. *Lanes* 1- 15 are DNA of *Dactylis* accessions, the lane farthest right (*C*) control with no DNA present, left most lane (M) is DNA ladder (Kappa 1Kb DNA Ladder, kit code; KK6302).


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 C
bp
1000
500

Results

Nucleotide diversity of the trnD – trntW intergenic region

A total of 37 samples of the 57 listed in Table 1 were amplified using the *trn*D – *trn*tW primer pair. The highest nucleotide diversity observed for samples amplified with the *trn*D – *trn*tW primers was the *D. glomerata* subsp. *smithii* population ($\pi = 0.00484$). The lowest nucleotide diversity was observed in the *D. glomerata* subsp. *glomerata* population ($\pi = 0.00073$). The nucleotide diversity for the genus ($\pi = 0.00139$) was lower than some of the individual populations. *D. glomerata* subsp. *smithii* had the most polymorphic sites (5), and *D. glomerata* subsp. *woronowii* had the lowest with a single polymorphic site. Waterson's θ calculation suggests that the *D. glomerata* subsp. *smithii* population had the highest mutation rate ($\theta = 0.00484$), while the *D.glomerata* subsp. *glomerata* population was seen to have the lowest mutation rate ($\theta = 0.00087$). The total Waterson's θ estimate for the genus was $\theta = 0.00255$. None of the populations were significant related to Fu and Li and Tajima tests.

Table 2: Nucleotide diversity estimates of the *trn*D – *trn*tW intergenic region of cpDNA in the *Dactylis glomerata* subspecies studied.

Population	N	S	h	π	θ	Tajima's	Fu and Li D	Fu and Li F
Genus	37	11	10	0.00139	0.00255	-1.4097	-2.8149	-2.7811
subsp. *glomerata*	16	3	4	0.00073	0.00087	-0.4941	-1.1215	-1.0921
subsp. h*ispanica*	5	4	4	0.00213	0.00186	0.9571	0.9571	0.9742
subsp. *lobata*	4	2	3	0.00097	0.00106	-0.7099	-1.1215	-0.6043
subsp. *lusitanica*	2	2	2	0.00193	0.00193		0.9571	
subsp. *smithii*	2	5	2	0.00484	0.00484			
subsp. *woronowii*	2	1	2	0.00097	0.00097			

Phylogenetic analysis of the trnD – trntW intergenic region

A distance analysis of the *trn* intergenic region sequences, with *Festuca arundinacea* as an outgroup was completed. A 50% majority rule consensus UPGMA tree using the Tamura – Nei (1993) substitution model with bootstrap values is shown in Figure 2. A single monophyletic clade is observed with a 66% bootstrap value containing all but one accession, a single *D. glomerata* subsp. *smithi* (PI441034) accession branched off the original monophyletic clade. *D. glomerata* subsp. *hispanica* (PI231544 & PI221085), formed a separate group with a 50% bootstrap value. Bayesian inference analysis resulted in a slightly different tree (estimated mean marginal likelihood values –1757.23 and –1787.82) (data not shown) shown in Figure 3. *D. glomerata* subsp. *hispanica* (PI231544 & PI 221085), along with a single *D. glomerata* subsp. *glomerata* (PI201887), and *D. glomerata* subsp. *woronowii* (PI283243) formed a separate group with a 91% posterior probability. *D. glomerata* subsp. *santai,* and a single *D. glomerata* subsp. *lusitanica* (PI634265) and *D. glomerata* subsp. *woronowii* (PI237610) form another distinct group with an 86% posterior probability.

Figure 2: A 50% majority rule consensus tree using 1000 bootstrap replications derived from the *trn*D – *trn*tW intergenic region sequences using the UPGMA distance method for 37 *Dactylis* accessions. *Festuca arundinacea* was used as an outgroup.

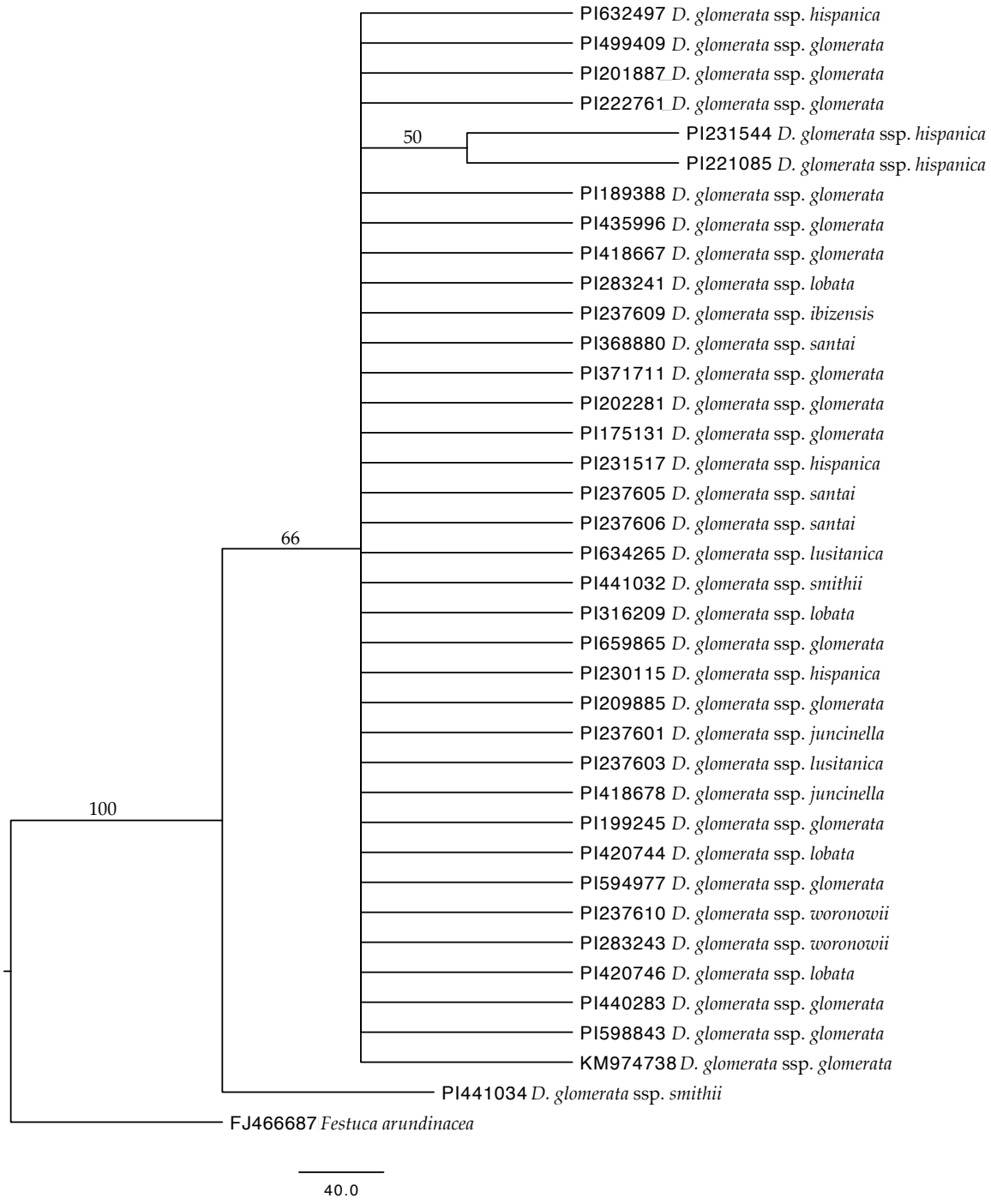
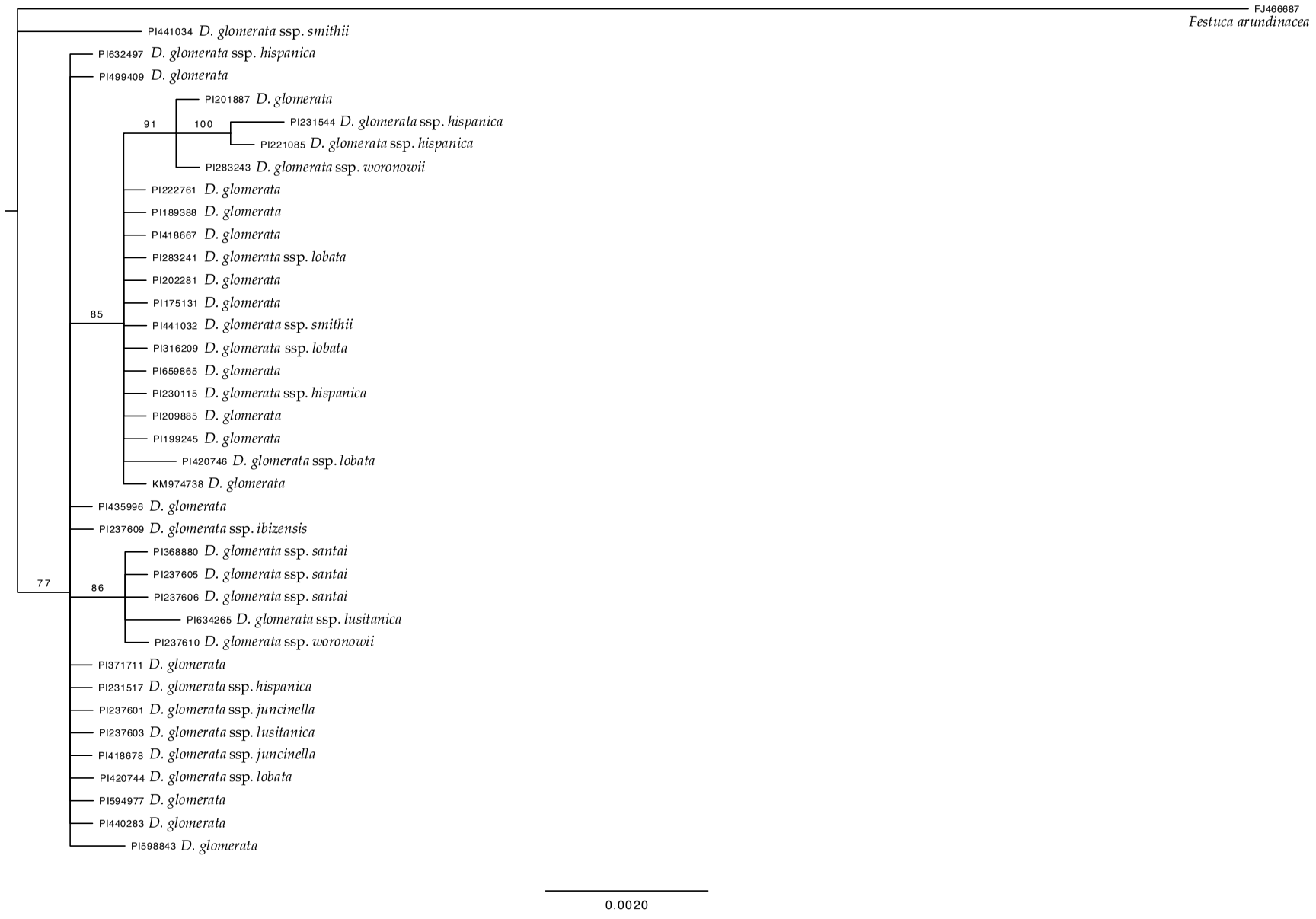

PI632497 D. glomerata ssp. hispanica
PI499409 D. glomerata ssp. glomerata
PI201887 D. glomerata ssp. glomerata
PI222761 D. glomerata ssp. glomerata
50
PI231544 D. glomerata ssp. hispanica
PI221085 D. glomerata ssp. hispanica
PI189388 D. glomerata ssp. glomerata
PI435996 D. glomerata ssp. glomerata
PI418667 D. glomerata ssp. glomerata
PI283241 D. glomerata ssp. lobata
PI237609 D. glomerata ssp. ibizensis
PI368880 D. glomerata ssp. santai
PI371711 D. glomerata ssp. glomerata
PI202281 D. glomerata ssp. glomerata
PI175131 D. glomerata ssp. glomerata
PI231517 D. glomerata ssp. hispanica
PI237605 D. glomerata ssp. santai
PI237606 D. glomerata ssp. santai
66
PI634265 D. glomerata ssp. lusitanica
PI441032 D. glomerata ssp. smithii
PI316209 D. glomerata ssp. lobata
PI659865 D. glomerata ssp. glomerata
PI230115 D. glomerata ssp. hispanica
PI209885 D. glomerata ssp. glomerata
PI237601 D. glomerata ssp. juncinella
PI237603 D. glomerata ssp. lusitanica
PI418678 D. glomerata ssp. juncinella
100
PI199245 D. glomerata ssp. glomerata
PI420744 D. glomerata ssp. lobata
PI594977 D. glomerata ssp. glomerata
PI237610 D. glomerata ssp. woronowii
PI283243 D. glomerata ssp. woronowii
PI420746 D. glomerata ssp. lobata
PI440283 D. glomerata ssp. glomerata
PI598843 D. glomerata ssp. glomerata
KM974738 D. glomerata ssp. glomerata
PI441034 D. glomerata ssp. smithii
FJ466687 Festuca arundinacea
40.0

Figure 3: Bayesian analysis consensus tree with 95% majority rule from the *trn*D – *trn*tW intergenic region sequence data using a GTR model. *Festuca arundinacea* was used as an outgroup. Posterior probability (PP) values are shown above the branches.


FJ466687
Festuca arundinacea
PI441034 D. glomerata ssp. smithii
PI632497 D. glomerata ssp. hispanica
PI499409 D. glomerata
PI201887 D. glomerata
91
100
PI231544 D. glomerata ssp. hispanica
PI221085 D. glomerata ssp. hispanica
PI283243 D. glomerata ssp. woronowii
PI222761 D. glomerata
PI189388 D. glomerata
PI418667 D. glomerata
PI283241 D. glomerata ssp. lobata
PI202281 D. glomerata
PI175131 D. glomerata
85
PI441032 D. glomerata ssp. smithii
PI316209 D. glomerata ssp. lobata
PI659865 D. glomerata
PI230115 D. glomerata ssp. hispanica
PI209885 D. glomerata
PI199245 D. glomerata
PI420746 D. glomerata ssp. lobata
KM974738 D. glomerata
PI435996 D. glomerata
PI237609 D. glomerata ssp. ibizensis
PI368880 D. glomerata ssp. santai
PI237605 D. glomerata ssp. santai
77
86
PI237606 D. glomerata ssp. santai
PI634265 D. glomerata ssp. lusitanica
PI237610 D. glomerata ssp. woronowii
PI371711 D. glomerata
PI231517 D. glomerata ssp. hispanica
PI237601 D. glomerata ssp. juncinella
PI237603 D. glomerata ssp. lusitanica
PI418678 D. glomerata ssp. juncinella
PI420744 D. glomerata ssp. lobata
PI594977 D. glomerata
PI440283 D. glomerata
PI598843 D. glomerata
0.0020

Nucleotide diversity of the rps16 intron

For the *rps*16F/R primers, 40 samples of the 57 listed in Table 1 were amplified successfully. The highest nucleotide diversity was detected in the *D. glomerata* subsp. *lusitanica* and *D. glomerata* subsp. *woronowii* populations, both had a Tajima's $\pi = 0.00393$. Additionally, the lowest nucleotide diversity was in the *D. glomerata* subsp. *glomerata* population with $\pi = 0.00011$. The nucleotide diversity for the genus ($\pi = 0.00009$) was the lowest value for nucleotide diversity calculated. The *D. glomerata* subsp. *lusitanica* and *D. glomerata* subsp. *woronowii* populations had the most polymorphic sites (3), and the *D. glomerata* subsp. *santai* and *D. glomerata* subsp. *lobata* populations had the lowest number of polymorphic sites (0). Waterson's θ estimate suggests that *D. glomerata* subsp. *lusitanica* and *D. glomerata* subsp. *woronowii* populations had the highest mutation rate ($\theta = 0.00393$), while the *D. glomerata* subsp. *glomerata* population was seen to have the lowest mutation rate ($\theta = 0.00036$). For the genus, the Watterson's θ estimate was $\theta = 0.00096$. For this primer pair none of the populations showed departure from neutrality that was significant regarding Fu and Li and Tajima tests.

Table 3: Estimates of nucleotide diversity of *rps*16 gene of cpDNA in the *Dactylis glomerata* subspecies studied.

Population	N	S	h	π	θ	Tajima's	Fu and Li D	Fu and Li F
Genus	40	1	2	0.00009	0.00041	-1.1241	-1.774	-1.8351
subsp. *glomerata*	26	1	2	0.00011	0.00036	-1.1556	-1.6338	-1.7271
subsp. *lusitanica*	2	3	2	0.00393	0.00393			
subsp. *smithii*	3	1	2	0.00096	0.00096			
subsp. *woronowii*	2	3	2	0.00393	0.00393			

Phylogenetic analysis of the rps16 intron

A distance analysis of the rps16 intron cpDNA sequences, with *Festuca arundinacea* as an outgroup was completed. A 50% majority rule consensus UPGMA tree using the Tamura – Nei (1993) substitution model with bootstrap values is shown in Figure 4. A single monophyletic clade was observed with a 100% bootstrap value; two groupings of the monophyletic clade were witnessed as well. A group of four accessions containing *D. glomerata* subsp. *glomerata* (PI202281 & PI308542), *D. glomerata* subsp. *lusitanica* (PI237603), and *D. glomerata* subsp. *woronowii* (PI 283243) formed a subgroup with a 64% bootstrap value. One accession of *D. glomerata* subsp. *glomerata* (PI636535) and *D. glomerata* subsp. *woronowii* (PI237610) were grouped together with a 61% bootstrap value. A separate Bayesian analysis resulted in a slightly different tree (estimated mean marginal likelihood values –1265.92 and –1286.70) (data not shown) shown in Figure 5. A single group consisting of six accessions was distinct to the rest of the samples with a posterior probability of 58%. Moreover, further branching distinguished two groups consisting of *D. glomerata* subsp. *glomerata* (PI202281 & PI308542), *D. glomerata* subsp. *lusitanica* (PI237603), and *D. glomerata* subsp. *woronowii* (PI 283243) with 80% posterior probability and *D. glomerata* subsp. *glomerata* (PI636535) and *D. glomerata* subsp. *woronowii* (PI237610) were grouped together with 93% posterior probability.

Figure 4: A 50% majority rule consensus tree from 1000 bootstrap replications derived from the *rps*16 intron sequences using the UPGMA distance method for 40 *Dactylis* accessions. *Festuca arundinacea* was used as an outgroup.

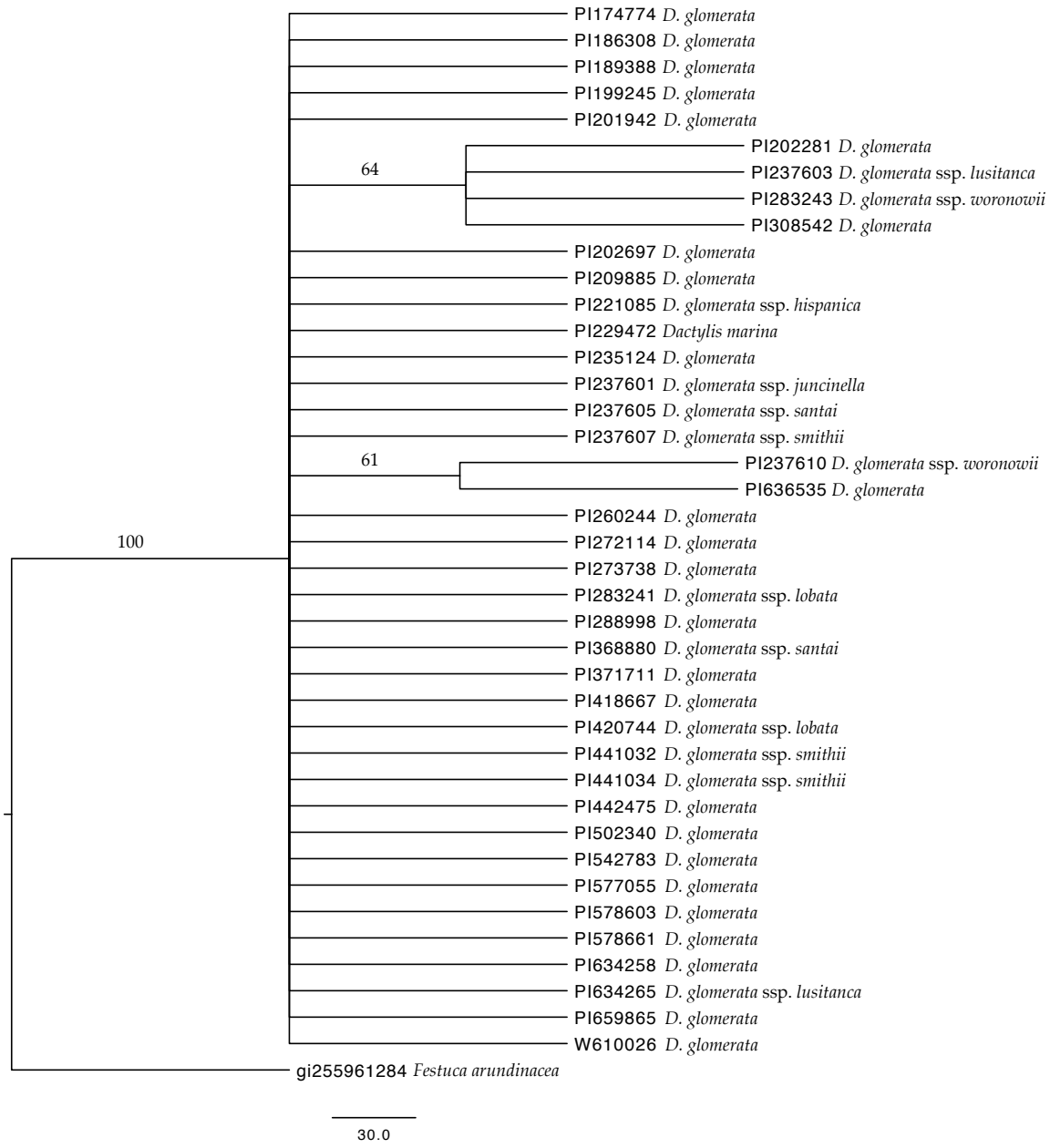
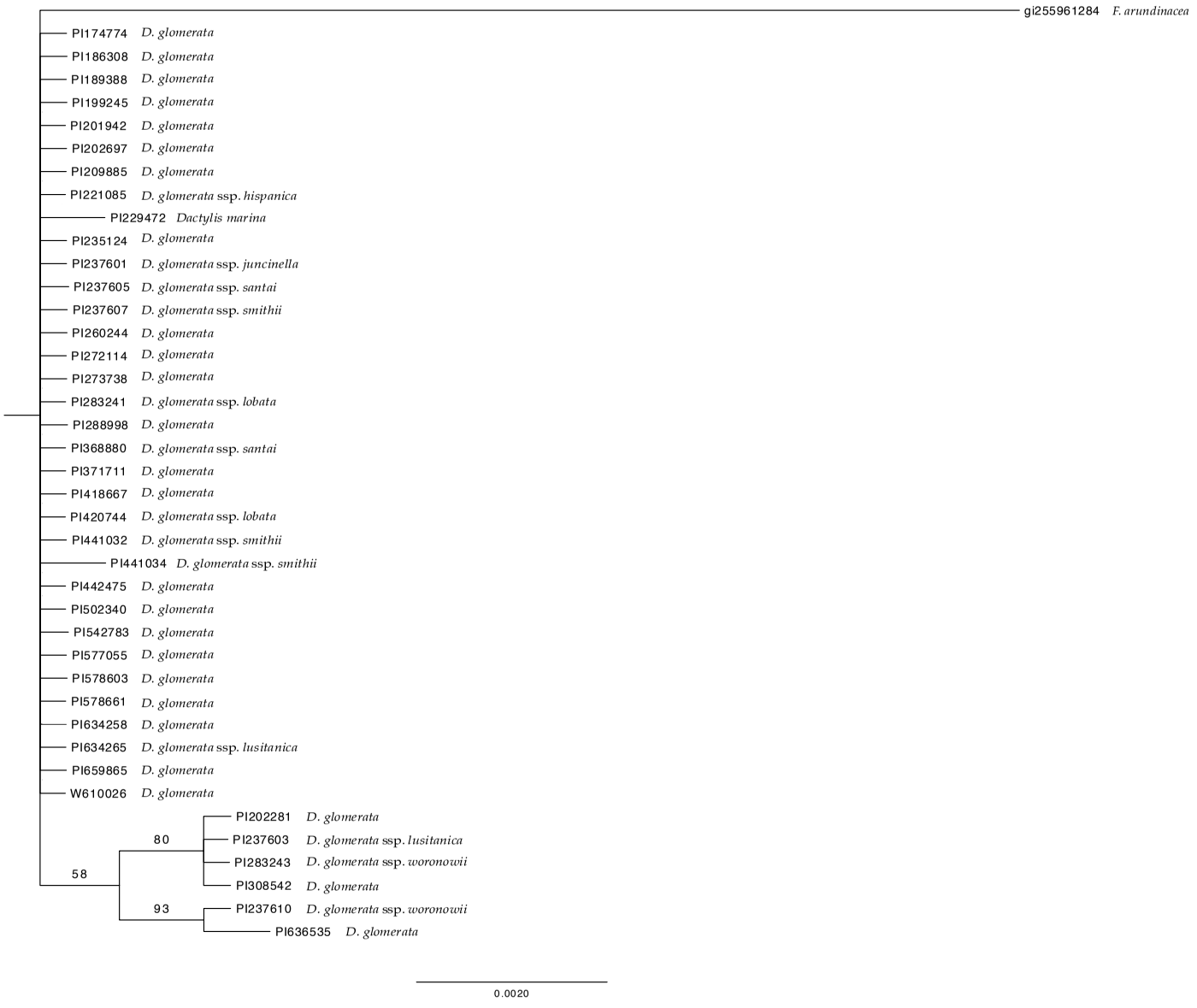

PI174774 D. glomerata
PI186308 D. glomerata
PI189388 D. glomerata
PI199245 D. glomerata
PI201942 D. glomerata
PI202281 D. glomerata
PI237603 D. glomerata ssp. lusitanca
PI283243 D. glomerata ssp. woronowii
PI308542 D. glomerata
PI202697 D. glomerata
PI209885 D. glomerata
PI221085 D. glomerata ssp. hispanica
PI229472 Dactylis marina
PI235124 D. glomerata
PI237601 D. glomerata ssp. juncinella
PI237605 D. glomerata ssp. santai
PI237607 D. glomerata ssp. smithii
PI237610 D. glomerata ssp. woronowii
PI636535 D. glomerata
PI260244 D. glomerata
PI272114 D. glomerata
PI273738 D. glomerata
PI283241 D. glomerata ssp. lobata
PI288998 D. glomerata
PI368880 D. glomerata ssp. santai
PI371711 D. glomerata
PI418667 D. glomerata
PI420744 D. glomerata ssp. lobata
PI441032 D. glomerata ssp. smithii
PI441034 D. glomerata ssp. smithii
PI442475 D. glomerata
PI502340 D. glomerata
PI542783 D. glomerata
PI577055 D. glomerata
PI578603 D. glomerata
PI578661 D. glomerata
PI634258 D. glomerata
PI634265 D. glomerata ssp. lusitanca
PI659865 D. glomerata
W610026 D. glomerata
gi255961284 Festuca arundinacea
64
61
100
30.0

Figure 5: Bayesian analysis consensus tree with 95% majority rule from the *rps*16 intron sequences data using a F81 model. *Festuca arundinacea* was used as an outgroup. Posterior probability (PP) values are shown above the branches.


gi255961284 F. arundinacea
PI174774 D. glomerata
PI186308 D. glomerata
PI189388 D. glomerata
PI199245 D. glomerata
PI201942 D. glomerata
PI202697 D. glomerata
PI209885 D. glomerata
PI221085 D. glomerata ssp. hispanica
PI229472 Dactylis marina
PI235124 D. glomerata
PI237601 D. glomerata ssp. juncinella
PI237605 D. glomerata ssp. santai
PI237607 D. glomerata ssp. smithii
PI260244 D. glomerata
PI272114 D. glomerata
PI273738 D. glomerata
PI283241 D. glomerata ssp. lobata
PI288998 D. glomerata
PI368880 D. glomerata ssp. santai
PI371711 D. glomerata
PI418667 D. glomerata
PI420744 D. glomerata ssp. lobata
PI441032 D. glomerata ssp. smithii
PI441034 D. glomerata ssp. smithii
PI442475 D. glomerata
PI502340 D. glomerata
PI542783 D. glomerata
PI577055 D. glomerata
PI578603 D. glomerata
PI578661 D. glomerata
PI634258 D. glomerata
PI634265 D. glomerata ssp. lusitanica
PI659865 D. glomerata
W610026 D. glomerata
PI202281 D. glomerata
80
PI237603 D. glomerata ssp. lusitanica
PI283243 D. glomerata ssp. woronowii
58
PI308542 D. glomerata
93
PI237610 D. glomerata ssp. woronowii
PI636535 D. glomerata
0.0020

Discussion

Various genetic markers have been used to assess *Dactylis glomerata* subspecies population structures, including: random amplified polymorphic DNA, amplified fragment length polymorphism, microsatellite markers, sequence related amplified polymorphism, sequence codon targeted markers, and single nucleotide polymorphisms (Bushman et al., 2011; Hirata et al., 2011; Jiang et al., 2014; Kölliker et al., 1999; Madesis et al., 2014; Peng et al., 2008; Reeves et al., 1998; Tuna et al., 2004; Xie et al., 2012, 2011; W. G. Xie et al., 2010; Zeng et al., 2014, 2008, 2006). Previous studies of orchardgrass that used genetic and genomic – scale molecular markers showed abundant diversity within the orchardgrass genus *Dactylis* and its related genera. Molecular markers have been used to detect the genetic diversity among samples of *D. glomerata* collected from China and other countries (Jiang et al., 2013, 2014; Kölliker et al., 1999; Last et al., 2014; Madesis et al., 2014; Peng et al., 2008; Reeves et al., 1998; Tuna et al., 2004; W.-G. Xie et al., 2010; Xie et al., 2012; W. G. Xie et al., 2010; Zeng et al., 2014, 2008). For example, Tuna et al. (2004) RAPD analysis of orchardgrass populations in the Thrace region of Turkey revealed that only 32% were polymorphic among the entire population studied. The highest genetic distance reported for the orchardgrass population studied was 0.1936, stating it appears that there is a high level of gene flow among natural orchardgrass populations and therefore genes were distributed quite

homogeneously throughout the region (Tuna et al., 2004). Kölliker et al. (1999) compared the genetic variability of *Dactylis glomerata* L., *Lolium perenne* L., and *Festuca pratensis* Huds., using random amplified polymorphic DNA. Results showed *Dactylis glomerata* displayed an average distance for pair-wise comparison of genotypes (Euclidean distance) of 20.3, and an 85% of total genotype variance within the cultivar. Reeves et al. (1998) looked at two populations of *Dactylis glomerata* selected from the French and Italian altitudinal transects to determine if there was a negative correlation between DNA C-value and altitude (Reeves et al., 1998). Their AFLP results showed that these populations were genetically distinct (Zeng et al., 2014). Peng et al. (2008) found high level of polymorphism (80.5%), low level of genetic similarity coefficient between accessions (0.69-0.93), and genetic distance between accessions (0.13 – 0.19), indicated high genetic diversity in the wild orchardgrass germplasm. The range of genetic similarity coefficients seen in the results of Peng et al., (2008) were consistent with the range of ISSR-based genetic similarities (0.61 – 0.93) for the orchardgrass populations studied by Zeng et al. (2006). Xie and colleagues looked at 16 accessions collected from China and other regions. Their SSR results (143 polymorphic alleles) from 21 SSR primers showed the average genetic diversity by the polymorphic information content (PIC) was 0.33, the average polymorphic rate was 90.7% (Xie et al., 2010). In 2012, they investigated the molecular variation and structure of cultivar populations,

subspecies and advanced breeding lines to determine whether there is still sufficient genetic diversity within currently used cultivars. Their SSR results showed the polymorphic rate was 100%, and the mean genetic diversity was 0.20 among the 120 individuals studied (Xie et al., 2012).

High genetic variability has been proven to be detected by microsatellites in *E.alaskanus* populations. These repetitive DNA sequences are important in the way plants adapt to environmental pressures (Rogers and Bendich, 1987). The high genetic variability seen in previous studies can be attributed to geographic range; widely distributed plant species have to adapt to their large distribution range which gives rise to high genetic diversity. Additionally, low genetic variation has been found on islands due to small founding populations and inbreeding; founder effects play a considerable role in governing patterns of genetic variability (Sun and Salomon, 2003). Sahuquillo and Lumeret (1999) studied 21 *Dactylis glomerata* populations from the Macaronesian islands using RFLP analysis. Results suggest that cpDNA introgression has occurred more than once from the Mediterranean material into the subtropical population, and may indicate that colonization between the mainland and islands probably played a major role in the geographical pattern observed by that marker (Sahuquillo and Lumaret, 1999). Moreover, our study only examined two regions of the chloroplast, while previously mentioned studies looked at the nucleus genome as well. Stewart and

Ellison (2011) examined the *trn*L intron of the chloroplast, and nuclear ITS mutations, and results showed no more than 2-7 ITS mutations and only 0 or 1 *trn*L intron mutations in any of the diploid *Dactylis* lineages (Stewart and Ellison, 2011). Their results showed one of the closest genera to *Dactylis* is *Lamarckia*, and *L. aurea* diverged from *Dactylis* with 1-2 *trn*L intron chloroplast differences (Stewart and Ellison, 2011). Yamane et al. (2006) observed that chloroplast genome *trn*L intron mutation rates are usually 3 – 8 times slower than nuclear ITS, with one per 200,000 years in rice and maize, and one per 90,000 years in the most rapidly changing perennial *Phleum* lineage (Stewart et al., 2009; Yamane et al., 2006).

In this study, the highest level of diversity was found in *D. glomerata* subsp. *Smithii* for *trn* intergenic region and *D. glomerata* subsp. *woronowii* and *D. glomerata* subsp. *lusitanica* for *rps*16; meanwhile, the lowest levels of diversity were found in *D. glomerata* subsp. *glomerata* for both regions examined. For the cpDNA intergenic region, the highest diversity was 0.00484, while the lowest was 0.00073. The highest for the *rps*16 cpDNA intron was 0.00393, and the lowest was 0.00011. The level of nucleotide diversity in theses regions is comparable to those found in a study using the same regions in *Elymus* populations. Adèle Joyce's (2014) results showed the highest nucleotide diversity for the cpDNA intergenic sequence was 0.00199 for *E. alaskanus*, and 0.02311 for *E. fibrosus*, *E. mutabilis* (Joyce, 2014). For the *rps16* intron, the highest nucleotide diversity was 0.00052 for *E. alaskanus* lowest was 0; or *E.*

fibrosus 0.0041 was the highest while 0.0030 was the lowest. *E. mutabilis* showed high diversity at 0.00156, and low diversity of 0.00078 (Joyce, 2014). The low levels of diversity shown in our study can be explained due to low evolutionary rate of chloroplast sequences in these regions.

It is clear that polyploids have developed from diploids and the likely reason why the tetraploids have such a broad range of distribution is that they have been much more successful in recolonizing northern regions after the last glacial event (Stewart and Ellison, 2015). Stewart and Ellison (2011) stated the molecular divergence within *Dactylis* divides, *D. glomerata* subsp. *Judica, D. glomerata* subsp. *himalayensis* and the paternal side of *D. glomerata* subsp. *parthiana* from the remaining diploids. This is consistent with an origin in Central Asia for *Dactylis* and an early geographic speciation leaving the ancestors spread over Western Asia (Stewart and Ellison, 2011). Stewart and Ellison (2015) examined an ITS region using the primer EC-1 and EC-2, the results showed a close affinity of Chinese *Dactylis* to European forms of the subspecies *D. ashersoniana* rather than to the nearest geographic *D. himalayensis*. This suggests that they have developed from ancestral "pre-European" type *D. ashersoniana* population which has migrated to China at some stage via a route close to the Himalayas (Stewart and Ellison, 2015). The remaining diploids displayed a second divergence with with Spanish forms; *D. glomerata* subsp. *lusitanica, D. glomerata* subsp. *juncinella,* and *D.*

glomerata subsp. *ibizensis* differentiating from the group. The early European form has since migrated into North Africa and across to China, as identical molecular profiles are seen in *D. glomerata* subsp. *aschersoniana, D. glomerata* subsp. *santai,* and *D. glomerata* subsp. *smithii,* with *D. glomerata* subsp. *woronowii* being derived from this group (Stewart and Ellison, 2011).

The low diversity of *D. glomerata* subsp. *glomerata* in both regions examined may be due to the hypothesis that it may have developed from the intercotypic hybridization between *D. glomerata* subsp. *aschersoniana* and *D. glomerata* subsp. *woronowii*, as artificial hybrids are intermediate between the two and indistinguishable from subsp. *glomerata* (Stebbins and Zohary, 1959; Stewart and Ellison, 2011). This again is suggestive of migration of subspecies *ashersoniana* into China (Stewart and Ellison, 2015). Stebbins also notes that as *D. glomerata* subsp. *reichenbachii* is similar to *D. glomerata* subsp. *woronowii* in many features it is possible that some subspeices *glomerata* have developed from the hybridization of *D. glomerata* subsp. *reichenbachii* and *D. glomerata* subsp. *aschersoniana* in the alps where they occur together (Stewart and Ellison, 2011). Further, it can be postulated that due to the low diversity of *D. glomerata* subsp. *glomerata* in both regions in comparison to other subspecies examined that *D. glomerata* subsp. *glomerata* may be an ancestral lineage to other diploid forms examined in this study.

In summary, *D. glomerata* subsp. *smithii* displayed the highest nucleotide diversity while *D. glomerata* subsp. *glomerata* displayed the lowest for *trn* intergenic region. For the *rps*16 intron *D. glomerata* subsp. *woronowii* and *D. glomerata* subsp. *lusitanica* equally had the highest nucleotide diversity while *D. glomerata* subsp. *glomerata* displayed the lowest. The intergenic region of the cpDNA yield a higher resolution in both the UPGMA and Bayesian inference trees, perhaps dividing *D. glomerata* subsp. *santai* and *D. glomerata* subsp. *hispanica* into a separate clade of their own. Classification of the *Dactylis glomerata* subspecies is very complex with various origins and polyploidy of subspecies; future studies should concentrate on mapping and evaluating the nuclear genome to support present and past phylogenetic and evolutionary relationships. Likewise, the use of big data mining may prove fruitful in combining large datasets of previous studies which can be used to characterize populations and support phylogenies.

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