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**Article:**

Ali, N., Ullah, Z., Ali, A. et al. (2026) DNA barcoding and phylogenetic relationship in Pakistani species of Aveneae-type plastid DNA clade (Pooideae, Poaceae) based on nuclear and chloroplast DNA markers. *Genetic Resources and Crop Evolution*, 73 (2). 89. ISSN: 0925-9864

<https://doi.org/10.1007/s10722-025-02716-1>

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1 **DNA Barcoding and Phylogenetic Relationship in Pakistani Species of Aveneae-type**  
2 **Plastid DNA Clade (Pooideae, Poaceae) based on nuclear and chloroplast DNA markers**

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19

20 **Abstract**

21 The Aveneae-type plastid DNA, clade belonging to the tribe Poeae s.l within the subfamily  
22 Pooideae (Poaceae), includes economically, nutritionally and ecologically important  
23 grasses. However, due to morphological similarity and prevalence of polyploidy, species  
24 boundaries and phylogenetic relationship among its taxa remain uncertain. This study  
25 aimed to evaluate, the species discrimination power of three universal plant DNA barcode  
26 loci (*ITS*, *matK* and *rbcL*) in 20 representative species of the Aveneae-type plastid DNA  
27 clade. Genetic distances were computed using MEGA-X software, while tree based  
28 maximum likelihood (ML) analyses were performed to infer the phylogeny. On the basis  
29 of percent discrimination rate at a significance level of  $p < 0.05$  *matK* and *rbcL* barcodes  
30 were 1.5% more successful than non-coding *ITS* region. However, in terms of barcode gap  
31 *ITS* marker showed highest barcode gap (0.075), than *matK* (0.029), and *rbcL* (0.008).  
32 Maximum Likelihood (ML) tree-based analyses identified 82.35% species using *rbcL*, 80%  
33 species with *matK*, and 43.75% species with *ITS* marker. Most subtribes and genera, except  
34 *Agrostis* and *Phalaris*, formed well-supported monophyletic clades (bootstrap > 90%). This  
35 study endorses the effectiveness of all the three barcode regions and provide novel DNA  
36 sequences of previously unsampled Himalayan grasses for global Poaceae barcoding  
37 initiatives and phylogenetic studies.

38 **Keywords:** Poaceae, DNA barcoding, Aveneae, *rbcL*, *matK*, Barcode gap, *p*-distances

39 **1. Introduction**

40 The subfamily Pooideae Benth., is the largest subfamily in Poaceae, having 15 tribes  
41 represented by 202 genera and 3968 species (APG, 2016; Soreng et al. 2022). The members  
42 of Pooideae are cosmopolitan in distribution but most abundant in temperate and alpine  
43 regions (Schneider et al. 2011). Major evolutionary lineages have been identified within  
44 the Pooideae as tribes and subtribes (Bouchenak-Khelladi et al. 2008; Schneider et al. 2011;  
45 Soreng et al. 2017). Among the tribes of Pooideae, tribe Poeae s.l. is the most species rich  
46 lineage with about 2600 species, distributed in Mediterranean, cool temperate and arctic  
47 regions of the world (Soreng et al. 2015, 2017).

48           The taxa now included in tribe Poeae s.l. (Saarela et al. 2018) were previously  
49           classified into two large tribes Poeae and Aveneae, (Soreng et al. 2007) mainly on the basis  
50           of morphological differences (Clayton & Renvoize 1986). Two major plastid lineages have  
51           been identified within Poeae s.l. taxa with Poeae-type plastid DNA and those with  
52           Aveneae-type plastid DNA (Soreng and Davis 2000). Saarela et al. (2010) studied the  
53           phylogeny of Aveneae-type plastid and nuclear ribosomal DNA clades. Subsequent studies  
54           found that it is not a single monophyletic tribe, and accordingly five sub tribes were  
55           recognized within this clade, i.e. (Aveninae, Agrostidinae, Brizinae, Phalaridinae, and  
56           Torreyochloinae). Later on, Soreng et al. (2017, 2022) expanded this classification to  
57           include eight sub tribes i-e Aveninae Dumort, Koeleriinae Rouy. Agrostidinae Fr, Brizinae  
58           Dumort, Phalaridinae Fr, Calothecinae Soreng & L.J.Gillespie, Torreyochloinae Soreng &  
59           L.J.Gillespie and Anthoxanthinae Tzvelev.

60           Among the five subtribes, Brizinae and several genera of Aveninae, including  
61           *Avena* and *Helictotrichon* have been the focus of phylogenic studies (Rodionov et al. 2005;  
62           Nikoloudakis and Katsiotis 2008). The few investigations that have extensively sampled  
63           the Aveneae-type plastid DNA lineage have primarily come from studies examining the  
64           relationships within the larger Poeae group (Soreng et al. 2007; Doring et al. 2007;  
65           Quintanar et al. 2007; Saarela et al. 2018). Among these, Quintanar et al (2007) broadly  
66           analyzed species of the Poeae s.l. complex based on ribosomal ITS and plastid trnT-F  
67           markers. Their findings were similar to subtribal classification proposed by Soreng et al  
68           (2007), but many of their clades lack strong support.

69           Agrostidinae is the most species rich lineage consisting of 16 genera and 550 species  
70           (Clayton and Renvoize 1986), remains under studied despite of their wide ecological  
71           distribution (Saarela et al. 2010). It includes complex genera *Agrostis* and *Calamagrostis*,  
72           whose taxonomy remained contentious due to polyploidy and apomixes. The major  
73           taxonomic problem in Agrostidinae is that it shares several characters (including ligule  
74           morphology, inflorescence structure, spikelet morphology and disarticulation pattern of the  
75           glumes) with the genera *Bromus* and *Muhlenbergia*, which belongs to distantly related  
76           tribes Bromeae and Muhlenbergeae respectively (Columbus et al. 2010; Saarela et al.  
77           2007).

78        Given the non-monophyletic nature of Aveneae s.s. here, we have used DNA  
79        barcoding, a recent molecular approach extensively used in many branches of science for  
80        example taxa identification (Wang et al. 2022), recognizing host-parasite relationship,  
81        analysis of food items (Staudacher et al. 2011), and detecting the herbal components of  
82        medicine (Travadi et al. 2023). Recent studies have explored potential of DNA barcoding  
83        in identifying species in flora and communities (Burgess et al. 2011; Elliot et al. 2014;  
84        Birch et al. 2017). The Consortium for the Barcode of Life (CBOL) Plant Working Group  
85        declared *rbcL* and *matK* as core barcodes, as well as *trnH-psbA* and *ITS*, an intergenic and  
86        nuclear gene as the supplementary barcodes (Hollingsworth 2009). Kress (2017), identified  
87        accurately 98% species within 50 ha forests using the core barcodes loci (*rbcL*, *matK*, *trnH-*  
88        *psbA*, and *ITS2*).

89        This study was aimed to produce a DNA barcode library and to infer phylogenetic  
90        relationship among Pakistani species of Aveneae-type plastid DNA clade based on  
91        universal plant DNA barcode markers i.e. *rbcL*, *matK* and *ITS*.

## 92        **2. Materials and Methods**

### 93        **Samples collection**

94        Twenty species belonging to Aveneae-type DNA clade were collected from various  
95        ecological regions of Pakistan (**Table 1**). The species were identified based on  
96        morphological features using different identification sources like Flora of Pakistan (Cope,  
97        1982), Flora of China, and online sources Tropicos, Grassbase. The nomenclature of  
98        species names is in accordance with Plants of the World Online Kew database  
99        (<https://powo.science.kew.org/>). Voucher specimens have been placed in Swat University  
100        Herbarium (SWAT). Field photographs of the species were taken using DSLR field camera  
101        Nikon 5200, Japan. Genomic DNA was isolated from about 2-4 cm<sup>2</sup> leaf blades dried in  
102        silica gel contained in paper envelopes. The silica dried leaf blades were kept in zip lock  
103        bags and were stored in air-tight container for prolong freezing in -20°C freezer. After  
104        drying the silica were removed and the leaf tissue inside the paper envelope were stored in  
105        a dry place (Peterson et al. 2014).

106

107 **Table 1.** List of species included in Aveneae-type plastid DNA clade along with collection  
 108 number, collection locality, and NCBI gene bank accession numbers of the three barcode  
 109 regions.

S. No.	Plant name	Collection No.	Collection Locality	Accession number <i>matK</i>	Accession number <i>rbcL</i>	Accession number <i>ITS</i>
1	<i>Agrostis gigantea</i> Roth	NA-1035	Barikot Swat, Deosai	PX098901	PX098865	PV866849
2	<i>Agrostis munroana</i> Aitch. & Hemsl.	Na-441, 447, 458	Miandam Swat	PX098893, PX098896, PX098891	PX098858, PX098860, PX098856	PV866854, PV866856, PV866858
3	<i>Agrostis pilosula</i> Trin.	Na-446, 453, 457	Miandam Swat	PX098895, PX098894, PX098892	PX098859, PX098857	PV866852, PV866853, PV866857
4	<i>Agrostis stolonifera</i> L.	Na-261, 1172	Utror Kalam Swat	PX098882, PX098906	PX098869	PV866842, PV866843
5	<i>Agrostis vinealis</i> Schreb.	Na-271, 1038, 1039	Utror and Shahi Bagh Kalam Swat	PX098883, PX098903, PX098902	PX098847, PX098867, PX098866	PV866851, PV866855, PV866859
6	<i>Avena fatua</i> L.	NA-29	Faisalabad Punjab	PX098872		PV866871
7	<i>Avena barbata</i> Pott ex Link	Na-68, 98, 115	Kanju Swat, Umarzai Charsada	PX098875	PX098840, PX098845	PV866873
8	<i>Avena sterilis</i> L.	Na-152	Quetta	PX098881	PX098846	PV866872
9	<i>Calamagrostis stolizkae</i> Hook. f.	Na-301, 433, 1050, 1050a	Astor, Deosai, Shahi Bagh Kalam Swat	PX098890, PX098884, PX098904, PX098905	PX098855, PX098848, PX098868	PV866860, PV866861, PV866862, PV866863
10	<i>Calamagrostis emodensis</i> Griseb.	Na-329	Deosai Skardu	PX098888	PX098852	PV866864
11	<i>Trisetopsis virescens</i> (Nees ex Steud.) Röser & A.Wölk	Na-905, 907	Miandam Swat	PX098898, PX098899	PX098862, PX098863	PV866868, PV866877
12	<i>Hierochloe laxa</i> Hook.f.	Na-322, 340	Deosai Skardu	PX098886, PX098889	PX098850, PX098853	PV866869, PV866870
13	<i>Phalaris minor</i> Retz	Na-30, 46	Kanju Town Swat	PX098873	PX098837	PV866874
14	<i>Phalaris paradoxa</i> L.	Na-114	Dargai Malakand	PX098880	PX098838, PX098844	PV866876, PV866875
15	<i>Polypogon hissaricus</i> (Roshev.) Bor	Na-1190	Utror Kalam Swat	PX098907	PX098870	PV866850
16	<i>Polypogon monspeliensis</i> (L.) Desf.	Na-102, 109, 935	Faisalabad Punjab	PX098877, PX098900, PX098879	PX098841, PX098864, PX098843	PV866840, PV866846, PV866847
17	<i>Polypogon fugax</i> Nees ex Steud.	Na-53, 901	Faisalabad Punjab and Miandam Swat	PX098874, PX098897	PX098839, PX098861	PV866839, PV866848
18	<i>Rostraria cristata</i> (L.) Tzvelev	Na-73, 108, 936	Swat University campus Charbagh	PX098878, PX098876	PX098842	PV866841, PV866844, PV866845
19	<i>Trisetum clarkei</i> (Hook.f.) R. R. Stewart	Na-1912, 352	Miandam Swat, Deosai Skardu	PX098908	PX098871, PX098854	PV866866
20	<i>Trisetum spicatum</i> (L.) K. Richt.	Na-313, 327	Deosai Skardu	PX098885, PX098887	PX098849, PX098851	PV866865, PV866867

110 **DNA extraction, PCR amplification, and sequencing success**

111 The silica gel dried leaf samples were used to extract DNA following modified CTAB  
112 protocol (Doyle, 1990), as well as Qiagen DNeasy Plant Mini Kit. The extracted and  
113 quantified DNA was stored in -20 °C for further analysis. The targeted DNA regions (*rbcl*,  
114 *matK* and *ITS*) genes were amplified (**Table 2**) following Peterson et al (2014).

115 **Table 2.** Regions studied and primers quality for polymerase chain reaction and  
116 sequencing.

Barcode Region	Primer	Sequence (5'-3')	Temperature (°C)	Reference
<i>matK</i>	<i>matK</i> -454F	CATATAGARATACCYTAYCCTATC	50-52°C	Peterson et al (2014)
	<i>matK</i> -1315R	GCTAAAGTTCTAGCRCATGAAAG		
<i>rbcl</i>	<i>rbcl</i> -926R	CATACGCAATGCTTTAGCTAATACACG	55-60°C	Peterson et al (2014)
	<i>rbcl</i> -109F	TGGCAGCATTCCGAGTAASTCCT		
<i>ITS</i>	<i>ITS</i> -4R	TCCTCCGCTTATTGATATGC	55°C	Stanford et al. (2000)
	<i>ITS</i> -5AF	CCTTATCATTAGAGGAAGGAG		White et al. (1990)

117

118 PCR amplification was performed using optimized conditions for each marker. For *rbcl*,  
119 *matK* and *ITS*, the general PCR program included initial denaturation at 95° followed by  
120 30-40 cycles at (94°C for 30 s), marker specific annealing at (49–56°C for 30–60 s), and  
121 extension (72°C for 1–1.2 min), with a final extension of 72°C for 10 min. Minor  
122 adjustment to annealing temperature were applied in case of *matK* and *ITS* to improve  
123 amplification efficiency due to difference in primer performance and template quality.  
124 Amplified PCR products for all the three markers were checked on 1 % agarose gel  
125 electrophoresis for their respective bands.

126 Sequencing was performed on the MinION Mk1B device using the MinKNOW software  
127 (v.23.07) for data acquisition and real-time base calling. The run was conducted for  
128 approximately 72 hours, with data streamed and processed in real-time (Jain et al. 2020).

129 **BLAST search comparison in NCBI and BOLD**

130 The *rbcl*, *matK*, and *ITS* sequences were compared using BLASTn searches against the  
131 NCBI GenBank and BOLD databases in order to confirm the species identity. Sequences

132 that were deemed accurate matches have a similarity of at least 98%. Database differences  
133 were utilized to evaluate reference coverage and find gaps in species' barcode data (Burgess  
134 et al. 2011).

### 135 **Comparison of sequence divergence**

136 DNA barcoding uses sequence data to identify specimens by comparing nucleotide  
137 similarities through pairwise sequence comparisons, expressed as genetic distances or  
138 substitution rates per site (Hebert et al. 2003). The genetic distances are presented as  
139 uncorrected *p* distances, which indicates the proportion of nucleotide differences between  
140 two sequences rather than statistical significance (Collins et al. 2012; Srivathsan and Meier  
141 2012). In this study, the effectiveness of the three barcodes regions was evaluated by  
142 calculating average *p*-distance values and assessing their ability to discriminate among taxa  
143 following established DNA barcoding threshold (Meier et al. 2006; Peterson et al. 2013).  
144 The software MEGA X was employed to generate a matrix for these distance analyses  
145 (Kumar et al. 2018).

146 Intra and interspecific distances were calculated from distance matrix to find out the  
147 barcode gap. The barcode gap was then calculated using Microsoft Excel, which is the  
148 difference between mean inter specific and mean intraspecific values. Barcode gap analysis  
149 identify the gap between specimens of the same species and then identify gap between  
150 different species. The difference between maximum intraspecific and minimum  
151 interspecific distance is known as barcode gap.

### 152 **Tree based discrimination within Aveneae type plastid DNA clade**

153 A DNA BLAST search is commonly used to identify unknown taxa by comparing  
154 sequences against a database of known sequences (Altschul et al. 1997). However, BLAST  
155 does not provide clear guidance for selecting among multiple best matches (Munch et al.  
156 2008). To address this, phylogenetic trees were constructed to identify species based on  
157 their phylogenetic placement within clades, Maximum Likelihood method was used  
158 (Hebert et al. 2003; Munch et al. 2008; Saarela et al. 2013). To identify the most suitable  
159 substitution model, a model selection test was conducted using MEGA. Several models

160 (Jukes-Cantor, Kimura 2-parameter, Tamura 3-parameter, and Tamura-Nei) were  
161 compared, and the Tamura-Nei model was selected based on the lowest AIC value,  
162 indicating the best fit for all three barcode datasets. Tree based identification was done  
163 using three universal plant barcode markers (*ITS*, *matK* and *rbcL*).

### 164 3. Results

165 A total of 20 species in 9 genera belonging to four subtribes of the Aveneae-type Plastid  
166 DNA clade, including Agrostidinae, Aveninae, Anthoxanthinae and Phalaridinae were  
167 evaluated in this study. In the final analyses, 30 accessions representing 17 species were  
168 included in the *ITS* barcode analysis, 36 accessions representing 20 species in the *matK*  
169 analysis, 31 accessions representing 17 species in the *rbcL* analysis and 38 accessions  
170 representing 20 species in the *matK+rbcL* analysis.

#### 171 Sequence variability

172 The sequence divergence (average uncorrected *p*-value) in the MEGA-X matrix was  
173 calculated as follows: 0.07 for *ITS*, 0.03 for *matK*, 0.01 for *rbcL* and for *matK+rbcL* was  
174 0.02 (Figure 1). The percent discrimination rate at a significance level of  $p < 0.05$  highlights  
175 the clear superiority of coding regions (*matK* and *rbcL*) over non coding regions (*ITS*)  
176 (Figure 2).

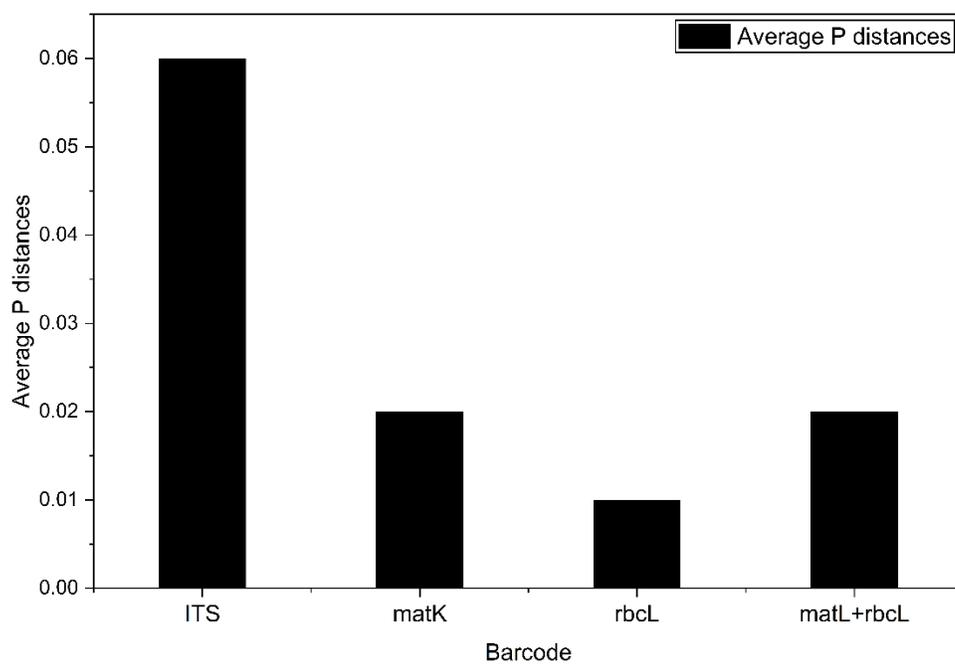
#### 177 Barcode gap analysis

178 Our barcode gap analysis revealed a positive gap in 20 species, indicating effective species  
179 discrimination. In these cases, the interspecific genetic distances significantly exceeded the  
180 intraspecific distances, confirming a clear separation between species. This positive  
181 barcode gap underscores the reliability of our chosen genetic markers for distinguishing  
182 among the Aveneae type plastid DNA Pakistani species, enhancing the accuracy of our  
183 phylogenetic assessments and contributing to a robust DNA barcode library for this clade  
184 (Table 3; Figure 3).

185 **Table 3.** Barcode gap analysis (41 accessions of 20 species) based on *ITS*, *rbcL* and *matK*  
186 barcodes showing inter and intra-specific genetic distances and barcode gap.

Barcode Marker	Mean interspecific distances	Mean intraspecific distances	Barcode gap
----------------	------------------------------	------------------------------	-------------

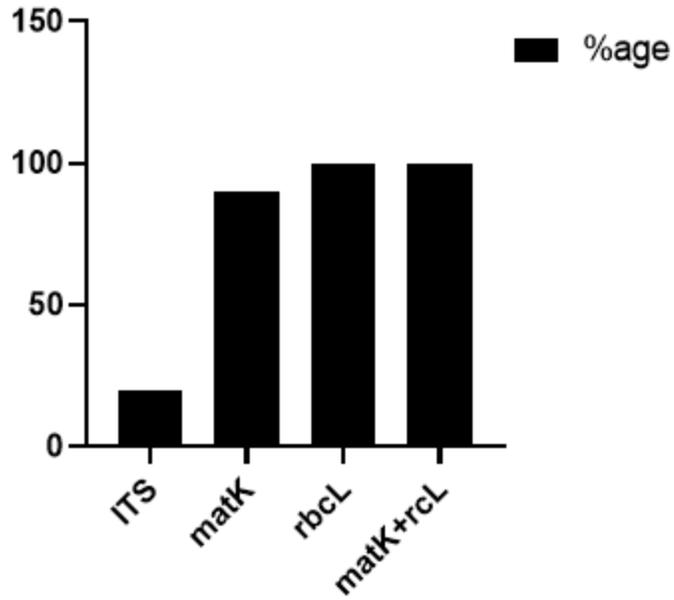
<i>ITS</i>	0.08	0.005	0.075
<i>rbcL</i>	0.01	0.001	0.008
<i>matK</i>	0.03	0.001	0.029



187

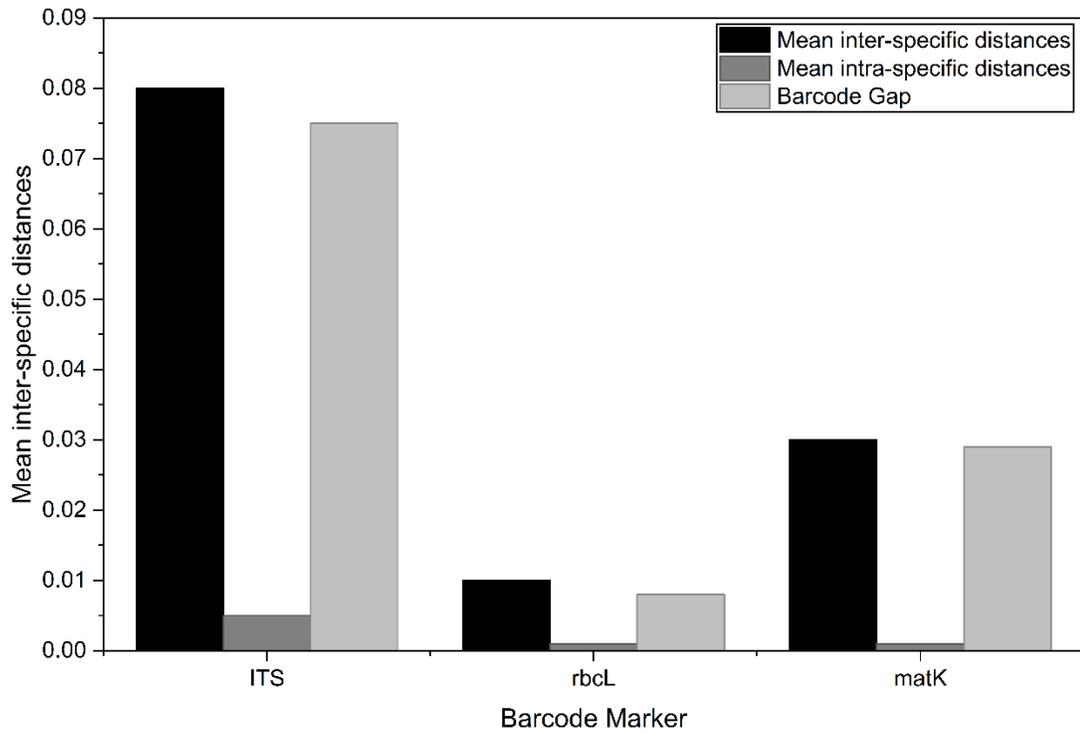
188 **Figure 1.** Average mean sequence divergence (uncorrected  $p$ -value,  $p < 0.05$ ) among the  
 189 three DNA markers of Aveneae type plastid DNA clade.

190



191

192 **Figure 2.** Percent determination rate of *ITS*, *rbcL*, *matK*, and *matK+rbcL* barcode  
 193 regions based on uncorrected *p*-values.



194

195 **Figure 3.** Inter-specific and intra-specific pairwise genetic distances calculated using K80  
196 model for the *ITS*, *rbcL* and *matK* markers for the 20 species of Aeneae Type plastid DNA  
197 clade from Pakistan.

### 198 **Tree based identification success**

199 In our tree-based analysis *rbcL* and *matK* barcodes correctly identified 82.35% and 80%  
200 species respectively, followed by 76.19% identification rate by a combination of *rbcL* and  
201 *matK*, while *ITS* was least successful with a low score of 43.75% (Table 4). Generic level  
202 identification was achieved almost equally by all the markers ranging from a low rate of  
203 77.77% for *rbcL+matK* to a higher value of 88.88% for *matK* barcode.

204 The *ITS* barcode correctly identified 87.7% genera (7 out of 8) of the Aeneae-type plastid  
205 DNA clade with a Bootstrap value >90. The three accessions of *Trisetum* are grouped in a  
206 monophyletic clade with (BS = 98). However, *Trisetum clarkei* is represented by a single  
207 accession, so its species level resolution cannot be confirmed in the present data set. The  
208 three accessions of *Rostraria cristata* formed a sister clade to *Trisetum*, but not forming its  
209 own group and not identified here. The two accessions of *Hierochloe laxa* formed a  
210 monophyletic group (BS = 100), while three species of *Avena* formed a sister group (BS =  
211 100). Within *Avena* two accessions of *A. fatua* formed a monophyletic group (BS=100),  
212 while *A. barbata* and *A. sterilis* are sister to it. The genus *Polypogon* (BS = 99) and *Agrostis*  
213 (BS = 100) formed their respective monophyletic clades. Within *Polypogon*, *P.*  
214 *monspeliensis* and *P. fugax* are monophyletic, while one accession of *P. monspeliensis* is  
215 sister to it. Within *Agrostis* three accessions of *A. munroana*, (BS = 87) two accessions of  
216 *A. pilosula* (BS = 71) and two accessions of *A. vinealis* (BS = 84) formed monophyletic  
217 groups. Within *Calamagrostis*, the two accessions of *C. stoliczkai* formed monophyletic  
218 group (BS = 96) while *C. emodensis* is sister to it. Within *Phalaris*, the two accessions of  
219 *P. minor* formed monophyletic group (BS = 89) while single accession of *P. paradoxa* (BS  
220 = 100) is sister to it (Figure 4). Furthermore, all the subtribes formed well resolved  
221 monophyletic clades with bootstrap value more than 98 except for Agrostidinae.

222 Similar results were also shown by *matK* barcode with relatively lower bootstrap value.  
223 Within *Agrostis*, accessions of *Agrostis munroana* (BS = 96), *Agrostis gigantea* (BS = 68),  
224 *Agrostis stolonifera* (BS = 97) and *Agrostis vinealis* (BS = 82) formed single group.

225 Accessions of *Polypogon fugax* and *Polypogon monspeliensis* formed their monophyletic  
 226 groups nested within *Agrostis*. Accessions of the genera *Avena*, *Trisetopsis*, *Rostraria* and  
 227 *Trisetum* formed their respective monophyletic groups. *Phalaris minor* is grouped with  
 228 *Phalaris paradoxa* (BS = 98). Accessions of *Hierochlo laxa* (BS = 100) and *Phleum*  
 229 *alpinum* (BS = 93) formed monophyletic groups (Figure 5). *matK* barcodes resolved the  
 230 Aveneae type plastid DNA group into subtribes with boot strap value greater than 95  
 231 (Figure 5).

232 Almost all the genera formed their separate groups based on *rbcL* barcode marker with  
 233 relatively lower bootstrap values. Accessions of *Agrostis* formed three groups sister to each  
 234 other with bootstrap value <50, *Rostraria* (BS = 63), *Trisetum* (BS = 41), *Phalaris* (BS =  
 235 34), *Avena* (BS = 53) and *Hierochloe* (BS = 69). The *rbcL* is also able to resolve all the  
 236 species into their respective subtribes and genera. Similarly, the combined *matK+rbcL* also  
 237 provides strong support to all the groups with bootstrap value > 90 for *Agrostis*, *Polypogon*,  
 238 *Rostraria*, *Avena*, *Helictotrichon*, *Phalaris* and *Hierochloe* (Figure 6 and 7).

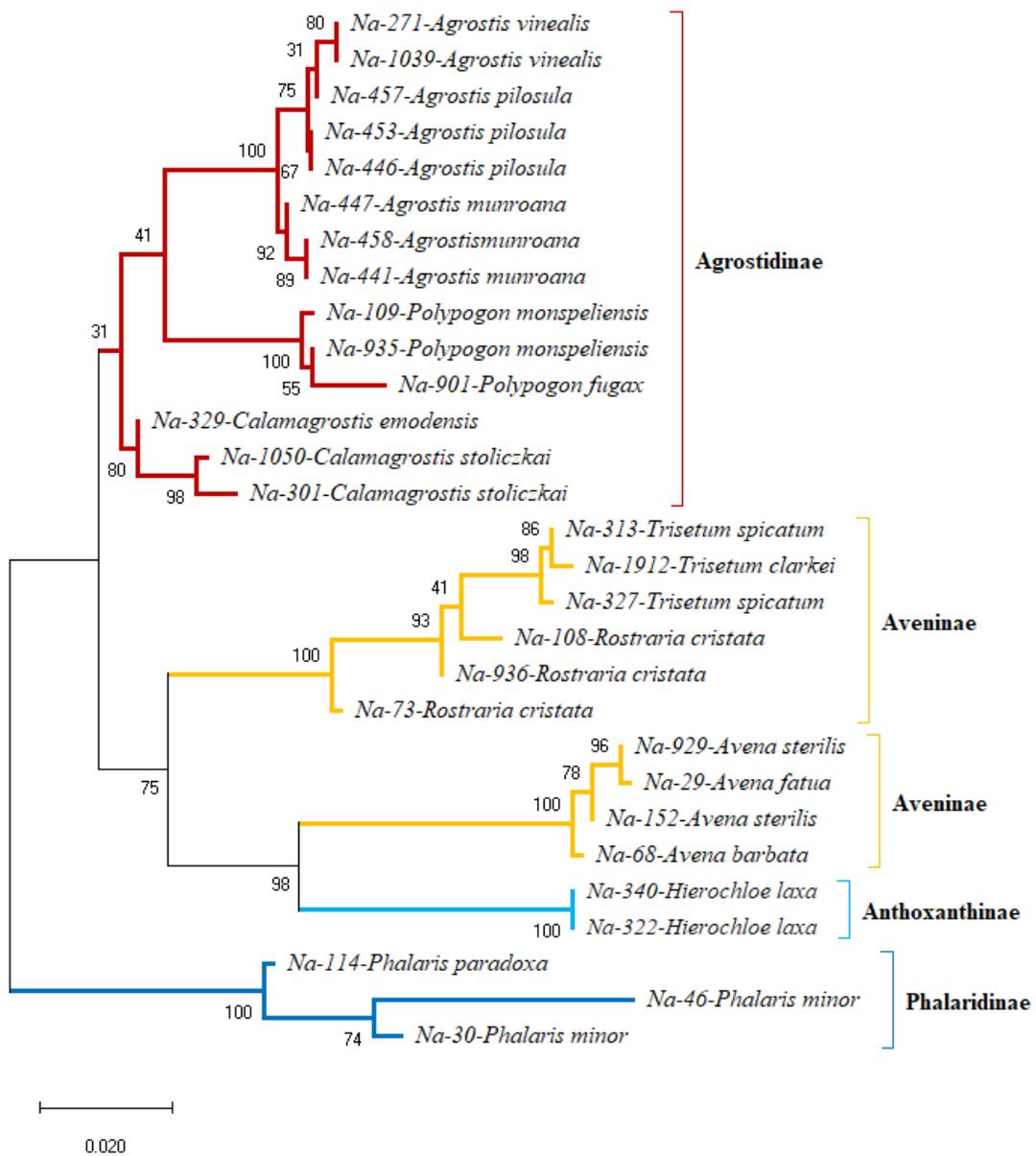
239 Over all the three markers were able to resolve species of Aveneae-type plastid DNA clade  
 240 into subtribes and genera. The total number of nodes successfully recovered with strong  
 241 support (BS = 70–100) for the barcode markers is as follows: *rbcL* with 12 nodes, *matK*  
 242 with 9 nodes, *ITS* with 11 nodes and *matK+rbcL* with 8 nodes.

243 **Table 4. Maximum Likelihood (ML) tree-based identification success of the three barcode**  
 244 **loci.**

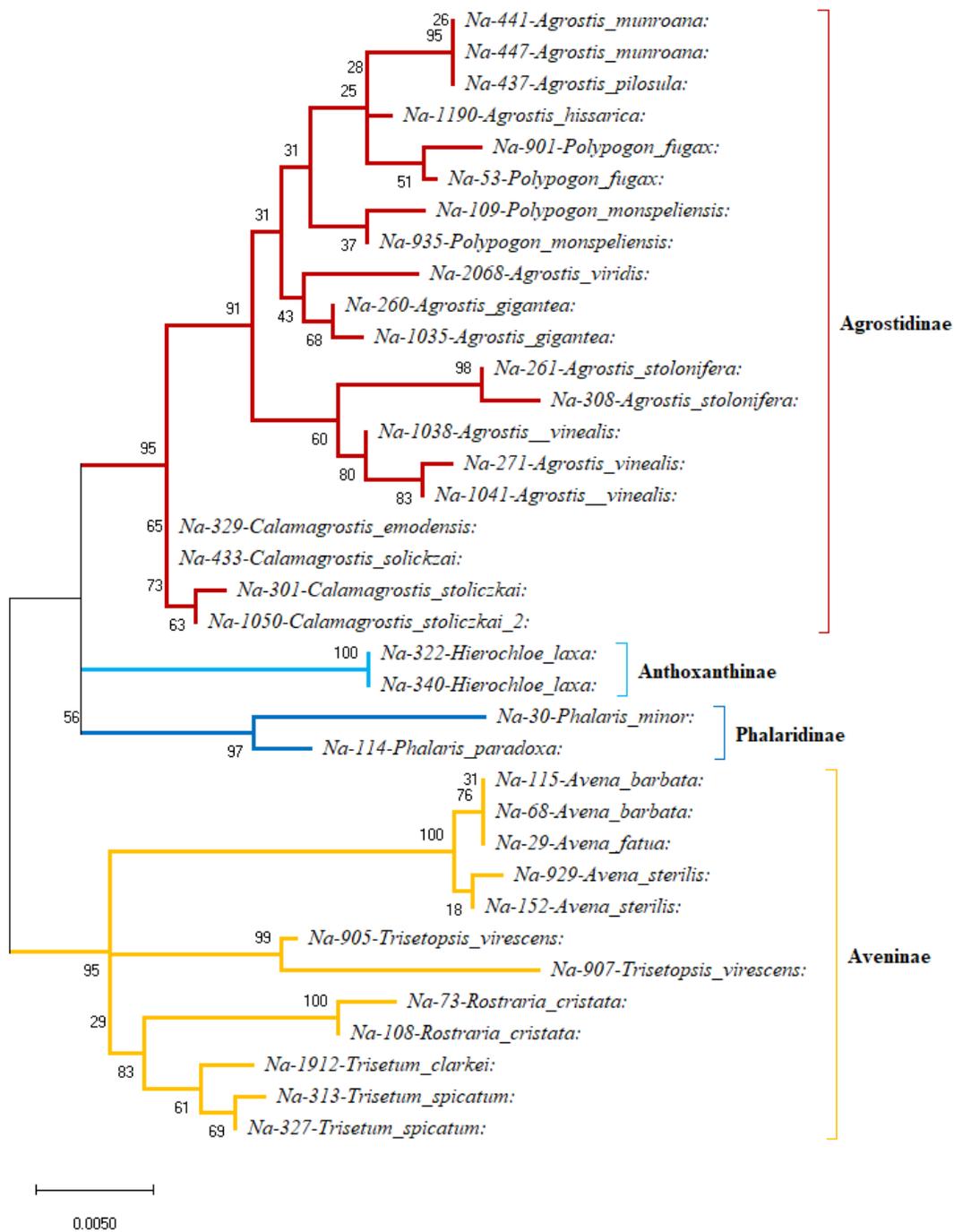
Taxon	Specimen identification (%)			
	<i>ITS</i> (8 genera, 16 species, 29 specimens)	<i>rbcL</i> (8 genera, 17 species, 31 specimens)	<i>matK</i> (9 genera, 20 species, 36 specimens)	<i>rbcL+matK</i> (9 genera, 21 species, 38 accessions)
	Correct/ incorrect	Correct/ incorrect	Correct/ incorrect	Correct/ incorrect
Genera	87.5%, 12.5%	87.5%, 12.5%	88.88%, 11.11%	77.77%, 22.22%
Species	43.75%, 56.25%	82.35%, 17.65%	80.0%, 20.0%	76.19%, 23.80%

245

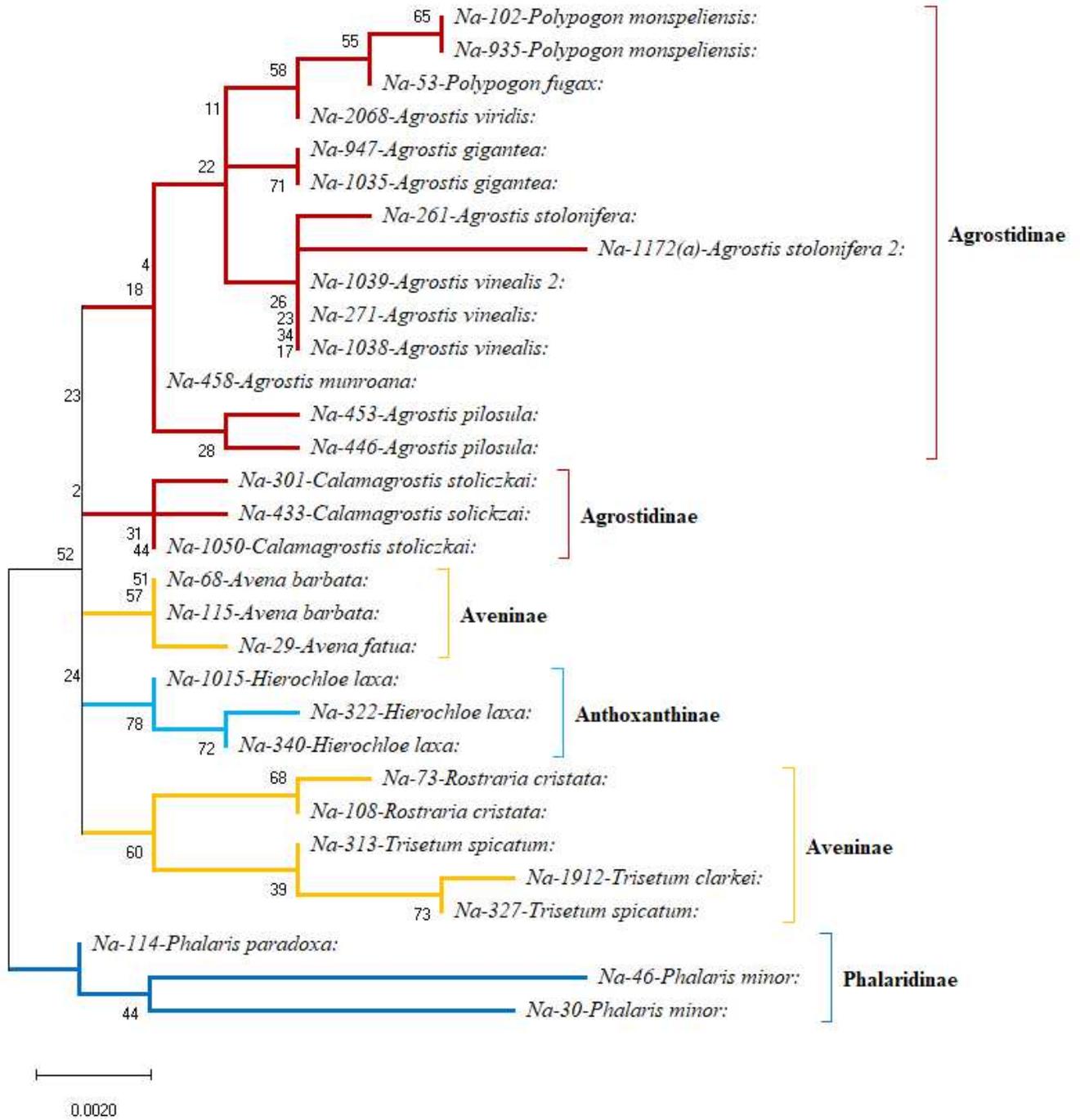
246



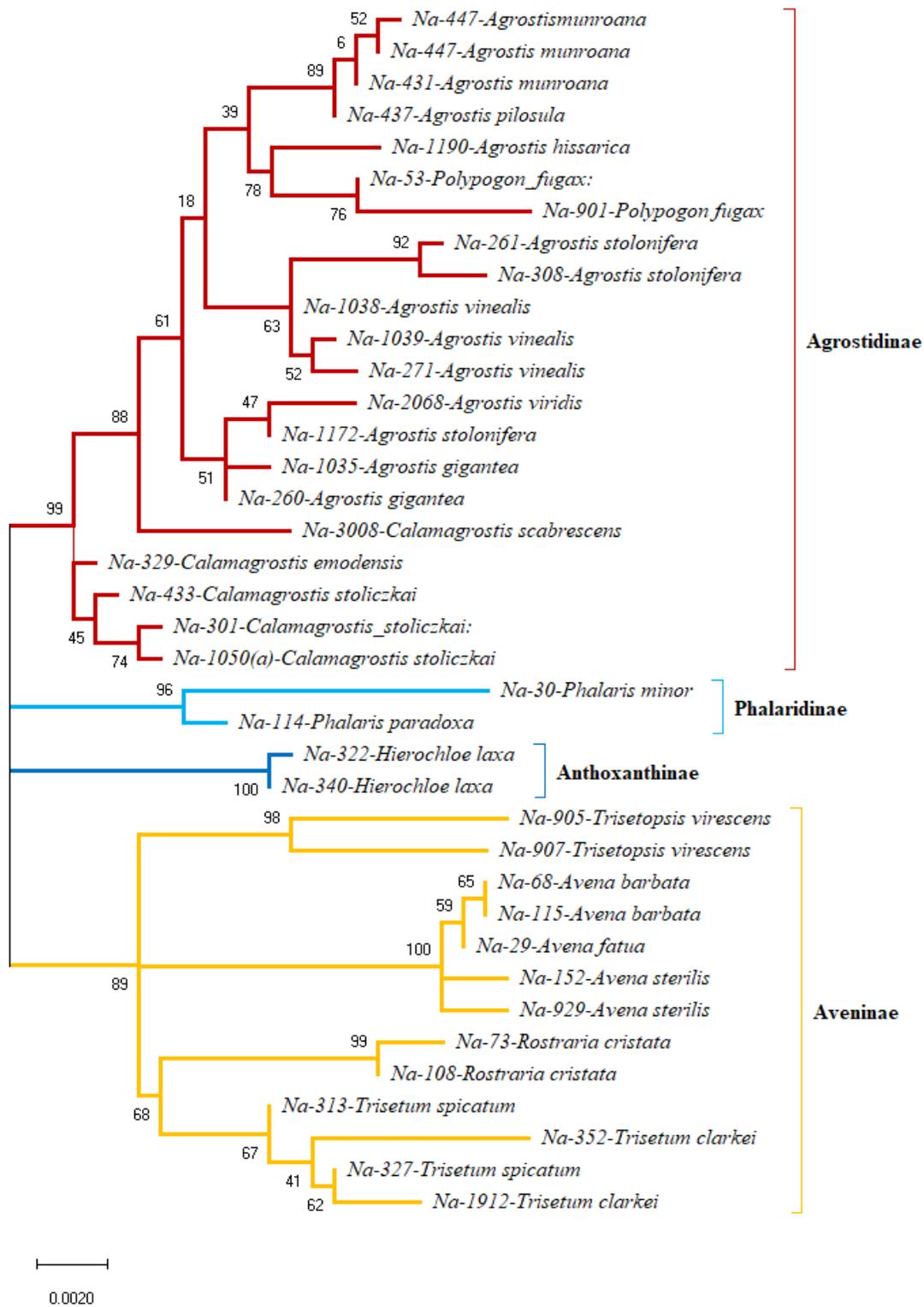
247 **Figure 4.** Maximum-likelihood tree of Aveneae-type plastid DNA clade based on ITS barcode sequence data.  
 248 Branch colors indicate subtribes: Agrostidinae (red), Anthoxanthinae (light blue), Phalaridinae (dark blue)  
 249 and Aveninae (yellowish)



250 **Figure 5.** Maximum-likelihood tree Aveneae-type plastid DNA clade based on *matK* data, rooted on  
 251 *Calamagrostis* spp. Branch colors indicate subtribes: Agrostidinae (red), Anthoxanthinae (light blue),  
 252 Phalaridinae (dark blue) and Aveninae (yellowish).



253 **Figure 6.** Maximum-likelihood tree of Aveneae-type plastid DNA clade based on *rbcL* data, rooted on  
 254 *Phalaris* spp. Branch colors indicates subtribes: Agrostidinae (red), Anthoxanthinae (light blue), Phalaridinae  
 255 (dark blue) and Aveninae (yellowish).



256 **Figure 7.** Maximum-likelihood tree of Aveneae-type plastid DNA clade on the basis of combined  
 257 *rbcL*+*matK* sequence data

258       **4. Discussion**

259       Our comprehensive analysis using *ITS*, *matK*, and *rbcL* DNA barcodes, individually and  
260       in combination (*rbcL+matK*), successfully identified most of the genera and species, and  
261       provided significant insights into the evolutionary relationships within the Aveneae-type  
262       Plastid DNA clade.

263       **Sequence variability and barcode gap analysis**

264       The sequence divergence (average uncorrected *p*-value) in the MEGA-X matrix was  
265       calculated as follows: 0.07 for *ITS*, 0.03 for *matK*, 0.01 for *rbcL* and for *matK+rbcL* was  
266       0.02. Our results are in agreement with that of (Peterson et al. 2014). The percent  
267       discrimination rate at a significance level of  $p < 0.05$  highlights the clear superiority of  
268       coding regions (*matK* and *rbcL*) over non coding regions (*ITS*). Our barcode gap analysis  
269       revealed a positive gap in 20 species, indicating effective species discrimination. In these  
270       cases, the interspecific genetic distances significantly exceeded the intraspecific distances,  
271       confirming a clear separation between species. This positive barcode gap underscores the  
272       reliability of our chosen genetic markers for distinguishing among the Aveneae type plastid  
273       DNA Pakistani species, enhancing the accuracy of our phylogenetic assessments and  
274       contributing to a robust DNA barcode library for this clade.

275       **Tree based identification success of different barcode regions**

276       All barcode markers demonstrated robust support for the monophyly of all the genera  
277       within the Aveneae type Plastid DNA clade, (except *Agrostis* and *Polypogon* complex)  
278       with strong bootstrap support exceeding 90%. Furthermore, our results also support  
279       monophyly of all the subtribes included in this clade.

280       In this study Agrostidinae appeared as monophyletic based on all three barcodes, which is  
281       in congruence with Saarela et al (2007, 2018), however the genus *Polypogon* is nested  
282       within *Agrostis* in all the ML trees of the three barcode regions. These results are in  
283       agreement with the recent studies (Rosen and Tkach 2024; Peterson et al. 2025), which  
284       suggest merger of these two genera. The subtribal clades in this study are in congruence  
285       with (Saarela et al. 2007), but (Soreng et al. 2017) also observed intermixing of some

286 accessions of the genus *Agrostis* and *Calamagrostis* based on both nuclear and plastid  
287 DNA.

288 Similarly, Aveninae is monophyletic in our analysis based on all three barcodes and within  
289 the Aveninae all the genera are also monophyletic. Our result is in congruence with Soreng  
290 et al (2007, 2017) and Saarela et al (2007) except that the nuclear ribosomal DNA in their  
291 studies did not provide strong support based on Maximum Parsimony results, while  
292 Bayesian analyses provided strong support. Clayton and Renvoize (1986) and Soreng et al  
293 (2007) placed *Trisetum* and allies within Aveninae. Quintanar et al (2007) recovered  
294 monophyletic lineage for our species of *Trisetum* similar to our findings. Whereas, Saarela  
295 et al (2010) recovered polyphyletic lineage and found unexpected results for *Calamagrostis*  
296 species collected from Mexico, that were grouped with *Trisetum*. Most of the old World  
297 *Trisetum* have been grouped in Koeleriinae clade A (Quintanar et al. 2007; Saarela et al  
298 2010) while that of the new world are grouped in Koeleriinae clade B (Wölk and Röser  
299 2014). But still molecular and morphological data remains a priority for resolving these  
300 taxonomic complexities (Saarela et al. 2017). To maintain monophyly of *Trisetum*, Barberá  
301 et al (2020) suggested to limit the genus to its typical species. The genus *Rostraria* in our  
302 study is monophyletic but overall, the previous literature confirms that *Rostraria* is not  
303 monophyletic (Saarela et al. 2010, Soreng et al. 2017, Saarela et al 2017; Barberá et al.  
304 2020). Furthermore, Saarela et al (2017) worked on Aveninae and established two  
305 subclades. In the first clade *Avena* and *Trisetopsis* were unresolved based on plastid and  
306 nrDNA like the earlier work of Wölk & Röser (2017), where our plastid DNA resolved  
307 (Fig. 4-7).

308 Furthermore, our result support the monophyly of the subtribes Anthoxanthinae and  
309 Phalaridinae (each represented by single genus *Hierochloe* and *Phalaris*, resepctivley). Our  
310 findings are similar to the previous studies including Döring et al (2007), Saarela et al  
311 (2010), Soreng et al (2017) Saarela et al (2017).

312 The nuclear ribosomal *ITS* has been identified as a promising supplementary barcode Chen  
313 et al. 2010; Hollingsworth et al. 2011). However, several issues limit its widespread use in  
314 barcode studies. Paralogous copies are common in polyploids, rapid genetic mutation  
315 saturation complicates alignments beyond closely related genera, and PCR products often

316 contain fungal contamination (Jani et al. 2010). Despite these challenges, *ITS* is believed  
317 to be highly effective for distinguishing closely related taxa.

318 The combined analysis of *matK* and *rbcL* markers offered enhanced resolution compared  
319 to *rbcL* alone, supporting multiple clades with strong bootstrap values, which is consistent  
320 with the findings of Newmaster et al (2006), who advocated for multi-locus barcoding  
321 strategies to improve phylogenetic accuracy. However, Peterson et al (2014) reported weak  
322 resolution of some grasses and the strength of *rbcL* barcode is questionable. The standard  
323 *rbcL* DNA barcode primers specifically in grasses, often fail to produce the desired  
324 amplicons while sometimes, the PCR product is contaminated with a mitochondrial copy  
325 (a paralogous pseudogene) of the *rbcL* gene (Cummings et al. 2003), which leads to  
326 ambiguous chromatograms with double peaks. However, Peterson et al (2014) reported  
327 weak resolution of some the grasses and the utility of *rbcL* barcode is questionable.

328 The *matK* and *rbcL* markers were equally successful in identifying the species and genera,  
329 although with lower bootstrap values than the *ITS*. slightly less resolute, corroborated  
330 many of the *ITS* findings. It successfully delineated monophyletic groups within *Agrostis*  
331 and other genera, albeit with relatively lower bootstrap values. For instance, *Agrostis*  
332 *munroana* (BS = 26) and *Agrostis gigantea* (BS = 68) were clearly resolved. This  
333 consistency across markers underscores the reliability of *matK* as a supplementary barcode,  
334 as noted in prior studies (Kress et al. 2005). The rapidly evolving coding region of *matK*  
335 has been extensively used to construct the phylogeny of Poaceae (Hilu et al. 1999; Hilu  
336 and Alice, 2001; GPWGII 2012). In our research, *matK* exhibited greater variability than  
337 *rbcL* and proved useful in our tree-based analyses. However, the primary challenge with  
338 using *matK* as a barcode marker is its low sequencing success rate (Hollingsworth et al.  
339 2011).

## 340 **5. Conclusion**

341 Our findings conclude that DNA barcoding is a promising tool for species identification  
342 and for understanding evolutionary relationship within Aveneae type plastid DNA clade.  
343 This study endorses the usefulness of universally recommended plant DNA barcode  
344 markers (*rbcL* and *matK*) and the supplementary barcode *ITS* for DNA barcoding studies  
345 in grasses. *ITS* marker performed best in terms of sequencing recovery, and barcode gap,

346 while *matK* and *rbcL* were better than ITS on the basis of tree-based identification. All the  
347 three barcodes successfully identified species within the subtribes Agrostidinae, Aveninae,  
348 Phalaridinae, and Anthoxanthinae with strong bootstrap support for the monophyly of most  
349 of the tribes and genera. On average all the markers were +80% successful in identifying  
350 the species and genera.

351 Beyond taxonomic resolution, these findings have broader implication in grass systematics,  
352 biodiversity conservation and agriculture resource management. DNA barcoding can aid  
353 in the verification of germplasm collection, identification of invasive species and  
354 monitoring of endangered taxa.

### 355 **6. Funding**

356 Princess Nourah bint Abdulrahman University Researchers Supporting Project number  
357 (PNURSP2025R318), Princess Nourah bint Abdulrahman University, Riyadh, Saudi  
358 Arabia.

### 359 **Funding**

360 Princess Nourah bint Abdulrahman University Researchers Supporting Project number  
361 (PNURSP2025R318), Princess Nourah bint Abdulrahman University, Riyadh, Saudi  
362 Arabia.

### 363 **Acknowledgements**

364 The authors gratefully acknowledge the support of Higher Education Commission (HEC)  
365 of Pakistan through NRPU project (6932) and IRSIP program. The authors are grateful to  
366 the people who worked at the College of Bioscience the University of Sheffield, Sheffield,  
367 UK for their assistance and cooperation throughout and providing lab facilities. Princess  
368 Nourah bint Abdulrahman University Researchers Supporting Project number  
369 (PNURSP2025R318), Princess Nourah bint Abdulrahman University, Riyadh, Saudi  
370 Arabia.

### 371 **Authors Contributions**

372 N.A., Z.U., and A.A., designed the sampling strategy. H.S., J.I., and L.D., designed the  
373 experiments. N.A, performed the experiment along with Z.U. H.S. provided materials and  
374 supervision. N.A. wrote the manuscript. K.M.A., F.A.S., and R.I, reviewed and edited the  
375 manuscript. All authors have read and agreed to the published version of the manuscript.

376 **Data Availability**

377 All the data related to this work can be sourced from the corresponding authors.

378 **Competing interests**

379 The authors showed no relevant financial or non-financial interests to disclose.

380

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