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CAPS DNA BARCODING FOR FIELD LABORATORY IDENTIFICATION OF GRASS SPECIES (BRITISH GRASSES AS A MODEL)

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The true grasses (*Poaceae*) comprise one of the largest plant families on earth. The group is peerless in its contribution to global agricultural production and its members dominate many of the world's most important habitats. However, morphological diagnosis of wild grasses is notoriously problematic and it is often impossible in the absence of flowering individuals. The advent of DNA barcoding provided a useful tool to address this problem for larger-scale or longer-term studies but the need for sequencing precludes its use in a field laboratory context or in situations where either funding or time is limited. Here, a chloroplast DNA (cpDNA)-based Cleaved Amplified Polymorphic Sequence (CAPS) system of molecular species diagnosis that has the capacity to address this problem is presented using British grasses as a model. First, PCRs were performed using universal primer pairs targeting 21 regions of the chloroplast genome in authenticated representatives of the 117 grass species from the British Isles, and universal amplification for all loci targeted was demonstrated. Second, 54 restriction enzymes were applied on amplification products generated from all species. There were 10 locus-enzyme combinations (with the highest variation) that had the best diagnostic utility for the 117 grass species.CAPS analysis on 16 representatives of three genera (*Calamagrostis*, *Phleum,* and *Agrostis*) was then used to illustrate the potential utility of the pipeline for establishing a field-laboratory screen of species identity. CAPS DNA barcoding system developed here may have ecological, conservation, and commercial applications. However, it has limited possibilities for intraspecific differentiation due to the highly conserved nature of loci targeted within species.

Key words: CAPS, DNA barcoding, identification, grasses

On one level, the grass family can be deemed of great importance among flowering plants simply for being one of the largest families. Indeed, the *Poaceae* includes over 12,000 species and 800 genera (Soreng *et al*. 2015) and is eclipsed in size only by the *Asteraceae* (24,000), *Orchidaceae* (20,000) and the *Fabaceae* (18,000) (Judd *et al.* 2008). The ecological importance of the group is even more remarkable, with grasslands accounting for over 40% of terrestrial land area and defining many of the world's most diverse natural ecosystems (White *et al.* 2000). It is in the economic realm, however, where the pre-eminence of the family becomes most apparent. Amenity grasses play a pivotal role in the sports, amenity and urban landscape arenas and fodder grasses form the primary food source for livestock throughout the world, either as sward, hay, silage or feed. However, it is in the field of human

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food production where the grasses arguably make their greatest contribution to humankind, with the cereal grasses representing four of the five most important staple food crops in the world (FAOSTAT 2014, http://faostat3.fao.org). For these reasons, grasses as a whole can be reasonably argued to be the most important group of plants both to mankind and the natural world. Given this intensity of interest in the group, it is perhaps unsurprising that there is a strong desire for a reliable diagnosis of the many species it contains.

Grasses are very difficult to identify by morphological characters due to phenotypic plasticity, with many overlapping characters and few distinguishable (Lucas *et al.* 2012; Saadullah *et al.* 2016). The reduced vegetative structures in grasses mean that features of the inflorescence form the primary source of diagnostic characters for grass species identification (Vegetti & Anton 1995). In the absence of flowering individuals during non-flowering seasons or in the face of excessive grazing or mowing, researchers often resort to anatomical features to effect identification (Haider 2011). Frequently, this requires additional time and effort and often fails to allow the diagnosis to species rank. For instance, Krishnan *et al.* (2000) used the size and shape of phytoliths (amorphous silicon dioxide (SiO2.nH2O)) inclusions abundant in leaves, internodes, and glumes of around 100 grass species from India to diagnose grasses and succeeded to separate many samples at the generic level. In contexts in which only a limited number of species are encountered, vegetative diagnosis of species is a tractable prospect. Fermanian and Michalski (1989) developed a computer software 'WEEDER' using the artificial intelligence system AGASSISTANT to enable species identification of 37 turf species and weed grasses found in the USA. For many more complex settings, however, reliable species identification of grasses only became a realistic prospect with the advent of molecular DNA barcoding.

Hebert *et al.* (2003) proposed the use of DNA sequences from conserved loci (so-called DNA barcodes) as a means of species identification. Initial success came from the use of a highly variable 648-bp region near the 5'end of the cytochrome oxidase subunit I (COI) gene led to the application of the approach on a wide diversity of animal species including birds (Hebert *et al.* 2004), amphibians (Vences *et al.* 2005), fishes (Ward *et al.* 2005) insects (Nelson *et al.* 2007), and mammals (Lorenz *et al.* 2005). However, the COI gene performed poorly for plant species diagnosis, partly because genes of the mitochondrial genome of plants evolve extremely slowly (Wynn & Christensen 2019) and so exhibit limited interspecific variation. Following an extensive worldwide search for alternative loci (Kress *et al.* 2005; Chase *et al.* 2007; Ford *et al.* 2009; Pennisi 2007; Ledford 2008), the plant working Group of the Consortium for the Barcode of Life (CBOL) finally agreed to designate variable regions of *rbcL* and *matK* as a dual universal DNA barcode for plants (Hollingsworth *et al.* 2011).

Saadullah *et al.* (2016) used the combination of both markers (*rbcl* + *matK*) to identify the grass family and constructed a well resolved monophyletic tree with a strong bootstrap threshold value. The authors, however, were not able to identify congeneric species because their sequence overlapped and showed zero interspecific distance between one another. Very few other attempts have been carried out to barcode grass species (e.g., Lucas *et al.* 2012; Wang *et al.* 2014; Tahir *et al.* 2018). Lucas *et al.* (2012) stressed that the DNA barcoding system they developed for seagrasses is not simple because a few complexes remained unsolved even when constructing a combined tree for all three loci (*trnH-psbA*, *matK* and *rbcL*). *ITS2*, that proved by Khan *et al.* (2019) as a cost-effective barcoding marker for verifying the authenticity of *Rhazya stricta* and other medicinal plants, was revealed by Tahir *et al.* (2018) to have the highest number of variable sites for tested *Poaceae* species when they compared six barcode regions (*ITS2, matK, rbcLa, ITS2+matK, ITS2+rbcLa, matK+rbcLa,* and *ITS2+matK+rbcLa*).

Chloroplast-derived Cleaved Amplified Polymorphic Sequence (CAPS) markers have also been reported to detect the interspecies variation of different plant species (e.g., Sakka *et al.* 2015), and hence it has been applied to identify several groups of plant species including grasses. For instance, Kim *et al.* (2018) reported that differences revealed between *Pinus sylvestris* and *P. densiflora* based on cpDNA-CAPS can be used to distinguish between these two species. CpDNA-CAPS markers were

also developed to discriminate *Solanum chacoense* from other *Solanum* species (Kim & Park 2019). As for grasses, Haider and Nabulsi (2008) were also able to identify *Aegilops* L. species and *Triticum aestivum* L. based on CAPS and sequencing applied on cpDNA.

DNA barcoding (when successful) represents something of a landmark in DNA-based species identification of plants in the many situations in which DNA sequencing is practical. There are nevertheless some contexts in which there is a desire for low-cost, low-volume but high-speed molecular diagnosis of species, including as a teaching aid on field courses, protracted field studies based in isolated rudimentary laboratories and in commercial or research settings in which resources and/or time are limited. In these cases, there is a need for a sequence-free approach to DNA-based plant identification. Therefore, the primary objective of this study is to identify variable CAPS markers that have potential worth for the diagnosis of grasses at the species level using British grasses as a model. Accordingly, variation in SNP alleles should be relatively high within taxonomic groups above these ranks but should be largely invariant at lower ranks. Thus, the aim is to produce markers that vary between species but not between populations of the same species.

MATERIALS AND METHODS

Plant collection, DNA extraction, and PCR

All laboratory experiments were carried out in laboratories of `School of Biological Sciences (SBS)`, the University of Reading (RDG), UK. Leaf-blade material representing 117 species of native British grasses was collected. See Haider (2016) for names, sources, and year of collection (for fresh samples) or preservation (for dry samples) of all grass species used in this study. Two sources of leaf material were used: 1) fresh leaves of flowering living plants obtained from field collections, and 2) dried leaves taken from herbarium specimens held in the herbarium of SBS. In both cases, great care was taken to ensure the authenticity of species identity. The identity of all specimens was verified or established using the diagnostic keys given by Stace (1997) for the *Poaceae* family. Plant material of three British native species (*Festuca huonii* Auquier, *Festuca armoricana* Kergue`len, and *Poa humilis* Ehrh. Ex Hoffm) were unavailable and so were excluded from this study.

DNA was extracted from leaf fine powder (0.5 g) using the 'DNeasy 96 Plant Kit' (Qiagen, UK) according to the manufacturer's instructions. Recovered DNA pellets were dried under the laminar flow and then resuspended in 150 ml of doubled distilled and sterilized water. DNA was quantified using

Table 1

DNA sequences, annealing T/ºC, and expected product size of five grass-universal primers (Haider unpublished data) used to amplify five chloroplast regions. Numbers 1 and 2 refer to the portion of locus.

Locus name	Primer sequence/5`–3`	Annealing T/ºC
rpoA.1.F	ctcaaacactacagtggaagtg	52
rpoA.1.R	cagcatctataggataacttcc	
infA-rps11-rpoA.F	gtggaagtgtgttgaatcaag	54
infA-rps11-rpoA.R	tcttccgcatgtaggaaagg	
psbH.F	ggatctacgaaaagatcgtg	55
psbH.R	tccgtccagtaaaacggaag	
psbK-psbI-trnS.2.F	ccaggacggaatcctggg	55
psbK-psbI-trnS.2.R	cctatatggaaatgcccgc	
rpl23&rpl2.1.F	gtcgaatcaggatcaactagg	55
rpl23&rpl2.1.R	gtatgcatttcgattagggtcg	

Gene Quant Spectrometer (Amersham Biosciences, UK) and the concentration of each DNA template was set at 10 ng/µL.

PCRs were performed in 20 µl reaction mixes comprising of 20 ng template DNA (2 µl @ 10 ng/µl), 15m M primer, and other reagents described by Haider (2015). We first used 16/19 universal primer pairs that were developed by Haider (2015) for targeting 16 regions of 13 genes of the chloroplast genomes of grasses to test for amplification on template DNAs from 117 British native grasses. These were: *rps.4, psbE&psbF, rpoA.2, rbcL.1, ndhF.1, ndhF.2, ndhF.3, 23S,4.5S&5S, rpl2&trnH.1, rpl2&trnH.2, psbE-psbF-orf38-orf40, clpP&rps12, psbK-psbI-trnS.1, rpl23&rpl2.2, pcbC* and *orf62.* Another set of 5 universal primer pairs (Table 1) developed by Haider (unpublished data) was applied on the same DNA templates for targeting five coding loci of the cpDNA. These were: *rpoA.1, infA-rps11-rpoA, psbH, psbK-psbI-trnS.2,* and *rpl23&rpl2.1.* All samples were subjected to the following thermocycling regime: thirty-five cycles of 94°C for 30 s, 52 – 58.5°C for 1 min, and 72°C for 1 min, followed by 5 min at 72°C. For amplicon visualization, 2 µl of each PCR product was loaded into 1.5% agarose gel and run at 120 V for 30 min.

CAPS analysis

CAPS analysis was performed on a single representative of 117 British native grass species (Haider

Table 2

Chloroplast loci and enzymes used for CAPS applied on 117 grasses studied. Expected sizes of amplification products are according to Haider (unpublished data; 2015).

Chloroplast locus name	Expected product size (bp)	Enzymes used for restriction
rps.4	383	-*Hind* II, -*Apa* I, and -*Ava* I
psbE&psbF	508	-*Hinf* I, -*Ava* II, -*Hpa* II, and -*Mva* I
rpoA.1	495	-*Hae* II, -*EcoR* V, and -*Ban* II
rpoA.2	489	-*Dde* I
rbcL.1	524	-*Ava* I, -*Ban* II, and -*Ava* II
ndhF.1	298	-*Mva* I and -*Taq* I
ndhF.2	316	-*Taq* I and -*Rsa* I
ndhF.3	393	-*Sca* I, -*Hinf* I, -*Bam* HI, -*Kpn* I, and -*Ava* II
infA-rps11-rpoA	642	-*Dde* I and -*Hae* II
psbH	285	-*Xba* I, -*Bln* I, and -*Ava* I
23S,4.5S&5S	393	-*Hae* III, -*Hinf* I, -*Nde* I, and -*Cla* I
rpl2&trnH.1	272	-*Nco* I
rpl2&trnH.2	564	-*Nco* I and -*Nde* I
psbE-psbF-orf38-orf40	365	-*Ban* II
clpP&rps12	608	-*Hpa* II, -*Rsa* I, -*Kpn* I, and -*Xba* I
psbK-psbI-trnS.1	534	-*Sca* I, and -*Pst* I
psbK-psbI-trnS.2	463	-*Hae* III
rpl23&rpl2.1	498	-*Mva* I, -*Nco* I, and -*Cla* I
rpl23&rpl2.2	353	-*EcoR* V, and -*Cla* I
pcbC	487	-*MvaI*, -*AvaI*, and -*Ban* II
orf62	414	-*Dde* I, -*Hpa* II, and -*EcoR* I

2016) using locus-enzyme combinations (1 – 5 restriction enzymes per locus) as listed in Table 2.

For the digestion of PCR products, restriction enzymes generating polymorphic (length) fragments were selected according to sequences retrieved from the NCBI database for grasses. Two aspects of the CAPS markers were examined prior to evaluation for species diagnosis. First, the selected primers should be able to generate consistent amplification products that contain recognition sites for targeted restriction enzymes to develop informative markers for species identification. Second, genetic changes in the recognition sites of restriction enzymes must be available in NCBI sequences aligned for each locus targeted.

Screen for the interspecific variation of CAPS markers within three genera

The ability of the most variable CAPS markers that had diagnostic utility for the 117 species was evaluated to diagnose species within the same genus for three genera. These are *Calamagrostis* (*C. epigejos, C. canescens, C. purpurea, C. stricta,* and *C. scotica*), *Phleum* (*Ph. arenarium, Ph. bertolonii, Ph. alpinum, Ph. Phleoides,* and *Ph. pratense* L. cultivar Latima), and *Agrostis* (*A. gigantea, A. stolonifera, A. curtisii, A. canina, A. vinealis,* and *A. capillaris*). DNA was extracted from all representative species as described earlier. The most variable 10 locus-enzyme combinations (six loci and 10 enzymes) were applied on these three genera representatives. These were: *rps4-Hind* II, *-Apa* I and *-Ava* I; *rpoA.1-Eco*R V and *-Ban* II; *rpoA.2-Dde* I; *rbcL.1-Ava* II; *ndhF.1-Mva* I; and *clpP&rps12-Hpa* II and *-Rsa* I.

Screen for the intraspecific variation of CAPS markers

In order for the markers identified to have utility for species diagnosis, it is vital that variation observed between species is not matched by variation within the species. Conservation of intraspecific

Table 3

Representative samples (1 – 26) of the grass species *D. glomerata* used in this study. The table shows source and year of preservation of samples 1 – 19 that were collected from the herbarium of School of Biological Sciences. Plant material of the remaining seven samples was seeds provided by Institute of Grassland and Environmental Research, Genetic Resources Unit.

D. glomerata
1. Flora of the British Isles, MJP. Scannell, (1992)
2. Flora of France- 16683, E. Berger, (1992)
3. ssp. hispenica. Flora of France- 17711, G. Certa, (1995)
4. Portugal-12, SE. Cross, (1975)
5. Crete, CG. Hanson, (1999)
6. Spain-Optima Itter VI 593, (1994)
7. Giblartar-147, VH. Heywood, DM. Moore *et al.* (1969)
8. Flora of Norway-No. 14.T.F., (1972)
9. Flora of Spain-379, B. ValdËs, MF. Watson *et al.* (1988)
10. Flora of Portugal-Optima Itter VI 1056, (1994)
11. Flora of Italy-Optima Itter VIII 54, (1997)
12. Flora of Belgique-00/Co/192, J. Lambinon, J. Margot and B. Stouff, (2000)
13. Flora of Morocco, SL. Jury, (1997)
14. ssp. hispenica (Ruth) Koch. Flora of Jordan, Dawud Al-Eisawi and Pasmi Jasrrar, (1978)
15. Flora of Turkey-1209, RMA. Nesbitt, (1987)
16. Flora of Palestine (Israel)-44-24/1522, A. Danin, SG. Knees *et al.* (1989)
17. Flora of Greece (East Aegean Islands), JR. Akeroyd 331, (1983)
18. Flora of Belgique, Leg. G. Van Buggenhout Det. J. Lambinon, (1996)
19. Flora of Northern Morocco, MA. Mateos, E. Ramos and J. Willarreal, (1995)
20. IGER/ABY-BC 7044.1980U
21. IGER/ABY-BC 7045.1980U
22. IGER/ABY-BC 7047.1982U
23. IGER/ABY-BC 7048.1980U
24. IGER/ABY-BC 7050.1981U
25. IGER/ABY-BC 7051.1981U
26. IGER/ABY-BC 7341.1998U

CAPS profiles was surveyed in six species. These are: *Dactylis glomerata, Phleum pratense, Nardus stricta, Molinia caerolia, Festuca rubra,* and *Lolium perenne.* For this purpose, 3 (*F. rubra*) – 26 (*D. glomerata)* representatives of each species were selected from a wide range of geographical regions. The sources and origins of all representatives of the six species screened are given in Tables 3 – 7. In each case, the sample that was examined earlier was used as a positive control. PCR products of each of the six species were subjected to restriction using only those locus-enzyme combinations required to

Table 4

Source, year of preservation, and collector of representative samples of the grass species *Ph. pratense* used in this study. Samples were numbered 1 – 19, and plant material was collected from the herbarium of School of Biological Sciences.

Ph. pratense
1. South Wales, RW. Rutherford, (1992)
2. ssp. bertolonii, Spain-10979, SL. Jury, (1992)
3. Iceland-88064, PMD. Etherington, (1988)
4. Norway, HJM. Bowen, (1977)
5. Italy-Optima Itter VIII 1839, (1997)
6. North America-5279, HJM. Bowen, (1987)
7. Falkland Islands-15, CD. Young, (1967)
8. Argentina, (1973)
9. Flora of Turkey-2857, RM. Nesbitt and DJ. Samuel, (1989)
10. Flora of Poland, Leg. M. Matoga, Det. T. Tacik, (1972)
11. Flora of Spain-2906, P. Valdes, MF. Watson *et al.* (1988)
12. Flora of Germany-5478, HJM Bowen, (1981)
13. Flora of Norway, No. 4T. F., (1972)
14. Flora of Argentina-4460R, N. Goodall, (1972)
15. Flora of France-54, RM. Salinon, (1971)
16. Flora of Italy-Optima Itter VIII 1972, (1997)
17. Flora of the British Island-37, DA. Waroell, (1980)
18. Flora of Hertfordshire-61, DW. Nelson, (1979)
19. France, Leg et det. S. Pignatti, (1985)

Table 5

Source, year of preservation, and collector of representative samples (1 – 19) of *N. stricta* species used in this study. Plant material was collected from the herbarium of School of Biological Sciences.

N. stricta
1. Spain-1757, Christoph Dobes, Ernst Vitek, (1998)
2. Orrtugal-Optima Itter VI-1370, (1994)
3. Flora of British Isles, Surry-22284, ST. Blake, (1964)
4. Flora of British Isles- ref SP 177425, G. F. Hotge 44, (1980)
5. North America-No. 66-1925, Carlo Hansen, (1966)
6. Norway-N13, Bokstindan Biological Research, (1972)
7. Flora of Italy-Optima Itter VIII 978, (1997)
8. Flora of the British Isles, Trongh nr Fortan, Lames, (1971)
9. Flora of Portugal-Optima Itter VI 1105, (1994)
10. Spain-882, B. Valdes, UF. Watson *et al.* (1998)
11. Scotland-ref. 009929, no. 120, RW. Hambrook, (1971)
12. Hampshire-ref. Su 621623, GJ. Ieach, (1957)
13. N. Wales-116-7214, JR. Gbloomfield, (1966)
14. Spain -Exped. 169, K. Retley, (1969)
15. Finland, Hiitonen-Ihnari, (1955)
16. France- 11013, KL 37554, Leg. et det. K. and SS. Larsen, (1983)
17. Central Pyrenees, RE. Langton, (1961)
18. Russia, Moscow province, HM. Pewethukoba, NM. Reshetnikova, (1994)
19. Italy-5626, SP. Brooks, RS. Hadded and SL. Jury, (1984)

yield species-specific CAPS profiles.

Plant material used for DNA extraction comprised of either dry leaves that were taken from specimens held in the Herbarium of School of Biological Sciences (SBS) at University of Reading (RDG) (*D. glomerata, Ph. pratense, N. stricta,* and *M. caerolia*) or else fresh leaves taken from plants grown in SBS glasshouses from seeds provided by the Genetic Resources Unit (GRU) at the Institute of Grassland and Environmental Research (IGER) for *F. rubra* and *L. perenne*.

DNA extraction, PCR and restriction of PCR products

DNA was extracted from leaf fine powder (0.5 g) of all representatives of the above species using the

Table 6

Source and year of collection of representative samples (1 – 19) of *M. caerulea* species used in this study. Plant material was collected from the herbarium of School of Biological Sciences.

M. caerulea
1. ssp. arundinacea, Flora of Austria-2299, HJM. Bowen, (1981)
2. ssp. litoralis France- 8977, (1977)
3. ssp. arundinacea, Franc- 16719, W. Lippert and F. Schuhwerk, (1995)
4. Norway, HJM. Bowen, (1977)
5. Spain, VH. Heywood and DM. Moore, (1973)
6. Germany-5481, HJM. Bowen, (1981)
7. France-62576 14813, P. Alankon, (1991)
8. Morocco, RU Biol. Exped, P. Crane, (1975)
9. Flora of West Suffolk Cool-No. 667, PJO. Trist, (1986)
10. Wales-AC.-33, Esgair Elan, AGDR. Channer, (1984)
11. Scotland-260, PF. Cannon, (1978)
12. ssp. arundinacea., Spain, SL. Jury and B. Molestorth-Alen, (1993)
13. Norway-N4F, (1972)
14. Spain-572, DJ. Goyder and SL. Jury, (1982)
15. URSS, (1972)
16. Greece, (1988)
17. Museum Botanicum Hauniense Kell Dansholt, Kjeld Holmen and Jette Svendsen, (1970)
18. Flora of the British Isles-SU 193041, Alastair Culham, (1984)
19. British Isles-199, SL. Jury (1972)

Table 7

Representative samples of *L. perenne* and *F. rubra* (seeds were provided by the Institute of Grassland and Environmental Research (IGER), Genetic Resources Unit).

L. perenne	*F. rubra*
1. IGER/ABY-BA 9790.1981U	1. IGER/ABY-BL 2690.1983U
2. IGER/ABY-BA 9792.1981U	2. IGER/ABY-BL 2813.1995U
3. IGER/ABY-BA 9830.1980U	3. IGER/ABY-BL 2888.1998U
4. IGER/ABY-BA 9958.1996U	
5. IGER/ABY-BA 9965.1982U	
6. IGER/ABY-BA 9966.1981U	
7. GER/ABY-BA 10096.1995U	
8. IGER/ABY-BA 10297.1983U	
9. IGER/ABY-BA 10299.1983U	
10. IGER/ABY-BA 10414.1995U	
11. IGER/ABY-BA 10818.1986U	
12. IGER/ABY-BA 11142.1989U	
13. IGER/ABY-BA. 13210.1998U	
14. IGER/ABY-BA 13229.1998U	
15. IGER/ABY-BA 13232.1998U	
16. IGER/ABY-BA 13243.1997U	

'DNeasy 96 Plant Kit' (Qiagen, UK) according to the manufacturers' instructions. Chloroplast loci used for CAPS-based species identification were amplified by PCR as described above. The following seven loci were targeted: *rps4, rpoA.1, rpoA.2, clpP&rps12*, *ndhF.1, ndhF.3,* and *rbcL.1*.

PCR products of each of the six species were subjected to restriction using only those enzyme-combinations required to yield species-specific CAPS profiles (Table 8). Representatives of the six species considered earlier for the generation of CAPS markers diagnostic for these species were used as a positive control in each restriction experiment.

RESULTS

All representative plant materials exhibited an appropriate phenotype to yield the corresponding diagnosis when passed through the identification keys used and also to match with the detailed description provided by Stace (1997). All herbarium specimens used were authentic specimens (collected by the authority who first described the species), topotypes (collected from the type locality), or else were iso- or lectotypes.

All 21 primer pairs tested generated a single, strong amplification product with the sizes (bp) reported by Haider (unpublished data; 2015) (see Ta-

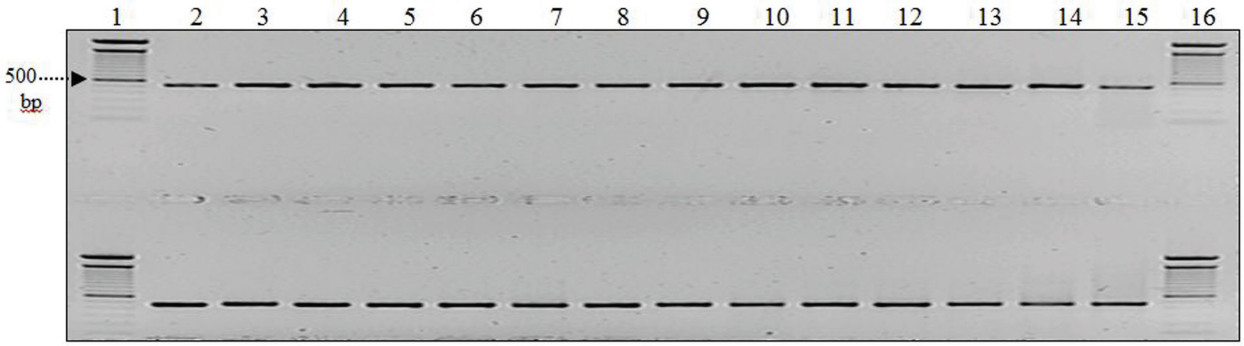

Figure 1. Amplification of loci *rpoA.2* and *psbC* in 14 grass species. A. Lanes 1&16 (A&B), 100 bp DNA ladder; lanes 2-15A and 2-15B, amplicons of loci *rpoA.2* and *psbC* in 14 grasses, respectively.

Table 8

CAPS markers specific to 6 grass species. The table gives species names and locus-enzyme combinations that allowed the discrimination of each of these species from the remaining grass species examined.

Grass species	Specific-specific CAPS markers	Grass species	Specific-specific CAPS markers
D. glomerata	*rps4-Ava* I *rpoA.1-EcoR* V *rpoA.2-Dde* I	*Ph. pratensis*	*rps4-Hind* II *rbcL*.1-*Ava* II *clpP&rps12-Hpa* II
L. perenne	*rps4-Hind* II *rpoA.1-Ban* II *rpoA.2-Dde* I	*M. caerolia*	*rps4-Hind* II and -*Ava* I *clpP&rps12-Hpa* II and -*Xba* I *rpoA.1-Ban* II
F. rubra	*rbcL.1-Ava* II *ndhF.1-Mva* I *rpoA.1-Ban* II *clpP&rps12-Rsa* I *ndhF.3-Hinf* I *rps4- Hind* II	*N. stricta*	*rps4-Ava* I *rbcL.1-Ava* II *rpoA.1-Ban* II

ble 2) as visualised by agarose gel electrophoresis when targeted chloroplast loci were amplified by PCR in all species used in the screen carried out to evaluate the universality of these primers on grasses (e.g., Figure 1).

PCR and CAPS analysis for developing variable markers with diagnostic utility for the grass species targeted

Single and strong products were generated by PCR for every primer pair using all template DNA samples, including those extracted from dry material. There was no size variation among the analysed species samples for all 21 regions amplified.

In each case, the incubation of the amplification product in the presence of an appropriate restriction enzyme yielded one or two smaller restriction products in at least some species. In some of the combinations, however, variation was observed in the nature of the restriction profile produced. For instance, restriction of the targeted region of gene *rps4* with *Hind* II showed three different profiles in different samples. Other locus-enzyme combinations that generated variable restriction profiles among the grass species were: *rps4*-Ava I, *rpoA.2*-Dde I, and *clpP&rps12*-Hpa II and -Rsa I. Such chloroplast loci were the most useful of generating CAPS markers for grass species identification. Five locus-enzyme combinations (*rps4-Hind* II, *rpoA.2-Dde* I, *ndhF.1-Mva* I and *clpP&rps12-Hpa* II and -*Rsa* I) were very polymorphic among grasses studied and three combinations (*rbcL.1-Ava* II, and *clpP&rps12-Kpn* I and -*Xba* I) displayed very little variation.

There was considerable redundancy amongst the set used, with the majority of sample pairs being distinguished by more than one locus-enzyme combination. There were 10 locus-enzyme combinations (with the highest variation) that had the best diagnostic utility for the 117 grass species. These are: *rps4*-Hind II, -Apa I and -Ava I; *rpoA.1*-EcoR V and -Ban II; *rpoA.2*-Dde I; *rbcL.1*-Ava II; *ndhF.1*-Mva I; and *clpP&rps12*-Hpa II and -Rsa I.

As there was more than one species representative of 24 of the 48 British grass genera studied, it was possible to observe the level of the allele conservation among congeneric species. The genera *Glyceria, Festuca*, *Puccinellia*, *Agrostis*, and *Alopecurus* showed the highest level of CAPS markers

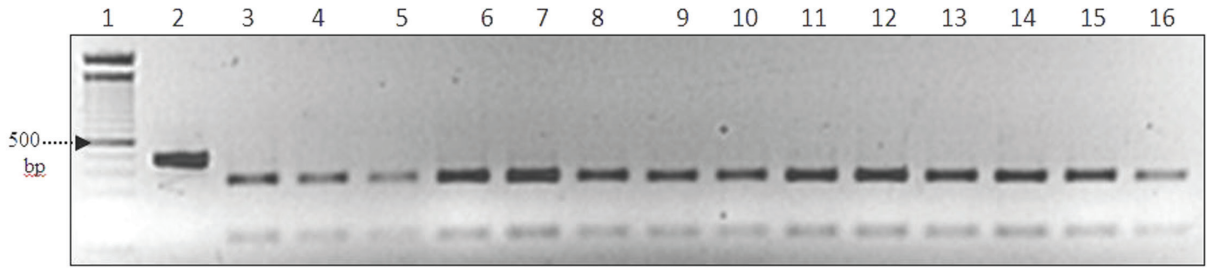

Figure 2. Restriction of locus *rpoA.2* amplicons with *Dde* I in 14 accessions of *L. perenne*. Lane 1, 100 bp DNA ladder; lane 2, template amplicon of locus *rpoA.2*; lanes 3-16, restriction products of 14 *L. perenne* accessions.

Table 9

CAPS markers proved useful for the diagnosis of species within three grass genera (*Calamagrostis, Phleum,* and *Agrostis)*

Genus/species	Useful CAPS markers
Calamagrostis epigejos, C. canescens, C. purpurea, C. stricta, and *C. scotica*	*ndhF.3-Rsa* I; *rps.4-Hind* II, -*Apa* I; *rpoA.1 -Ban* II; *clpP&rps12-Hpa* II
Phleum arenarium, Ph. bertolonii, Ph. alpinum, Ph. Phleoides, and *Ph. pratense* L. cultivar Latima	*rps.4-Hind* II; *rpoA.1-EcoR* V; *ndhF.1-Mva* I; *clpP&rps12-Hpa* II, -*Rsa* I
Agrostis gigantea, A. stolonifera, A. curtisii, A. canina, A. vinealis, and *A. capillaris*	*rps.4 - Apa* I, -*Hae* III; *rpoA.1-Ban* II; *rbcL.1-Ava* II; *ndhF.1-Mva* I; *clpP&rps12-Hpa* II

conservation among species belonging to each of those genera. In some cases, CAPS markers were conservative both within the genus or tribe and some others were polymorphic.

Screen for the interspecific variation of CAPS markers within three genera

The ability of CAPS markers screened to diagnose species within the same genus was screened for three genera (*Calamagrostis, Phleum,* and *Agrostis*). It was possible to differentiate the five species of *Calamagrostis* using only five enzymes for the restriction of amplification products of loci *ndhF, rps.4,* and *clpP&rps12*. As for *Phleum*, restriction of *rps.4, rpoA.1, ndhF.1,* and *clpP&rps12* amplification products with five enzymes allowed the diagnosis of its five species. Only six CAPS markers were useful for the diagnosis of species within *Agrostis (rps.4, rbcL.1, rpoA.1, rbcL.1, ndhF.1,* and *clpP&rps12)* (see Table 9).

Intraspecific variation of CAPS markers

Amplification of chloroplast loci in accessions and representative species collected from various geographical regions resulted in a single band of identical size within each species for all loci involved.

For four (*D. glomerata*, *Ph. pratense, M. caerulea,* and *L. perenne*) of the six species examined for intraspecific variation, no polymorphisms were observed among species representatives screened for any of the CAPS markers used for identification of corresponding species. One example is provided in Figure 2.

Polymorphism was observed, however, among representatives of *F. rubra* and *N. stricta*. Among the three representatives of *F. rubra,* accession numbers 1 and 2 (Table 7) possessed different restriction profiles from the other two *F. rubra* representatives in four of the six markers applied (Table 9). Figure 3 provides an example. The conserved CAPS markers were *rps4-Hind* II and *rbcL.1-Ava* II. As for *N. stricta*, polymorphism was observed in the CAPS *rps4-Ava* I combination, while the other two CAPS markers screened (*rbcL.1-Ava* II and *rpoA.1-Ban* II) were monomorphic between samples.

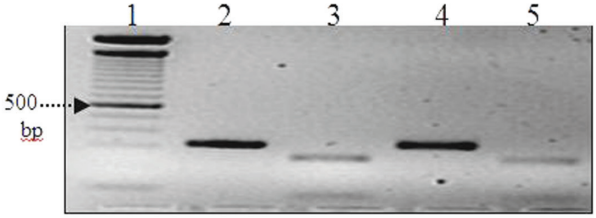

Figure 3. Restriction of locus *ndhF.1* with *Mva* I in three accessions of *F. rubra*. Lane 1, 100 bp DNA ladder; lane 2, template amplicon; lanes 3 – 5, restriction products of accessions numbered 1, 3 and 2 (Table 7).

DISCUSSION

As for gasses, molecular-based techniques have been employed mainly to evaluate genetic relationships and study the origin and diversity of species and genera. Haider (2015) referred to the few examples that have used molecular markers for grasses identification based on either nDNA (ISSR, SSR, RAPD, SCAR, and single-copy nuclear gene GBSS1) or cpDNA (*trnS-psbC*, *trnL*, *trnT-F,* and SSR) which has been proved to be the most suited for plant species identification (Ford *et al.* 2009). These studies either used markers that are less potentially species-specific such as those based on nDNA or non-coding cpDNA loci (e.g., Ridgway *et al.* 2003) or limited to certain genera (e.g., Parani *et al.* 2001).

The molecular method that can be effective for large scale identification of grass species, however, must be fast, reliable, and cost-effective high throughput method that reveals enough variability to distinguish species and at the same time generates highly conserved markers within species and implies specific amplification of DNA (Haider 2015). CAPS was proved useful for identification of several groups of plant species such as those of *Aegilops* L. and *T. aestivum* L. (Haider & Nabulsi 2008), *Orchidaceae* (Haider *et al.* 2010), and *Vicia* subgenus (*Fabaceae,* Haider *et al.* 2012).

For large scale identification of plant species using the CAPS technique, universal primers facilitate the amplification of a target region in all species to be identified. Therefore, the set of cpDNA-specific universal primers published by Haider (unpublished data; 2015) for grasses was used in this study. Interestingly, all products of the amplification of the same locus from different species were of identical size. This is consistent with the consensus view that

chloroplast genes are highly conserved. It was largely possible to generate locus-specific PCR products using the same conditions across the different primers used. This opens the way to the possibility of multiplex amplification using several sets of primer pairs in a single reaction mix.

In order to screen for variable CAPS markers that can allow the diagnosis of grasses, CAPS was applied on 21 chloroplast loci in 117 British native grass species. All complete restrictions generated two bands that were smaller in size than the unrestricted PCR product. The clarity in the distinction between the restricted and unrestricted alleles allowed rapid scoring of the restriction results on agarose gels and perhaps opens the way for future automation using existing gel documentation and analysis software.

Generated markers collectively revealed sufficient polymorphisms that are useful for the diagnosis of species used in the study. The utility of this finding for species diagnosis rests largely on the consistency of these markers within each of the species studied.

To determine the extent to which the recovered CAPS markers are species-specific, within-species conservation of CAPS markers that identify 6 of the grass species involved in this study was assessed on different representatives of each species. For 4 of the 6 species examined, the putative diagnostic CAPS markers were entirely conserved between members of the same species (Haider 2016). If this finding applied generally, it can be reasoned that these markers have huge potential for large-scale, rapid, simple, easy-to-perform, accurate, and low-cost identification of grass species.

The intraspecific variation in CAPS marker profiles detected in two species (*F. rubra* and *N. stricta*) warrants attention. Representatives of *N. stricta* displayed a restriction profiles that were very distinct from the majority of species of the *Pooideae* (Haider unpublished data). This genus has been a continual source of controversy to taxonomists and in some phylogenies (Kellogg & Campbell 1987; Davis & Soreng 1993) has been isolated from the rest of the Pooideae. Genera like *Nardus* represent either the earliest and most primary vestiges of evolutionary lines, which were successful in the past and are now almost extinct or the culmination of evolutionary trends that have been successful in only one specialised ecological environment (Stebbins 1956). Both scenarios could lead to enhanced variability at subspecific ranks. Intraspecific variation within the taxonomically problematic *F. rubra* based on sequences of *petB&D* was also observed by Haider (2016) who referred to reasons behind this variation.

Sanger sequencing (Haider 2016) and the more complicated pyrosequencing (Haider 2015) have been used recently for identification of grass species for which we present here the alternative CAPS technique that is much cheaper, faster, less complex, and utilises the technologies of ubiquitous PCR, digestion with endonucleases and agarose gel electrophoresis. When choosing among these three techniques, factors to consider include sensitivity, specificity, reproducibility, the limit of detection, turnaround time, ease of data interpretation, and cost (including instrument, reagent, etc.) (Tsiatis *et al.* 2010). Other advantages of CAPS include the involvement of PCR requiring only low quantities of template DNA, and the high reproducibility (Wagner & Ulrich-Merzenich 2013). Generated markers are mostly co-dominant and locus-specific, easily scored and interpreted, and easily shared between laboratories (Shavrukov 2016).

CAPS is, however, limited by mutations that create or disrupt a restriction enzyme recognition site. It is also very susceptible to contamination so precautions should be taken against possible contamination. As for the problem of possible undigested PCR fragments, Minarovič *et al.* (2010) mentioned that cleavage inhibition due to methylation cannot occur since PCR products do not possess methylated nucleotides which occur in plasmids and genomic DNA. Availability of universal primers and screen a large number of locus-enzymes combinations also overcomes the problems of needing sequence data to design PCR primers and the difficulty to find SNPs due to the limited size of the amplified fragments (270−600 bp in this study).

CONCLUSIONS

DNA barcoding provides a useful tool for larger-scale identification of plant species. The need for sequencing, however, prevents its use when fund-

ing or time is limited. For addressing this problem, we presented in this study a cpDNA-based CAPS system and illustrated its potential utility for establishing species identity using grasses as a model. Polymorphisms that can be detected among grasses using developed CAPS markers can be used for various applications such as cpDNA capture, species origin, determination of maternal parent for hybrid species, interspecific variation, interspecific and intergenic phylogenetic relationships, and forensic botany. These markers may also have ecological, conservation, and commercial applications. It is worth noting that cpDNA-CAPS markers developed here are not useful for phylogenetic studies of grasses at the individual level or the detection of infraspecific variation.

Due to the conservative nature of cpDNA in plants, variable locus-enzyme combinations that proved useful in this study for the diagnosis of British grasses may also have utility for diagnosis of grass species of other origins or species in other plant groups.

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