Pak. J. Bot., 55(2): 549-554, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-2(2)

A TRANSCRIPTOMIC APPROACH TO DEVELOP A NOVEL SET OF LOW-COPY NUCLEAR GENE PRIMERS FOR THE SAND WHIP GRASS, *PSAMMOCHLOA VILLOSA* (TRIN.) BOR (POACEAE), A DOMINANT SPECIES FROM THE INNER MONGOLIA PLATEAU

TING LV[1], YU-PING LIU[2], TAO LIU[1], GUI FU[1], ZI-LAN MA[2], CHANG-YUAN ZHENG[2] AND XU SU[1,2,3,4*]

[1]*School of Geosciences, Qinghai Normal University, Xining 810008, P. R. China*
[2]*Key Laboratory of Medicinal Animal and Plant Resources of the Qinghai-Tibet Plateau in Qinghai Province, School of Life Science, Qinghai Normal University, Xining 810008, P. R. China*
[3]*Academy of Plateau Science and Sustainability, Xining 810016, P. R. China*
[4]*Key Laboratory of Education Ministry of Earth Surface Processes and Ecological Conservation of the Qinghai-Tibet Plateau, Qinghai Normal University, Xining 810008, P. R. China*
[*]*Corresponding author's email: xusu8527972@126.com*

Abstract

The sand whip grass, *Psammochloa villosa* (Trin.) Bor, is a species found in the Inner Mongolian Plateau and neighboring areas with economic and ecologic importance due to its abundance in sand dunes. Its tolerance to dry environments makes of this grass a species of interest for a potential source of genetic material to improve crops or as a forage plant for local livestock. Here, we designed a collection of primers suitable to amplify genes with the capacity to assess the genetic variability among populations of *P. villosa*. The primers were designed from a randomly selected subset of 72 low-copy nuclear genes retrieved from 300 orthologs amplified from transcriptomes. We tested the performance of the 10 primers using 15 individuals from five wild populations. The primers were able to amplify ten low-copy genes, whose aligned sequences ranged in length from 716 to 1221 base pairs. We used the resulting data to measure diversity of haplotypes (*H*d) and nucleotides (π), which varied from 0.402 to 0.979, and from 0.00064 to 0.006540, respectively. Meanwhile, a phylogenetic analysis showed topological consistency between the topologies of trees constructed using ML and BI methods, placing the genus *Achnatherum* as sister to *Psammochloa*. Our work demonstrated the success of these primer pairs amplifying low-copy nuclear genes in *P. villosa*, and their potential to perform large-scale studies assessing the genetic variation and evolution of this species. These primers may be also useful to conduct molecular studies of closely related taxa such as the numerous and economically important feather grasses in the genus *Stipa* L.

Key words: Haplotype diversity, Phylogenetic relationship, Population genetics, *Stipa*, Psammophyte.

Introduction

Since the last decade, low-copy nuclear genes have been proved to be useful in plants to resolve molecular phylogenies and to enrich population-level studies (Soltis *et al*., 1999; Mort & Crawford, 2004; Li *et al*., 2019; Bratzel *et al*., 2020). Recent efforts for sequencing low copy genes have relied on PCR techniques using primers designed from genes retrieved from transcriptomes or other next generation sequencing approaches (e.g., Zeng *et al*., 2014; Fu *et al*., 2016). The ability to amplify selected nuclear genes represent an advance in the field, as researchers are now able to incorporate nuclear genes in evolutionary studies with non-model organisms without being restricted by complex data processing or high sequencing costs (Zhang *et al*., 2012; Liu *et al*., 2015; Waselkov *et al*., 2018; Montes *et al*., 2019).

Here, we used transcriptome sequences to design primers targeting a series of low-copy nuclear genes in the sand whip grass *Psammochloa villosa* (Trin.) Bor (Poaceae). This rhizomatous grass is endemic to the open dessert areas of the Inner Mongolian Plateau and neighboring areas (Liu, 1985; Ma, 1994) and has economic and ecologic importance due to its abundance in sand dunes. *Psammochloa villosa* is an early colonizer of desert lands and has been demonstrated to be a crucial element to the natural succession in these habitats (Dong & Alaten, 1999; Lv *et al*., 2018). Its tolerance to dry environments makes of *P. villosa* a species of interest for a potential source of genetic material to improve grass crops in dry areas or as a forage plant for livestock. Thus, we believe that it is important to improve our knowledge on the genetic structure among populations of *P. villosa* through developing a robust set of custom genomic resources for the species such as low-copy nuclear genes. Besides increasing our learning on the genetic aspects for economical purposes, we are interested to understand about the history of this species to elucidate the timing and mechanisms of the formation of deserts in northwestern China. The creation of a collection of low-copy nuclear genes specifically designed for *P. villosa* would represent one of the first efforts of this kind for a clonal species of grass.

Materials and Methods

RNA extraction and sequencing: In the present study, we collected fresh leaves of *P. villosa* without evidence of disease or insect pests from naturally occurring plants in Minqin County of Gansu Province (39.17°N, 103.65°E; alt. 1250 m). Our sampling comprised three individuals each from five populations for a total of 15 individuals. From each population, we collected voucher specimens that were deposited in the Herbarium of the Northwest Plateau (HNWP), Institute of Biology at the Chinese Academy of Sciences in Xining, China (Table S1). Fresh leaves were preserved in liquid nitrogen in the field, and stored at -80°C upon arrival to the laboratory facilities.

Table S1. Localities and herbarium specimens representing each of the populations of *P. villosa* sampled in this study.

Population	Location	Latitude (N)	Longitude (E)	Altitude (m)	N	Voucher
1	Zhongwei City, NX	37°25′29.1"	104°40′5.00"	1707	3	X. Su, 16, 001
2	Hanggin Banner, NMG	40°01′59.2"	108°28′34.6"	1236	3	X. Su, 16, 021
3	Sonid Left Banner, NMG	43°40′36.5"	113°26′57.9"	1010	3	X. Su, 16, 043
4	Liangzhou District, GS	38°13′0.00"	103°18′0.00"	1459	3	X. Su, 18, 004
5	Otog Banner, NMG	38°53′0.00"	108°18′0.00"	1371	3	X. Su, 18, 019

Note: NX, Ningxia; NMG, Neimenggu; SX, Shaanxi. GS, Gansu. N = number of individuals sampled

We extracted total RNA from frozen leaves using a modified cetyltrimethylammonium bromide (CTAB) procedure (Ghangal *et al*., 2009). In order to evaluate the quality of total RNA, we performed automated microchannel electrophoresis on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA) and spectrophotometry on a NanoDrop-2000 (Thermo Fisher Scientific). We purified samples to remove poly(A)-tags using approximately 5 μg of RNAs and (dT)-conjugated beads (Life Technologies, Carlsbad, California, USA) and broke apart purified RNAs into fragments of 200 bp using divalent cations at a temperature of 75°C. Based on the RNAs, the first strand of cDNA was synthesized with a reverse transcriptase and random hexamer primers, while the second strand was synthesized using RNase H (Invitrogen, Ghent, Belgium) and Taq DNA polymerase I (New England BioLabs, Ipswich, MA, USA). Total genomic libraries were generated and sequenced on a single multiplex channel of the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, California, USA).

Transcriptome assembly and primer design: Raw reads were post-processed to remove adapters, filtered, and assembled into unigenes using the Trinity assembler, with default parameters (Grabherr *et al*., 2011). Based on the unigenes, we identified orthologous genes by mapping the reads of the needlegrass *Achnatherum splendens* (Trin.) Nevski (SRP068186, Liu *et al*., 2016) in Bowtie 2 (Langmead & Salzberg, 2012).

Based on orthologous unigene sequences, we detected low-copy nuclear genes using Microsatellite (MISA), which is a Perl script developed by Thiel *et al*., (2003) and available at http://pgrc.ipk-gatersleben.de/misa. Due to the introduction of multiple indels during *de novo* assembly, we deleted the ones shared by two orthologous unigenes and retained only sequences with indels at the 3' and/or 5' end of alignments. We used the resulting, vetted unigene sequences as candidates for primer design using the default settings in Primers 5 (Rozen & Skaletsky, 2000) for 72 randomly selected unigenes.

Testing developed primers: We tested ten primers (Table 1) on total genomic DNA extracted 15 individuals from five populations of *P. villosa* (voucher information provided in Table S1) using a CTAB method (Doyle & Doyle, 1987). We used the primers for amplification in standard PCR reactions comprising a 25 μL total volume, and containing 0.25 μL of *Taq* DNA polymerase (5 U·μL^{-1}; TaKaRa, Dalian, China), 2.5 μL of 10 × PCR buffer, 2 mM dNTPs, 1 μM of each primer, 2 μL template DNA, and distilled water up to the final volume. We performed a general PCR amplification program under the following thermocycling protocol: enzyme activations for 5 min at 94ºC; 37 cycles consisting of 50 s of denaturation at 94ºC, annealing at 52-64ºC for 1 min, and 1 min extension at 72ºC; and a final extension of 10 min at 72ºC. Reactions were verified using a 1% (w/v) agarose gel, confirming that all of the ten primers were able to successfully amplify target genes in *P. villosa*.

Data analysis: Multiple sequence alignments were performed using Clustal X (Thompson *et al*., 1997) and visualized in MEGA 5 (Tamura *et al*., 2011), with minor refinements applied when needed. The parameters of the ten targeted genes were analyzed with DnaSP V5.10.1 (Librado & Rozas, 2009) and include Tajima *D* test, haplotype diversity (*H*d), Fu and Li's *D** test statistic, number of segregating sites (*S*), average number of nucleotide differences (*K*), nucleotide diversity (π), and Fu and Li's *F** test statistic.

Sequences of species related to *P. villosa* were retrieved from the GenBank data repository available at http://www.ncbi.nlm.nih.gov/genbank/. All accession numbers for sequences analyzed in this study are listed in (Table S2). The phylogenetic tree containing both the ten pairs of low-copy nuclear genes and sequences from related species was constructed using a Bayesian Inference (BI) as well as a Maximum Likelihood (ML) method. The BI analysis was performed with the MrBayes V3.2.1 software (Ronquist & Huelseneck, 2003), while ML phylogenetic analysis was done in PhyloSuite (Zhang *et al*., 2020). Bootstrapping for the ML tree was done using 1000 replicates with the above-mentioned settings. The best-fitting evolutionary model for the BI analysis from ten genes was simulated by jModelTest V2.1.1 (David, 2008). The length for the Markov Chain Monte Carlo (MCMC) chains was set to 10,000,000, while the sampling frequency was 1000. We visualized the phylogenetic trees with FigTree V1.3.1 (Rambaut, 2009).

Table S2. Accession numbers from GenBank for the outgroup species of Poaceae analyzed using theten putative low-copy nuclear markers developed in this study.

Species	Y30	DHA	TOM	DAA	PP1	PP27	PP26	PP24	RGA	ORN
Achnatherum splendens	SRP068186	SRP068186	SRP068186	SRP068186	SRP068186	SRP068186	SRP068186	SRP068186	SRP068186	SRP068186
Aegilops tauschii	XM020316758.1	XM020341014.1	XM020300432.1	XM020314216.1	XM020324884.1	XM020291945.1	XM020342604.1	XM020329950.1	XM020310945.1	XM020342391.1
Brachypodiumdistachyon	XM003577557.4	XM003567240.4	XM037568004.1	XM003561910.4	XM024463548.1	XM010229035.3	XM003567220.4	XM010229602.3	XM010232922.3	XM003561785.4
Oryza minuta	-	-	-	-	-	HQ827835.1	-	-	-	-
Oryza brachyantha	XM006664766.2	-	XM015835880.1	XM006651054.2	XM006660355.1	-	XM006646416.2	XM015837998.1	-	XM015835386.1
Oryza sativa Indica	-	-	XM020300432.1	-	-	-	-	-	-	-
Oryza sativa Japonica	XM015760201.2	-	AK100276.1	XM015776719.2	XM015756621.2	XM015779451.1	XM015766718.2	XM015788593.2	XM015785220.2	XM015776575.2
Panicum hallii	XM025970902.1	-	-	XM025940566.1	XM025948592.1	XM025967338.1	XM025959214.1	XM025956990.1	XM025950250.1	XM025938440.1
Setaria italica	XM012848613.3	-	XM004970765.3	XM004985341.3	-	XM022824923.1	XM004972193.4	XM012845641.2	XM004961598.4	XM022823473.1
Setaria viridis	XM034713644.1	-	-	XM034719828.1	-	XM034729330.1	-	XM034734063.1	-	-
Sorghum bicolor	XM021461030.1	-	XM002458832.2	XM002465681.2	XM002461994.2	XM021462837.1	XM002458716.2	XM021449322.1	XM002439951.2	XM021447627.1
Triticum aestivum	AK336152.1	AK332720.1	-	AK447489.1	-	-	-	-	-	-
Triticum dicoccoides	XM037573403.1	XM037568004.1	XM037559295.1	XM037570529.1	XM037584723.1	XM037632036.1	XM037558881.1	XM037609838.1	XM037563926.1	XM037580080.1
Hordeum vulgare	-	-	AK357186.1	-	-	-	-	-	AF032682.1	AK371642.1
Phyllostachys edulis	-	-	FP095533.1	-	-	-	-	-	-	-
Saccharum hybrid	-	-	-	-	MF737053.1	-	-	-	KF184970.1	-
Avena strigosa	-	-	-	-	-	-	-	-	FJ829747.1	-

Results and Discussion

We detected a total of 300 putative low-copy nuclear genes in *P. villosa* based on the well-annotated genome of *A. splendens*, which is a close relative of *P. villosa* (Zhang *et al*., 2017). We randomly selected 72 of the low-copy genes to design primers, and tested them using 15 individuals from five natural populations of *P. villosa*. We successfully amplified ten pairs of primers which produced a single, well-defined target band.

Amplification and sequencing of ten target genes yielded products that ranged in length from 716 to 1221 aligned bp comprising all successfully amplified individuals. Among these alignments, we observed 2 - 24 parsimony informative sites (Tables 1-2). We inferred that these ten amplified markers represented four to 23 haplotypes, haplotype diversity (*H*d) ranged from 0.402 to 0.979, while the diversity of nucleotides (π) varied from 0.00064 to 0.00654 (Table 2). These results indicated that this species exhibited high genetic diversity as reported in Lv *et al*., (2021). Moreover, we found that D^*, F^* and Tajima *D* for most of the ten low-copy nuclear genes were positive but not significant, which suggested that this species have not experienced significant population expansions.

To test the phylogenetic signal produced by the ten low copy genes amplified, we build individual trees for each of them (Fig. 1). The topology of trees was largely consistent with the consensus phylogeny of Poaceae inferred from previous studies, indicating that these genes were likely orthologous without conflicting signals. A model test performed for the multiple sequence alignments from each of the ten genes suggested that HKY+F+G4 was the best-fitting evolutionary model for five of the genes (PP27, PP26, PP24, PP1, and DAA), whereas K2P + G4 were the selected models for genes Y30 and TOM. Meanwhile, model GTR + F + G4 was the best fit for genes RGA and ORN, and model K2P + I for the DHA gene. After comparing the resultant topologies from trees built using the ten individual genes, we did not found any major discrepancies neither in the ML nor the BI analyses (Fig. 1).

The phylogenetic trees suggested that all species from subfamily Pooideae – including *A. splendens* and *P. villosa* – clustered together with a well-supported value, while the remaining outgroups were clustered into other subfamilies (Oryzoideae and Panicoideae). Therefore, the candidate genes selected in this study show concordant phylogenetic signals with the rest of samples, suggesting that they might have evolved under similar evolutionary scenarios. More importantly, we found that *P. villosa* and *A. splendens* were clustered into one clade with high support values, which indicated that it was reliable to screen low-copy nuclear genes of *A. splendens* as the reference species.

In summary, this report represents the first set of primers developed for low-copy nuclear genes in *P. villosa* using transcriptomic data. These primers are a valuable tool to facilitate future studies of evolution and demographics of *P. villosa*, which is an ecologically important and regionally dominant species in the dessert habitats of northwestern China.

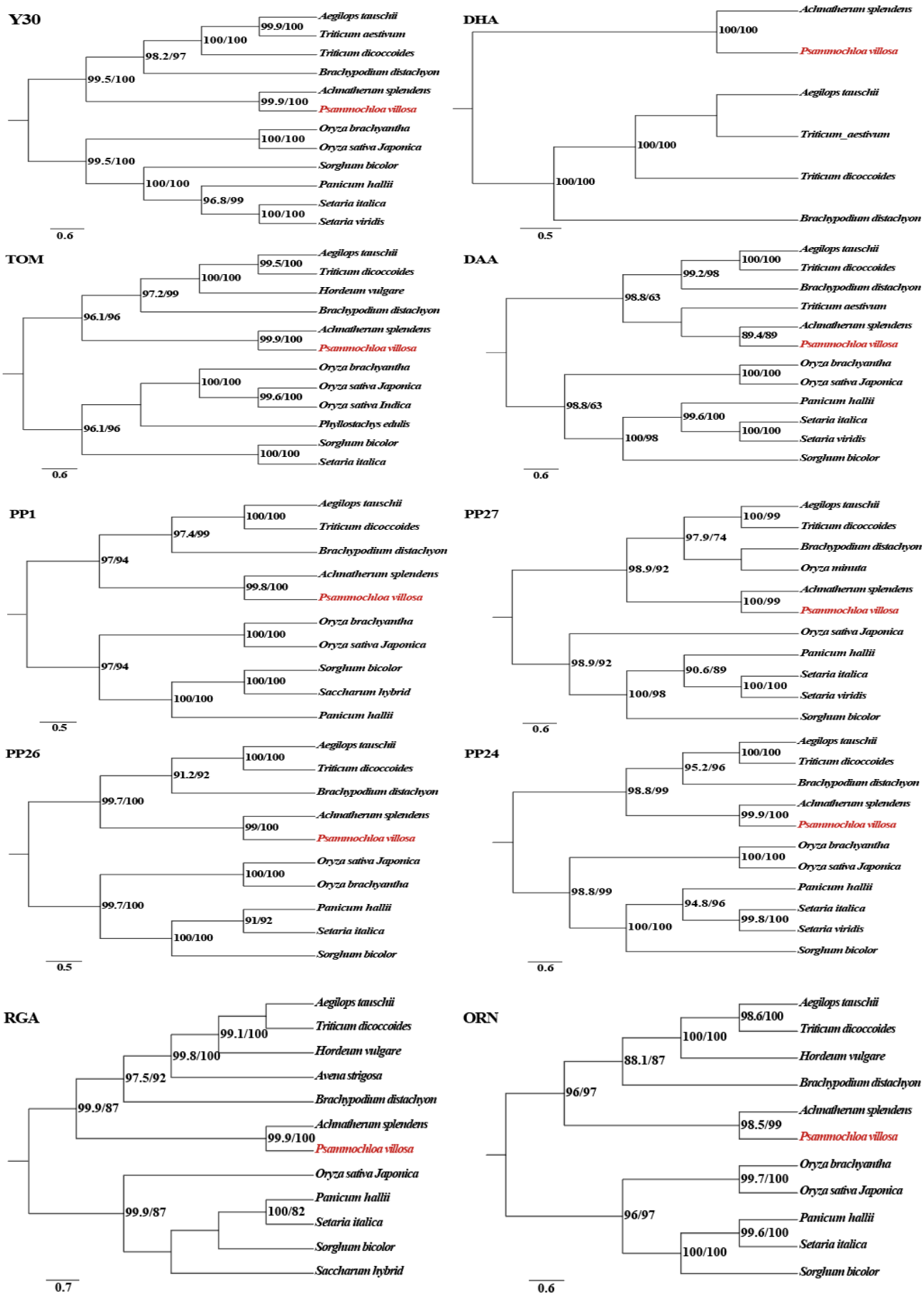

Fig. 1. Phylogenetic analyses of the low-copy nuclear markers for *P. villosa*. Numbers close to nodes indicate ML bootstrap values (LB). Bootstrap values under 70% are not shown.

Table 1. Characteristics of ten novel low-copy nuclear gene primers developed in P. villosa.

Locus	Primer Name	Primer sequences (5'-3')	AFL (bp)	LCR (bp)	Ta (°C)	Putative product
14031	Y30	F: TTCAGTGGCTATCCTGTTCC R: CTGCACGTTAAACTACTCACAAG	716	716	57.1	Probable inactive leucine-rich repeat receptor-like protein kinase At3g03770
14004	DHA	F: AGGTGAGGTGGGATTTGAGC R: AAGTGGGCGGATGGTTTC	736	736	61.5	Glycerol metabolism operon regulatory protein
13593	TOM	F: CCAGCTTAGTATGGCAGAGG R: CCACGCTTTGTTGATGTTTC	772	772	58.8	Tobamovirus multiplication protein 3
14111	DAA	F: CCTAACGGAAACGAGTGCC R: CCTCATCAGTTCCCGCAGAC	1221	1217	61.6	Dynein assembly factor 1, axonemal
14192	PP1	F: TGTCGGTAACCTGGATAGAGC R: TGATTAACTCCCTGACTGCTTC	815	807	59.1	Pentatricopeptide repeat-containing protein At1g79540
14928	PP27	F: GAGCGGTTTGTCCCTGAT R: GTCCAGCTCTAGTGCCTGTT	921	918	58.4	Pentatricopeptide repeat-containing protein At3g50420
14224	PP26	F: GCACTCAGTCACGGCAAAC R: AGCAACAGCCAGCTTCTCAC	891	884	60.9	Pentatricopeptide repeat-containing protein At3g46790, chloroplastic
14612	PP24	F: GATGCAGTGGCAGTGTTTG R: CTCTTACCGTCAGGCTCGT	887	887	57.1	Pentatricopeptide repeat-containing protein At3g18110, chloroplastic
14075	RGA	F: AGTTGCCAGTGGGTTTG R: CGAGCACATGGAGGTATCTT	1001	996	55.7	Disease resistance protein RGA2
13604	ORN	F: TCGCATTCCTCTTTCTCACCAAC R: CTATTCGACTTCCTCAGCTCCCT	742	735	60.7	Oligoribonuclease

Note: T_a = optimal annealing temperature, AFL: Aligned full length, LCR: Length of coding region

Table 2. DNA polymorphism data based on ten pairs of low-copy nuclear gene primers.

Locus	Primer name	*S*	*H*	π	H_d	D^*	F^*	Tajima's *D*
14031	Y30	5	7	0.00211	0.823	1.214	0.988	-0.01220
14004	DHA	5	8	0.00207	0.848	1.144	1.130	0.56146
13593	TOM	3	5	0.00165	0.756	0.950	1.318	1.60451
14111	DAA	24	15	0.00654	0.906	1.410*	1.547	1.11279
14192	PP1	2	4	0.00064	0.402	0.806	0.684	0.05032
14928	PP27	8	13	0.00197	0.869	0.690	0.447	-0.32308
14224	PP26	13	23	0.00550	0.979	1.497*	1.788*	1.59065
14612	PP24	7	12	0.00247	0.885	1.273	1.288	0.71262
14075	RGA	5	5	0.00199	0.828	1.144	1.475	1.56524
13604	ORN	4	9	0.00239	0.903	1.058	1.515	1.90455

Note: S, Number of polymorphic sites; *H*, Number of haplotypes; π, Nucleotide diversity; H_d, Haplotype diversity; D^*, Fu and Li's D^* test statistic; F^*, Fu and Li's F^* test statistic. Statistical significance: *, $p<0.05$; **, $p<0.01$

Acknowledgements

We are grateful to Dr. Wenchun Luo whose help to analyze an earlier data of our manuscript, and we greatly appreciated Prof. AJ Harris helping to improve an earlier draft of our manuscript. Funding agencies include the National Natural Science Foundation of China (grant # 32160297, 41761009), and the General Program of the Natural Science Foundation of Qinghai Province (grant #2022-ZJ-918).

References

Bratzel, F., S. Heller, N. Cyrannek, J. Paule and G. Zizka. 2020. The low-copy nuclear gene *Agt1* as a novel DNA barcoding marker for Bromeliaceae. *BMC. Plant Biol.*, 20(1): 111.

David, P. 2008. jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.*, 25(7): 1253-256.

Dong, M. and B. Alaten. 1999. Clonal plasticity in response to rhizome severing and heterogeneous resource supply in the rhizomatous grass *Psammochloa villosa* in an Inner Mongolian dune, China. *Plant Ecol.*, 141: 53-58.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochem. Bull.*, 19: 11-15.

Fu, Y., C.Z. He, M.M. Ju and B. Tian. 2016. The development of low-copy nuclear gene primers based on transcriptome for *Arnebia szechenyi*. *J. South. Fores. Univ.*, 36(3): 86-90.

Ghangal, R., S. Raghuvanshi and P.C. Sharma. 2009. Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites. *Plant Physiol. Bioch.*, 47(11): 1113-1115.

Grabherr, M.G., B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q.D. Zeng, Z.H. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F.D. Palma, B.W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman and A. Regev. 2011. Full-

length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.*, 29: 644-652.

Langmead, B. and S. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, 9: 357-359.

Li, X., T. Feng, C. Randle and G.M. Schneeweiss. 2019. Phylogenetic relationships in Orobanchaceae inferred from low-copy nuclear genes: consolidation of major clades and identification of a novel position of the non-photosynthetic *Orobanche* clade sister to all other parasitic Orobanchaceae. *Front. Plant Sci.*, 10: 902.

Librado, P. and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25(11): 1451-1452.

Liu, J.T., Y.L. Zhou, C.X. Luo, Y. Xiang and L.Z. An. 2016. *De novo* transcriptome sequencing of desert herbaceous *Achnatherum splendens* (Achnatherum) seedlings and identification of salt tolerance genes. *Genes*, 7(4): 12.

Liu, M., C.F. Zhang, J.X. Huang and H. Ma. 2015. Phylogenetic reconstruction of tribal relationships in Asteroideae (Asteraceae) with low-copy nuclear genes. *Chinese Bull. Bot.*, 50(5): 549-564.

Liu, Y.X. 1985. *Desert Flora of China*. Beijing: Science Press, 1: 34.

Lv, T., A.J. Harris, T. Liu, R.F. Liang, Z.L. Ma, Y.P. Liu and X. Su. 2021. Population genetic structure and evolutionary history of *Psammochloa villosa* (Trin.) Bor (Poaceae) revealed by AFLP marker. *Ecol. Evol*., 11(15): 10258-10276.

Lv, T., Y.P. Liu, Y.H. Zhou, T. Liu, X.Y. Zhang and X. Su. 2018. Germplasm collection and preliminary studies on genealogical differentiation of a desert species-*Psammochloa villosa*. *Acta Agrestia Sin.*, 26(3): 733-740.

Ma, Y.Q. 1994. *Flora of Inner Mongolia*. Hohhot: Inner Mongolia People's Publishing House, 5: 115-152.

Montes, J.R., P. Peláez, A. Willyard, A. Moreno-Letelier, D. Piñero and D.S. Gernandt. 2019. Phylogenetics of *Pinus* subsection *Cembroides* Engelm. (Pinaceae) inferred from low-copy nuclear gene sequences. *Syst. Bot.*, 44(3): 501-518.

Mort, M.E. and D.J. Crawford. 2004. The continuing search: low-copy nuclear sequences for lower-level plant molecular phylogenetic studies. *Taxon*, 53: 257-261.

Rambaut, A. 2009. FigTree v1.4: Tree Figure Drawing Tool. Available: http://tree.bio.ed.ac.uk/software/figtree/.

Ronquist, F. and J.P. Huelseneck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19(12): 1572-1574.

Rozen, S. and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers, 365-386. In Misener S and Krawetz SA, Methods in molecular biology. Bioinformatics methods and protocols, vol. 132.

Soltis, P.S., D.E. Soltis and M.W. Chase. 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature*, 402: 402-404.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28(10): 2731-2739.

Thiel, T., W. Michalek, R.K. Varshney and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, 106: 411-422.

Thompson, J.D., T.J. Gibson and F. Plewniak. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25(24): 4876-4882.

Waselkov, K.E., A.S. Boleda and K.M. Olsen. 2018. A phylogeny of the genus *Amaranthus* (Amaranthaceae) based on several low-copy nuclear loci and chloroplast regions. *Syst. Bot.*, 43(2): 439-458.

Zeng, L.P., Q. Zhang, R.R. Sun, H.Z. Kong, N. Zhang and H. Ma. 2014. Resolution of deep angiosperm phylogeny using conserved nuclear genes and estimates of early divergence times. *Nat. Commun.*, 5: 4956.

Zhang, D., F.L. Gao, L. Jakovlić, H. Zou, J. Zhang, W.X. Li and G.T. Wang. 2020. PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. *Mol. Ecol. Resour.*, 20: 348-355.

Zhang, N., L.P. Zeng, H.Y. Shan and H. Ma. 2012. Highly conserved low-copy nuclear genes as effective markers for phylogenetic analyses in angiosperms. *New Phytol.*, 195(4): 923-937.

Zhang, Z.S., L.L. Li and W.L. Chen. 2017. *Ptilagrostis contracta* (Stipeae, Poaceae), a new species endemic to Qinghai-Tibet Plateau. *PLoS One*, 12(1): e0166603.

(Received for publication 26 April 2021)