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Cross-Specific Amplification of SSR Markers in *Dalbergia latifolia* and *Dalbergia sissoo* - A Case for Hybrid Seed Production

Asif Javed Muhammad[1,2,*], Izhar-Ul-Haq[1,2], Nauman Ghafoor[1,2], Fazal-Ur-Rehman[1,2], Atif Ali[1] and Zia Bilal Muhammad[3]

[1]*Department of Forestry and Range Management, Faculty of Agriculture, University of Agriculture Faisalabad, Faisalabad 38000, Pakistan*

[2]*Department of Centre for Advanced Studies, Agriculture Food Security (CAS-AFS), University of Agriculture Faisalabad, Faisalabad 38000, Pakistan*

[3]*Division of Genetics, Pakistan Forest Institute, Peshawar 25130, Pakistan*

Abstract

Dalbergia sissoo and *Dalbergia latifolia* are the two most important timber wood species that are indigenous to the Indo-Pak subcontinent producing very high-quality timber. Its wood is used to produce high-quality furniture. Due to overexploitation and dieback disease, their production is seriously hampered and threatens their genetic diversity. Several ecotypes are tolerant to dieback thus offering an opportunity to develop a superior hybrid for the establishment of plantations for hybrid seed production. Hybrid evaluation can only be done by using DNA markers such as SSR markers. Cross-specific amplification of SSR markers is a cost-effective way of producing DNA markers for species lacking genetic information. Here, we report for the first-time successful cross-amplification of SSR markers in *Dalbergia latifolia* and added new SSR markers in *D. sissoo*. Cross-species amplification resulted in 13 successful SSR markers in *D. latifolia* and an addition of 14 markers in *D. sissoo* of expected sizes. Six SSR markers were further selected randomly to validate the breeding behavior of both species. A diverse DNA profile of seed progenies matched to different pollen donors deviated from the same mother suggested cross-pollination is the most likely mechanism of seed production in *D. sissoo* and *D. latifolia*. However, the results must be validated by using a large sample size and through controlled pollinations. SSR markers thus developed will be useful in the conservation and development of superior hybrids for sustainable development and production of commercial populations in *Dalbergia sissoo* and *D. latifolia*.

Key Words: *Dalbergia sissoo, Dalbergia latifolia*, SSR markers, breeding behavior, hybrid seed production

Introduction

The Dalbergia genus is a diverse group of 250 species that are distributed across various regions of the world, including Asia, Central and South America, Africa, and Madagascar (Kile 1993; Hartvig et al. 2017). In Pakistan, *Dalbergia sissoo* holds great significance as a native tree that is frequently encountered in the Himalayan subtropical valleys. The tree is commonly referred to as "shisham or talli" in the local area. The tree is widely distributed throughout Pakistan, thriving in various ecological regions, and cultivated for its versatile uses. The Dalbergia genus is re-

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Corresponding author: Asif Javed Muhammad
Department of Forestry and Range Management, Faculty of Agriculture, University of Agriculture Faisalabad, Faisalabad 38000, Pakistan
Tel: +92-419200161, E-mail: azurefromheavens@gmail.com

nowned for its robust and long-lasting wood, it boasts of its color, aroma, texture, and grain (Hartvig et al. 2017). It is frequently utilized as a species in agroforestry, and its wood possesses a significant calorific value. Nevertheless, the primary application of this species lies within the furniture industry, where its furniture is renowned globally for its exceptional durability and ability to withstand various environmental pressures (Sheikh 1989).

The species was introduced from the subtropical regions of the Himalayas to other parts of Pakistan such as semi-arid regions in 1886 mainly for wood fuel (Sheikh 1989). The species was seriously damaged and destroyed by shisham dieback in 1998 mainly in the newly introduced areas (Muhammad et al. 2023). The cause of dieback remained elusive until recently (Muhammad et al. 2023). This caused a serious shortage of raw materials for the allied industries. To replace the affected plantations of *D. sissoo* and to provide wood to the local industry, *Dalbergia latifolia* was introduced from Nepal. However, *D. latifolia* performed poorly showing a slow growth rate and low biomass in the local trials. A recent survey of native populations of the Himalayan region revealed a complete absence of the shisham dieback (Muhammad et al. 2023). Similarly, sissoo plantations in the Sindh province of Pakistan showed better tolerance to dieback as compared to northern populations (Muhammad et al. 2023). Most of the sissoo plants showed inferior traits such as multi-stemmed, crooked bole, and low biomass. Therefore, the new plantations must be established with improved and genetically superior clonal hybrids. The breeding behavior of the species is not clear but is considered intermediate i.e., self, and cross-pollinated (Tewari et al. 2002; Chauhan et al. 2004). Breeding dieback tolerant shisham genotypes or the production of the hybrid seed from the tolerant genotypes is the most plausible long-term solution to manage shisham plantations in Pakistan. Unfortunately, the pace at which genetic information has been gathered on tree species in Pakistan lags behind the rapid deforestation caused by human activities (Sodhi et al. 2004). Traditionally, tree breeding has been a challenging and time-consuming process, primarily because of the complexities involved in hybridization and phenotyping. Nevertheless, thanks to the presence of molecular markers, hybrids can be easily identified within a breeding program.

Microsatellites are widely regarded as the preferred genetic marker system. Furthermore, their remarkable polymorphism, the coexistence of alleles, their consistent reproducibility, wide distribution throughout the genome, and the convenience of screening through a rapid PCR test make them highly suitable for high throughput genotyping (Shekhar et al. 2021). SSR markers have been widely employed in various research areas, such as population genetic studies, linkage mapping, and hybrid identification in tree species (Muhammad et al. 2016). However, the widespread use of microsatellites is hindered by the requirement to create distinct markers for the focal species (Zane et al. 2002; Muhammad et al. 2016). Therefore, the utilization of SSR loci across closely related species groups (Muhammad et al. 2016) could significantly expedite the acquisition of marker data to conserve and manage taxonomically similar species.

Existing studies indicate that the microsatellite loci of Dalbergia are expected to be conserved throughout the genus (Favreau et al. 2007; Ribeiro et al. 2011; Liu et al. 2019; Li et al. 2021). Several SSR markers were created in species such as *Dalbergia oliveri*, *Dalbergia nigra*, and *Dalbergia monticola,* utilizing both genomic and EST resources (Favreau et al. 2007; Ribeiro et al. 2011; Liu et al. 2019; Li et al. 2021). The cross-amplification using SSR markers was successful in various species, except *D.*

Table 1. Dalbergia species names and geographical distribution

Species	Distribution	References
Dalbergia sissoo	Afghanistan, Pakistan, India, Nepal	Lakhey et al. 2020
Dalbergia latifolia	South - East India	Sukhadiya et al. 2020
Dalbergia oliveri	Myanmar, Thailand, Laos, Cambodia, Vietnam	Hung et al. 2020
Dalbergia nigra	Brazil	de Carvalho 1997
Dalbergia monticola	Madagascar	Du Puy et al. 2002

Table 2. SSR loci in *D. sissoo* and *D. latifolia*, forward and reverse primer sequences, allele sizes, and optimum primer annealing temperatures

Sr. no.	Primer sequences	Repeat motif	Cross amplification						Source species	References
			Annealing g temp	Allele size (bp)	*Dalbergia latifolia*	Annealing g temp	Allele size (bp)	*Dalbergia sissoo*		
Oli-1	F: AAATAAAACTTATATGTTGCCAAACTC R: TGGTACTAAGTTCCTGGACAACA	(CA)15	56.7	125-130	Yes	56.7	120-140	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-2	F: GGATCAATTGGAGCCATACG R: TTTGCGACTCTCGTGTAGTGA	(AG)16	60.6	130-145	Yes	56.7	135-140	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-3	F: ACTGGTAGGCCGACTTAACG R: AAAATAGGCCATCATTAGTTTGC	(TG)16	56.7	110-120	Yes	56.7	110-120	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-4	F: TGAAATAAACCTAGCATCGACA R: TGTTAGCATGAAGACAGGGTT	(TG)17	56.7	180-190	Yes	56.7	185-190	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-5	F: TTCATCAATGGTGGAGTTGG R: CTGGCCAACCAGAATGATCT	(CT)17	56.7	240-250	Yes	56.7	245-250	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-6	F: TAGAGGAGGGAAAGGAAGGG R: AGCTGCCCTTCAGTTACAGTAT	(AC)17	60.6	195-205	Yes	56.7	190-200	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-7	F: ATCATTCCATGTGGCTTCCA R: TTTGATACTGATCTAACCACTGTCTT	(TG)18	56.7	180-195	Yes	56.7	185-195	Yes	*Dalbergia oliveri*	Hartvig et al. (2017)
Oli-8	F: TTGATCATTACCCTTATTAGCCTT R: CAGGAAGAAATTACAACCCCA	(CTT)18	56.7	100-110	Yes	-	110-120	No	*Dalbergia oliveri*	Hartvig et al. (2017)
Dnig-22	F: TTCTCTTGGTTGAGGCTCGT R: CGGTCTCCTCTTGCTATCCA	(GA)8(GT)2(GA)8(GT)14	-	-	No	56.7	160-170	Yes	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig-23	F: CAAGAGCTCGTCGAAATGTG R: AACTGCATGATTATATCGCTATTGA	(TA)5TGT(AG)9	-	-	No	56.7	180-190	Yes	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig24	F: CTGAATTGCCGAGACGAAGT R: GCTTTACGCTATTTAGCCCTCA	(AT)10(AG)19	60.6	175-180	Yes	56.7	190-195	Yes	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig25	F: TTCCTTCATTCCACTCTATTTCAA R:TCAATTCTAATATCTCAAAATCAGTCA	(TA)8	60.6	170-175	Yes	56.7	195-205	Yes	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig26	F: GACCAAAGGCAACACTTACG R: CACCCATATACACGCATAGCA	(CA)10	-	-	No	-	-	No	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig-2	F: TAAAGGGACCAGAGGGAAGG R: CCCCATTCCCATAAACCTCT	(AT)9(AG)15	-	-	No	50.0	185-190	Yes	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig-3	F:TCTGTCATGTTTGGGTGGTG R:CATTCCCTCCTTACCCCATT	(GA)14	56.7	210-220	Yes	56.7	180-185	No	*Dalbergia nigra*	Ribeiro et al. (2011)
Dnig-4	F:CAAAAACCTGTTTGGCAAATTA R: TCTTGGGTTGTGGTGTTGAA	(TCA)5	56.7	190-200	Yes	-	-	No	*Dalbergia nigra*	Ribeiro et al. (2011)
Mcola-8	F: AGTTCTAAACGAGAGGAGG R: CTTTGGACAATACATCACC	(GT)7A(TG)6	60.6	250-260	Yes	-	-	No	*Dalbergia monticola*	Favreau et al. (2007)
Mcola28	F: ACAACCCCACACTCTCAATC R: CACCAGCAACACTTTCAGG	(TG)10	-	-	No	-	-	No	*Dalbergia monticola*	Favreau et al. (2007)
Mcola30	F: CTCTCTTCTCCAATCCCAC R: CTACCAGTCACTCACCCTTC	(GT)18	-	-	No	56.7	220-230	Yes	*Dalbergia monticola*	Favreau et al. (2007)

latifolia. Similarly, only a few SSR makers were reported for *D. sissoo*. Thus, the objective of this study was to explore the potential for efficiently transferring DNA markers in *D. latifolia* through the novel approach of cross-amplification of microsatellite loci. Additionally, the introduction of additional markers to *D. sissoo* was also considered. SSR markers successfully developed were further validated by investigating the breeding behavior of open-pollinated seed progenies of both species.

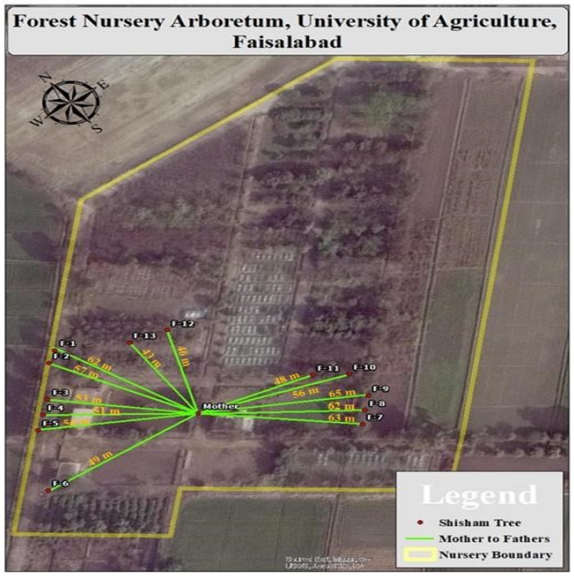

Fig. 1. Geospatial map of *Dalbergia sissoo* with a mother and the potential pollen donors from forest nursery arboretum of the University of Agriculture Faisalabad, Faisalabad Pakistan.

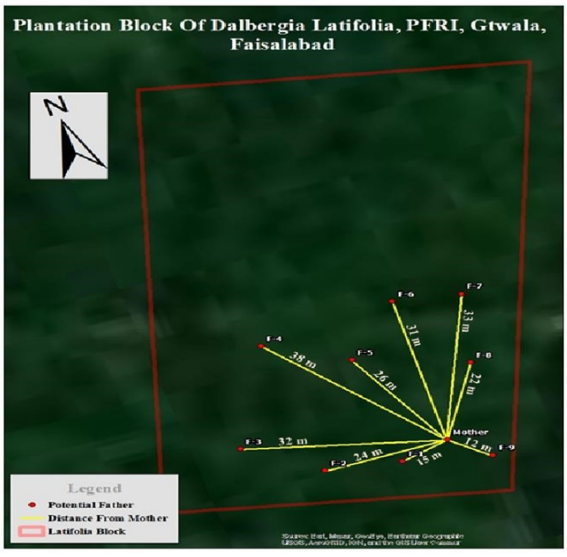

Fig. 2. Geospatial map of *Dalbergia latifolia* with a mother and the potential pollen donors from forest nursery arboretum of Punjab Forest Research Institute, Faisalabad Pakistan.

Materials and Methods

Leaf samples were gathered from 10-year-old trees of *D. sissoo* and *D. latifolia* that were planted in the arboretum of Pakistan Forest Institute (PFI) and Peshawar, Khyber Pakhtun Khawa (KPK). Genomic DNA was extracted using a ThermoScientific DNA extraction kit from ThermoFisher Scientific™. DNA extraction was carried out on 100 mg of fresh young leaf tissue following the manufacturer's protocol.

PCR assay

A total of nineteen SSR markers, chosen for their significant allelic diversity, were utilized to amplify the genome of both species (Table 1) (de Carvalho 1997; Du Puy et al. 2002; Hung et al. 2020; Lakhey et al. 2020; Sukhadiya et al. 2020). PCR reactions were conducted in a BioRad Thermocycler (BioRad™). PCR reactions were conducted using touchdown gradient cycles. The process began with an initial denaturation at a high temperature for 5 minutes. This was followed by 30 cycles of carefully timed temperature changes, ensuring optimal conditions for each step (Table 2) (Favreau et al. 2007; Ribeiro et al. 2011; Hartvig et al. 2017). Finally, a final extension was performed to complete the reaction. PCR amplicons were separated on an 8% polyacrylamide gel and stained with ethidium bromide.

Mating system analysis

For the validation of SSR markers in both species, leaf samples for *D. sissoo* were collected at the University of Agriculture Faisalabad's Nursery of Forestry Department

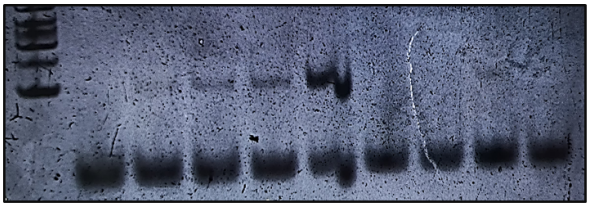

Fig. 3. Amplification and segregation of SSR markers with Oli-5 in *D. sissoo* progenies.

(Fig. 1), and Punjab Forest Research Institute (PFRI) in Faisalabad, Punjab, for *D. latifolia* (Fig. 2). Using a ThermoScientific DNA extraction kit (ThermoScientific™), DNA was isolated from 13 seeds and 10 leaf tissues to identify potential pollen contributors for each species.

Data analysis

The relationship between seed progenies, mother, and potential pollen donor was inferred by COLONY software (Jones and Wong 2010).

Results and Discussion

A total of fourteen markers (74%) were successfully cross-amplified for *D. sissoo*, while thirteen markers (68%) were cross-amplified for *D. latifolia* (Table 2) (Favreau et al. 2007; Ribeiro et al. 2011; Hartvig et al. 2017). Consistently replicable PCR products were amplified once the PCR conditions were standardized. Most of the primers that were amplified in both species are found in the order of *D. oliveri*, *D. nigra*, and *D. monticola*, as shown in Table 2 (Favreau et al. 2007; Ribeiro et al. 2011; Hartvig et al. 2017). The success rate is strongly influenced by the geographical distance between the species, as evidenced by studies conducted by de Carvalho (1997), Du Puy et al. (2002), and Hung et al. (2020). All markers were segregated in Mendelian ratio in both species, resulting in the production of only two alleles (Fig. 3). The limited range of allelic diversity observed may be a result of the relatively small sample size and the number of populations examined in this study. The seed genotypes in both species did not match the mother trees, suggesting that cross-pollination is the primary mode of pollination. This supports previous research that demonstrated varied breeding behavior, with outcrossing being the primary mode of pollination. Vasudeva and Sareen (2009) found that honeybees, beetles, butterflies, and thrips are commonly attracted to and involved in the pollination of *D. sissoo* flowers. These insects are primarily involved in cross-pollination. However, a small amount of self-pollination has also been reported, mainly due to Thrips. In a study conducted by Hartvig et al. (2017), it was found that *D. oliveri* had a 95% outcrossing rate, while *D. cochinchinensis* had an 82% outcrossing rate. However, the researchers also observed some degree of selfing in both species. With the use of COLONY, we can create seed arrays for the potential pollen donors. Regarding *D. sissoo*, six progenies were attributed to three pollen donors, while there was another group of five full-sib progenies that could not be linked to any of the pollen donors. Similarly, in the case of *D. latifolia*, six seed progenies were found to match with three pollen donors, while seven could not be assigned to any of the sampled pollen donors. The analysis revealed that the pollen originated from a donor located beyond the sampled area, indicating cross-pollination in both species.

Conclusion

One major drawback of microsatellite analysis is the requirement to generate informative loci for each species. Fortunately, with increasing efforts, there has been a noticeable increase in reported instances of cross-specific amplifications. These amplifications have been observed at the genetic level and, in some cases, even across different genera. Several studies have contributed to this growing body of knowledge (Konuma et al. 2000; Stacy 2001; Shepherd et al. 2002; Barbará et al. 2007; Muhammad et al. 2016; Shekhar et al. 2021). Being able to apply relevant findings from one species to the study of a diverse range of closely related species would be a major advantage. Given the availability of microsatellite loci in various species from different genera, cross-specific amplification can be a valuable tool for biologists working with limited economic resources. Based on the findings, it can be inferred that cross-pollination is the predominant method of pollination in *D. sissoo* and *D. latifolia*. This suggests that utilizing these species in the breeding process could lead to the development of superior hybrids.

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