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Review article

Approaches in development of DNA based identification system for industrially important timber species

Tanzeem Fatima[1]*, Ashutosh Srivastava[1], Vageeshbabu S. Hanur[2] and M. Srinivasa Rao[3]

[1]Genetics and Tree Improvement Division Institute of Wood Science and Technology, Bangalore-560003, Karnataka, India
[3]Department of Biotechnology, Indian Institute of Horticultural Research Hessarghatta, Bangalore-560089, Karnataka, India
[3]Forest Development Corporation of Maharashtra Limited, Nagpur-440036, Maharashtra, India

*Corresponding Author: tanzeem.fatima@gmail.com
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Abstract: Commercial illegal trade is the largest threat to important timbers in India and industrialized countries. Timber species valued for its wood and wood products are smuggled in the adulterated form that cannot be taxonomically identified. The development of DNA marker method to identify and control the origin of tree and tree products from tropical tree species would greatly contribute to distinguish legally from illegally harvested wood. Therefore, DNA barcoding has been anticipated as a reliable technique for wood species identification that can ensure that the tree harvested and traded are the same species/origin. The availability of DNA barcodes for increasing numbers of timber species allows rapid and accurate species identification. This is the first attempt to assemble all the timber barcodes which are available as a reference for the timber species of India. This paper describes whole DNA barcoding process from collection of plant material, to extract DNA and amplification as well as sequencing the amplified region to barcode generation.

Keywords: Adulteration - DNA barcoding - DNA markers - Timbers.

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INTRODUCTION

The identification of wood is very necessary within the forest trade in order to identify illegally harvested, traded timber and timber products (Lowe *et al.* 2016). Illegal work has been a subject of great national, regional and global concern for several decades, due to its serious impacts on forest biodiversity, wildlife habitat, and soil quality, access to water, poverty, greenhouse gas emissions and governance (Kidheghesho 2015, Ellison *et al.* 2017). DNA tracking technique potentially offers several advantages over current approaches for establishing the legitimate source of harvested timber products (Lowe 2007). The development of DNA marker method to identify the origin and species of timber and timber products would greatly contribute to distinguish legally from illegally harvested wood (Finkeldey *et al.* 2007). Wood DNA is a good candidate for forensic and illegal trading applications (Liepelt *et al.* 2006, Jiao *et al.* 2018). The ability of track timber resources from forest to market place would be critically important for successful management and proper regulation of the timber trade (Ramage *et al.* 2017). The ability to extract the DNA from wood sample would be a fundamental step in the application of genetic techniques to the timber trade (Porth & El-Kassaby 2014). Molecular marker ways is used at a range of levels to spot the wood from species identification, through regional and concession source verification, down to tracking individual logs (Lowe & Cross 2011). Both the overall amount and the spatial distribution of variation determine the usefulness of particular DNA bands for wood forensic and species identification and identification of the origin of plant material (Finkeldey *et al.* 2010). DNA barcoding is a reliable tool identifies tree species using a short section of DNA from specific genes. This

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technique made the large scale screening of DNA variation inexpensively and routinely with higher taxonomic resolution than morphological determination methods (Stein *et al.* 2014). It has the potential to provide a universal standard to identify positively all specimens of a timber species, no matter how far the timber is carried or if taxonomic discrepancies still persist For many of the high-value timber species, once species identification has been confirmed, the main issue is usually around verifying that the timber has come from a sustainable, or at least legal, source or that it has not been illegally sourced (Lowe 2007). Molecular marker methods currently being applied or developed for species identification, verification of source, either at the regional scale or population genetic assignment (Fig. 1). The availability of DNA barcodes for increasing numbers of species also potentially allows rapid DNA sequence identification and is an exciting recent development (Hartvig *et al.* 2015). The most acceptable molecular marker ways presently being applied or developed for species identification and for tracking individual logs or wood products (DNA fingerprinting) (Lowe & Cross 2011). In the light of above this paper aims to elucidate approaches to use of DNA based identification in important timber species.

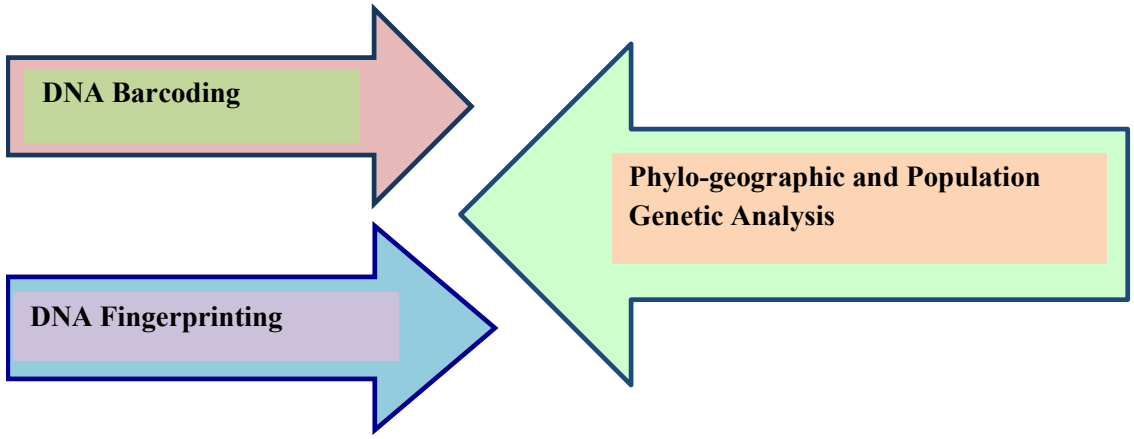


Figure 1. DNA-based methods for timber species identification.

DNA barcding and fingerprinting

DNA barcoding is an advantageous tool for tree species identification and phylogenetic construction (Kang *et al.* 2017). DNA fingerprinting can differentiate between individual trees that were harvested from the same concession even neighboring stumps (Nybom *et al.* 2014). It is based upon methods already used in criminal forensics and paternity testing. DNA fingerprinting of tree provides a truly independent, scientific verification of the tracking system. Introduction of DNA testing into these systems is not only an effective measure to deter document fraud, cutting off log laundering channels, but also to protect the certification brands, lower cost, facilitate uptake and increase transparency.

The process is simple to implement. Wood samples are taken from trees prior to harvest, during the forest inventory process. These samples are stored so that they can be tested and analyzed at a later date. During harvesting and processing, samples are taken from the same trees and logs, according to the tracking system documentation. This second set of samples is physically matched with the samples taken during the inventory. If the tracking documentation is correct, then the paired samples should come from the same trees. The targeted nature of DNA fingerprinting also allows auditors to reduce the intensity and frequency of regular physical audits. Since chain-of-custody audits make up a significant proportion of certification costs, it follows that a reduction in auditing time and effort all along the certified supply chain will reduce the overall cost of tree certification.

Wood DNA isolation

DNA extraction from wood is most difficult due to its highly degraded nature and the presence of high secondary metabolites. For DNA barcoding study we need high quality of genomic DNA. For wood genomic DNA isolation, very few studies have been validated (Fatima *et al.* 2018).

Mitochondrial and chloroplast DNA was isolated from wood species *Gonystylus bancanus* (Miq.) Kurz by using two methods Qiagen and CTAB with PTB and yielded high quality and quantity (20–50 ng μL^{-1}) of DNA (Asif & Cannon 2005). Both cpDNA and mtDNA region were successfully amplified and showed successful extraction wood DNA. Six DNA extraction treatments were used with12 cambial samples from mature trees. In treatment 6, the yield was highest with an average of 5.35–12.59 µg μL^{-1} (Tibbits *et al.* 2006). The isolated

DNA was amplified with three chloroplast microsatellite primers and genotyped as well as sequenced the amplified segment. Finkeldey *et al.* (2007) isolated the DNA from leaf and wood from the same unprocessed tropical timber Dipterocarpaceae by using Qiagen method applying the modification with three different concentration of PVP % (0, 2.6 and 5 (w/v) respectively.

Ten DNA extraction protocols were compared to isolate DNA from wood of European Aspen (*Populus tremula* L) **i.** SDS isolation **ii.** Protein precipitation **iii.** CTAB isolation **iv.** CTAB precipitation **v.** Guanidinium isothiocyanate **vi.** alkaline isolation **vii.** applichem **viii.** macherey-nagel **ix.** fermentas, innu PREP and **x.** Plant DNA kit (Analytikjena). The DNA concentration varies from 20–28 ng μL^{-1} from wood tissue and 100–310 ng μL^{-1} from leaf tissue (Verbylaite *et al.* 2010). Xiaoshu *et al.* (2011) extracted the DNA from different parts of dry wood by using three DNA extraction protocols **i.** Qiagen, **ii.** modified CTAB protocol and **iii.** modified CTAB with PTB protocol. They observed the quality and PCR amplification was successful in DNA extracted from the standardized protocol. The results for on the DNA. Jiao *et al.* (2012) isolated DNA from the wood tissues from *Cunninghamia lanceolata* (Lamb.) Hook. using two DNA extraction protocols, **i.** the modified CTAB method and **ii.** and the modified Qiagen method.

The reliability of the extraction method was confirmed by comparing fragment sizes and sequences after isolation of DNA from leaves and wood of the important tropical tree family Dipterocarpaceae trees. Average DNA yield of 2.2 µg $sample^{-1}$ was obtained from 50–100 mg $sample^{-1}$ of dried wood. They amplified the chloroplast DNA (cpDNA) fragments of different lengths by means of PCR, investigated key factors influencing PCR, and conducted inhibitor tests for a subset of the samples. It was found that the Amplification success was higher if DNA was isolated from outer sapwood (without cambium) in comparison to DNA isolated from the transition zone between sapwood and heartwood and the inner heartwood, (Rachmayanti *et al.* 2006). Rachmayanti *et al.* (2009) extracted DNA from wood with the DNeasy Plant Mini Kit (Qiagen) applying the same modifications to the surface tissues (including cambium and bark) of wood samples. The isolated DNA from three different zones of wood amplified and genotyped with three chloroplast microsatellite primers (ccmp2, ccmp3 and ccmp10). Fatima *et al.* (2018) standardized and validated the wood DNA extraction protocol of tree species which contains high amount of inflexible tissues, fibers, phenolic and secondary metabolites.

Universal DNA barcoding markers

Deguilloux *et al.* (2003) developed two chloroplast DNA markers for oak, the intergenic spacer *trn D- trnT* and the intron of *trnL* fragment. Primer pair I and primer pair II. Primer pair I was chosen due to its differentiating capacity among gymnosperms, while primer II is selected for the identification of angiosperm trees (Liepelt *et al.* 2006). There are several types of universal DNA barcoding markers are available *viz.*, rbcL, matK, ITS-trnH (CBOL 2009). Meullner *et al.* (2011) evaluated nuclear *rpoC1*, *rpoB*, *accD* and plastid markers *psbB*, *psbN*, *psbT* and the *trnS-trnG*) as well as the nuclear ribosomal spacer region (*ITS1-5.8S-ITS2*). Armenise *et al.* (2012) examined four marker regions (*trnH-psba*, *rbcL*, *rpoc1* and *matK*) to match conifer species. The standard barcode region, *rbcL*, *matK* and *trnH-psbA* chloroplast genomic sequences were analysed to distinguish wood adultrants of Sandalwood (*Santalum album* L.) (Dev *et al.* 2013). Mishra *et al.* (2017) identified four barcoding regions *rbcL*, *matK*, *ITS* and *psbA-trnH*. Yu *et al.* (2017) developed eight potential DNA barcodes *viz.*; *ITS2*, *matK*, *trnL*, *trnH-psbA*, *trnV-trnM1*, *trnV-trnM2*, *trnC-petN*, and *trnS-trnG*. Celinski *et al.* (2017) evaluated eight chloroplast DNA barcode regions *viz.*, *matK*, *rbcL*, *trnH-psbA*, *trnL-trnF*, *rpl20-rps18*, *trnV*, *ycf1*, *ycf2* in pines. Jiao *et al.* (2018) developed four conserved DNA barcodes, *ITS2*, *matK*, *ndhF-rpl32* and *rbcL*. Two primer pairs were designed to differentiate among forest tree genera. Each primer pair amplified a small fragment of the chloroplast trnL. Three barcodes were validated *rbcL*, *matK* and *ITS-trnH* (Fatima *et al.* 2019).

DNA Barcoding studies on few important timber species

Stalin *et al.* (2014) sampled 429 trees representing 143 tropical dry evergreen forest species, which included 16 threatened species. The barcoding approach accurately identified 136 species out of 143 species by using *rbcL* and *matK* markers. This high level of species resolution (95%) by *rbcL* and *matK* was largely due to the fact that the tree species were taxonomically diverse in the tropical dry evergreen forest. The ability of *rbcL*, *matK* and *trnH-psbA* plastid DNA barcoding markers to identify rain forest trees at two sites in Atlantic central Africa was assumed that a database were exhaustive in terms of species identification, but not necessarily within species (Parmentier *et al.* 2013). There are several important timber species, which have their standard barcodes (Table 1).

Table 1. Commercially important timber species in India.

S.N.	Forest Type	Scientific Name	Trade Name	Barcode
1.	Temperate forests	*Cedrus deodara* (Roxb.) G.Don	Deodar	Barcodes available (Laiou *et al.* 2013)
2.	Deciduous forests	*Casurina* species	Beach Oak, Iron wood	No barcodes available to distinguish at species level
3.	Dry mixed Forests	*Dalbergia latifolia* Roxb.	Rosewood	Barcodes available (Fatima *et al.* 2019)
4.	Deciduous forests	*Dalbergia sissoo* Roxb.	Sheesham	Barcodes available (Bhagwat *et al.* 2015)
5.	Dry mixed Forests	*Lagerstroemia parviflora* Roxb.	Bendara	No barcodes available to distinguish at species level
6.	Deciduous evergreen forests	*Lagerstroemia lanceolata* Wall.	BenTeak	Barcodes available (Fatima *et al.* 2019)
7.	Moist deciduous forest	*Melia dubia* Cav.	Malabar neem	Barcodes available (Sivaraj *et al*. 2018)
8.	Mixed deciduous forest	*Melia azedarach* L.	Chinaberry tree, bead-tree	Barcodes available (Sivaraj *et al*. 2018)
9.	Tropical Semi-Evergreen Forests	*Memecylon umbellatum* Burm. F.	Anjani	No barcodes available to distinguish at species level
10.	Mixed deciduous forest	*Quercus* species	Oak	Few species barcodes available (Fitzek *et al.* 2018)
11.	Montane Wet Temperate Forests	*Pinus* species	Pine	Few species barcodes are available (Celinski *et al*. 2017, Armenise *et al.* 2012)
12.	Tropical dry deciduous Forests	*Pterocarpus santalinus* L.f.	Red Sandars	Barcodes available (Jiao *et al.* 2018)
13.	Tropical deciduous forests	*Pterocarpus marsupium* Roxb.	Indian Kino tree	Barcodes available (Jiao *et al.* 2018)
14.	Tropical dry deciduous forests	*Shorea robusta* Gaertn.	Sal tree	Barcodes available (Tripathi *et al.* 2013, Hegde *et al.* 2018)
15.	Tropical Semi-Evergreen Forests	*Terminalia paniculata* Roth	Kinjal	No barcodes available to distinguish at species level
16.	Tropical Semi-Evergreen Forests	*Terminalia chebula* Retz.	Hirda	Barcodes available (Mishra *et al.* 2017)
17.	Moist Mixed deciduous Forests	*Terminalia tomentosa* Wight & Arn.	Ain	No barcodes available to distinguish at species level
18.	Tropical moist and dry deciduous forest	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Arjuna	Barcodes available (Sharma & Shrivastava 2016)
19.	Mixed deciduous forest	*Terminalia bellerica* (Gaertn.) Roxb.	Bibhitaki	Barcodes available (Mishra *et al.* 2017)

1. *Dalbergia* species

Dalbergia L.f. species belonging to Fabaceae families one of the most useful and high profile timber species due to its durability (Li *et al.* 2017). The entire genus on Dalbergia species placed that includes approximately 250 species in Appendix II of the CITES (The convention of International Trade of Endangered Species of Flora and Fauna). Yu *et al.* (2017) selected nine *Dalbergia* species Xylarium wood specimens from the Wood Collection of the Chinese Academy of Forestry amplification by eight potential DNA barcodes *viz.*; *ITS2*, *matK*, *trnL*, *trnH-psbA*, *trnV-trnM*, *trnV-trnM2*, *trnC-petN* and *trnS-trnG*. These barcodes were comparatively short regions (<350 bp) and amplification reactions were found with high success rate (≥90%). Hartvig *et al.* (2015) applied DNA barcoding methods to support conservation efforts of Dalbergia species in Indochina. They used *rbcL*, *matK* and *ITS* barcoding markers on 95 samples of 31 species of *Dalbergia* and found that ITS yielded the single highest discrimination rate (100%), but due to difficulties in achieving high-grade sequences from wood degraded samples, the better overall choice for *Dalbergia* species to be the standard *rbcL+matK* barcode which yielded discrimination rates up to >90%. Fatima *et al.* (2019) selected two plastid-specific conserved barcoding markers *rbcL*, *matK* and one nuclear-specific *trnH-ITS* to identify *Dalbergia latifolia* Roxb. from three different locations in Karnataka. Hassold *et al.* (2016) tested DNA barcoding with partial sequences of three plastid markers (*matK*, *rbcL* and *trnL*) to

distinguish between Dalbergia Malagasy from Madagascar and from other areas of its distributional range.

The two-locus combination *ITS2+trnH-psbA* markers showed the highest success rate for discriminating the eight *Dalbergia* species (He *et al.* 2018). Ten *Dalbergia* species were evaluated by using three regions in the plastid genome (*matK*, *rbcL* and *trnH-psbA*), a nuclear transcribed spacer (*nrITS*) and their combinations (Bhagwat *et al.* 2015).

2. *Tectona* species

The genus *Tectona* L.f. is represented by only three species *viz.*, *Tectona grandis* L.f., *Tectona hamiltoniana* Wall. and *Tectona philippinensis* Benth. & Hook.f. Among them *T. grandis* is one of the most valuable timbers yielding species (Palanisamy *et al.* 2009). Fatima *et al.* (2019) selected two plastid-specific *rbcL*, *matK* and one nuclear-specific *trnH-ITS* conserved barcoding markers to compare and identify the discrimination of *Tectona grandis* from three different regions in Karnataka.

3. *Pterocarpus* species

Pterocarpus Jacq. is a pan-tropical important genus of trees belonging to family Leguminosae with 35 species (Saslis-Lagoudakis *et al.* 2011) which are distributed throughout the tropics of Africa, the Indo-Malayan region and North America (Mabberley 2017). Jiao *et al.* (2018) selected 31 Xylarium wood specimens and 8 leaf samples of six important commercial species of *Pterocarpus* to investigate the authentication at the species level and to determine the feasibility of building wood DNA barcode reference libraries. Four DNA barcodes (*ITS2*, *matK*, *ndhF-rpl32* and *rbcL*) and their combination were tested to evaluate their discrimination ability for *Pterocarpus* species with both Taxon DNA and tree-based analytical methods. Results from this study verified not only the feasibility of building DNA barcode libraries using Xylarium wood specimens, but the importance of using wood rather than leaves as the source tissue, when wood is the botanical material to be identified.

4. *Lagerstroemia* species

The genus *Lagerstroemia* L. belongs to the family Lytharaceae with 20 species. Fatima *et al.* (2019) identified two-chloroplast specific *viz.*, *rbcL*, *matK* and a nuclear-specific *trnH-ITS* conserved barcoding marker to compare species discrimination of *Lagerstroemia lanceolata* Wall. from three different regions of Karnataka.

5. *Melia* species

The genus *Melia* L. belongs to Meliaceae family with two accepted species *Melia dubia* Cav. and *Melia azedarach* L. Total 10 accessions of *M. dubia* and *M. azedarach* by DNA barcoding using three chloroplast DNA markers (*rbcL*, *matK*, and *trnH–psbA*), and one nuclear marker (*ITS2*). The interspecific divergence between *M. azedarach* and *M. dubia* ranged between 0.3% (*rbcL*) and 4.7% (*ITS2*) respectively, and for the combined *rbcL+ITS2*, it was up to 8.5%. Among the markers, *ITS2* was found to be the most suitable marker for differentiating *M. azedarach* and *M. dubia* (Sivaraj *et al.* 2018).

6. *Terminalia* species

Terminalia L. genus belonging to family Combretaceae that comprises about 150 species and in India 14 species is found in India (Raman & Khan 2011). Mishra *et al.* (2017) estimated the possibility of using barcoding regions *rbcL*, *matK*, *ITS* and *psbA-trnH* in the *Terminalia*, a complex species taxonomy that exhibits various overlapping morphotypes with tropical distribution for *Terminalia bellerica* (Gaertn.) Roxb. and *Terminalia chebula* Retz.

7. *Swietenia* species

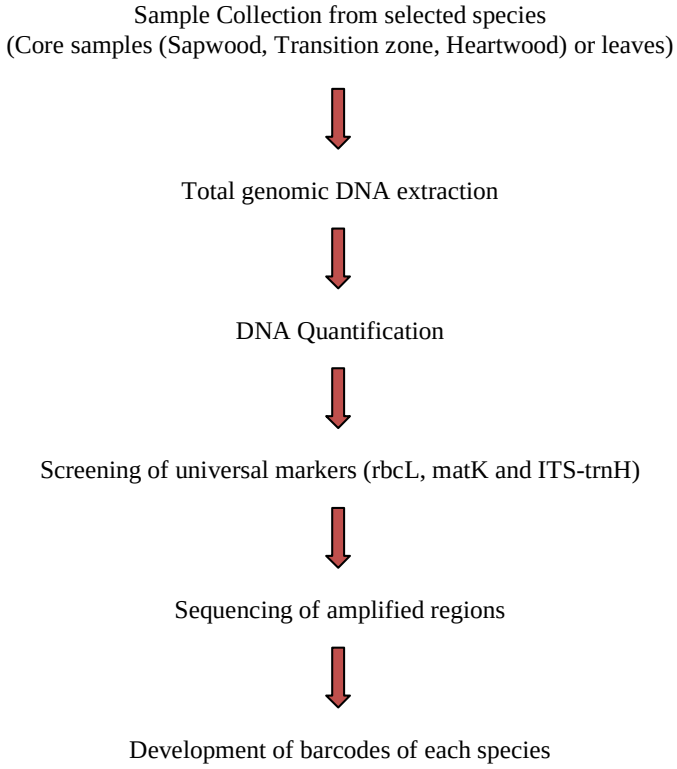
Swietenia macrophylla King (Mahogany) belongs to Meliaceae family is important timber called as a multipurpose tree due to moderate fast growth and remarkable wood qualities (Kumar *et al.* 2016). Muellner *et al.* (2011) evaluated main candidate plastid regions (*rpo*C1, *rpo*B, *acc*D) and extra plastid markers (*psb*B, *psb*N, *psb*T exons and the trnS-trnG spacer) further more nuclear ribosomal spacer region (ITS1-5.8S -ITS2) in a group of land plants belonging to the mahogany family, Meliaceae. Across these samples, only ITS region showed high levels of discrimination between the species listed in Endangered Species.

8. *Pinus* species

Pinus L. is the most common genus of the Pinaceae family and is the largest family of conifers. Five species of Pines are indigenous to India *viz.*, *Pinus roxburghii* Sarg., *Pinus wallichiana* A. B. Jacks., *Pinus kesiya* Royle ex Gordon, *Pinus gerardiana* Wall. ex D. Don and *Pinus merkusii* Jungh. & de Vriese (Sharma

et al. 2018). Twenty-five conifer species were studied discriminate by two-locus barcodes rbcL+ trnH-psbA (Armenise *et al*. 2012). Celinski *et al.* (2017) compared the genetic variation of eight DNA barcode regions in seventeen conifers in which three closely related pines from *Pinus mugo* complex and fourteen were representing two genera and four sections of the Pinaceae family.

Barcoding generation procedure flow chart



CONCLUSION

The present study evaluates DNA barcoding technique for the identification of important timber, which are illegally harvested and traded. From this study we can identify that on which timber species barcodes are available. DNA barcode identification can be more authentic by relying on integrated approach. The universal sequences of the multi locus barcodes provided accurate marker for the molecular identity of species. In this study we can depicts that among all the barcode loci ITS and rbcL+ ITS possess high rate of discrimination power which can be further access as core barcodes. The present data will promote the progress of DNA barcoding in designing species-specific molecular markers for facilitating the targeted use of important timers in timber trading.

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