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Geographical traceability of an important tropical timber (*Neobalanocarpus heimii*) inferred from chloroplast DNA

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ABSTRACT

The inbuilt unique properties of DNA within the timber could serve as tracking and monitoring tools to verify the legality of a suspected timber in the context of illegal logging, forest certification and chain of custody certification. By using *Neobalanocarpus heimii* (Dipterocarpaceae) as an example, a population identification database and haplotype distribution map in Peninsular Malaysia were generated for authenticity testing based on four chloroplast DNA markers (*trn*L intron, *trn*G intron, *trn*K intron and *psb*K-*trn*S spacer). Twenty one haplotypes were identified from 10 significant intraspecific variable sites. The results clearly revealed that only northern and southern regions of Peninsular Malaysia were distinguishable. Thus, this database could only be used to determine the wood lot of unknown origin at the regional level. Statistical procedure based on the composition of wood lot was used to test whether a suspected timber conforms to a given regional origin. Overall, the observed type I and II errors of the database showed good concordance with the predicted 5% threshold, which might indicate that the database is useful to reveal provenance and establish conformity of wood lot from northern and southern regions of Peninsular Malaysia. Applications of this database for timber tracking are discussed.

1. Introduction

New methods to match a timber log into its population of origin would signify an important forensic component in the context of stolen log traceability for the control of illegal logging and also the approach in chain of custody developed for the certification of timber from sustainably managed forests (Lyke, 1996; Chihambakwe et al., 1997). Indeed, illegal logging is a problem that not only destroys forest ecosystems in its own right but also threatens the viability of forest certification by depressing the price of timber and creating extremely low-priced competitor products (Cashore et al., 2004). Although only timber products with legality licenses are allowed to enter European markets (Commission, 2003), it is estimated that 50% of the tropical timbers traded in the European markets are illegal (Richert, 2003).

In response to the increasing concern on illegal logging, nearly 10% of commercial forests worldwide have been certified as being "well-managed" by the Forest Stewardship Council in 2005 (FSC, 2005a). Driven by the rapid growth of the forest certification, in some countries, forest certification has become a regular aspect in the logging industries, and the timber trades have become more transparent, since the origins of certified timbers are known (Visseren-Hamakers and Glasbergen, 2007). Similarly, chain of custody approach and ecolabelling schemes have also proliferated in recent years and have attracted widespread participation of forest landowners (FSC, 2005b; Global Ecolabelling Network, 2008).

The implementation of forest certification and ecolabelling contributes to halting deforestation and ensuring a sustainable use of forest resources globally. However, there may be products bearing ecolabels that do not actually meet the label's environmental standards (Global Ecolabelling Network, 2008). Most timber-auditing systems rely on tagging or certificates of origin issued in the source country to verify the legality of the timbers, but these methods are susceptible to falsification (Carr, 2007). Therefore, there is a need for a harmonized timber tracking system to trace and verify the origin of a suspected timber in order to reduce illegal timber trade and hence strengthening the cooperation between producers and consumer countries (Asia Forest Partnership, 2005).

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In the past, spectrometry and isotopic methods have been applied and proposed to differentiate wood samples from different geographical origins that will permit their geographical origins to be determined with varying degrees of certainty (Perez-Coello et al., 1997; Durand et al., 1999; English et al., 2001). However, these approaches are influenced by the local environment, variability of chemical composition and are limited by the fact that such markers can show a discrepancy between individuals from the same population or even between different tissues from the same individual (Hoffman et al., 1994; Towey and Waterhouse, 1996). Hence, this has led to major advances in the use of inbuilt unique properties of DNA within the timber to support the determination of identity and provenance (Asia Forest Partnership, 2005). The use of DNA track-back system, once thought to be impossible for wood, is now feasible, though in its infancy. A good example is shown in the European white oaks. The strong geographical structure and differentiation of western vs. eastern population were used for the oak wood traceability (Deguilloux et al., 2003).

There are two very different ways in which DNA could be applied in timber tracking and forensic forestry investigations. First, cpDNA markers showing enough geographical structure could be used to differentiate the origin of one source of timber from another. Second, a highly polymorphic nuclear short tandem repeat (STR) marker could be used to generate DNA profiling databases for individual identification, in which an illegal timber log could be matched into its original stump (Tnah, 2007). In combating illegal logging, both of these tools require rapid development of large comprehensive databases, detailing the distribution of genetic markers and incorporating these DNA-based techniques into the traceability systems. It is important that these databases can be established as soon as possible, in order to capture the 'natural' conditions, before the important patterns have been completely erased by human activity (Asia Forest Partnership, 2005).

In plants, molecular techniques using chloroplast DNA (cpDNA) marker have provided tools for studying the phylogeography or migration footprints of a species (Avise, 2000). Chloroplast DNA is thought to evolve slowly, with low mutation and recombination rates, and is known to be maternally inherited in most angiosperms. Maternally inherited DNA markers generally reveal much greater genetic structure in comparison with biparentally inherited nuclear markers (Petit et al., 1993a,b) and these markers have been successfully applied to identify possible glacial refugia and species migration routes of many plant species (Huang et al., 2004; Cheng et al., 2005; Fjellheim et al., 2006; Shephard et al., 2007). In principle, the geographical origin of wood samples can be checked with the cpDNA markers that show enough geographical structure. For example, a study on *Cedrela odorata* throughout Mesoamerica using cpDNA markers indicated a strong geographical pattern which can be used to determine the geographical origin of Mesoamerican *C. odorata* timbers (Cavers et al., 2003).

Neobalanocarpus heimii or locally known as chengal is endemic but widely distributed in Peninsular Malaysia. It is found in diverse localities, on low-lying flat land as well as on hills of up to 900 m (Symington, 1943). *N. heimii* produces a naturally, highly durable wood and is among the strongest timbers in the world. It is used for heavy constructions, bridges, boats, buildings, and wherever strength is considered essential (Thomas, 1953). Under the IUCN Red List of Threaten Species, it was assigned under the vulnerable category due to a decline in the area of its distribution, the extent of occurrence and/or quality of habitat, and actual or potential levels of exploitation (Chua, 1998). Owing to the high demand for its valuable timber, *N. heimii* is subjected to illegal logging and this species might become endangered in the near future. Therefore, this study was aimed to (1) provide a detailed picture of the distribution of chloroplast haplotypes of *N. heimii* throughout Peninsular Malaysia and (2) identify specific haplotypes that could be used to generate population identification database and serve as a tracking and monitoring tools in the context of illegal logging, forest certification and chain of custody certification.

2. Materials and methods

2.1. Sample collection and DNA extraction

In order to generate a comprehensive database of *N. heimii* for population identification, sample collection was conducted throughout the distribution range of *N. heimii* in Peninsular Malaysia. Thirty two natural populations of *N. heimii* from 29 forest reserves, with a total of 256 individuals of more than 10 cm diameter at breast height (eight samples per population) were investigated in this study (Table 1). The samples were collected either in the form of inner bark or leaf tissues. Total DNA was extracted using the procedure described by Murray and Thompson (1980) with modification, and further purified using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH).

2.2. PCR amplifications and sequencing

Twenty seven cpDNA universal primer pairs of higher plants (Heinze, 2007) were screened to identify intraspecific variability; four cpDNA markers (*trn*L intron, *trn*G intron, *trn*K intron and *psb*K-*trn*S spacer, Table 2) were proven to be informative in the characterization of haplotypes in *N. heimii*. The PCR amplifications were performed in 20 μL reaction mixture, consisting of approximately 10 ng of template DNA, 50 mM of KCl, 20 mM of Tris–HCl (pH 8.0), 1.5 mM of $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP, and 0.5 U of *Taq* DNA polymerase (Promega). The reaction mixture was subjected to amplification using a GeneAmp PCR System 9700 (Applied Biosystems), for an initial denaturing step of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 50–55 °C annealing temperature for 1 min, and 72 °C for 1 min. This was followed by further primer extension at 72 °C for 8 min.

The PCR products were purified using MinElute PCR Purification Kit (Qiagen) and sequenced in both directions using BigDye Terminator Sequencing Kit (Applied Biosystems) based on the standard dideoxy-mediated chain termination method. The sequencing thermal profile was 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min on a GeneAmp PCR System 9700. Sequencing reactions were purified using ethanol precipitation and run on the ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequencing data were edited and assembled using CodonCode Aligner version 2.0 (CodonCode Corporation). Haplotypes were determined from nucleotide substitutions and indels (insertions and deletions).

2.3. Population identification database and test of conformity of origin

As a result of their uniparental inheritance, cpDNA were expected to show pronounced levels of population differentiation and large proportion of population specific haplotypes. These haplotypes were used to generate a haplotype distribution map throughout Peninsular Malaysia. The population identification database was constructed based on the entire 256 samples dataset, which was fitted to classify the samples into their population of origin based on the significant intraspecific variable sites.

To test the conformity of origin, a statistical procedure based on either presence or absence of haplotype on wood sample was used to test whether a suspected wood lot confirms to a given geographical origin (Deguilloux et al., 2003). The null hypothesis H_O: 'the wood lot is of the presumed origin' is defined. For each