PHYLOGEOGRAPHICAL PATTERNS OF *NEOBALANOCARPUS HEIMII* (DIPTEROCARPACEAE) FOR THE EVOLUTIONARY HISTORY AND CHAIN OF CUSTODY CERTIFICATION

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CORAK FILOGEOGRAFI *NEOBALANOCARPUS HEIMII* (DIPTEROCARPACEAE) UNTUK SEJARAH EVOLUSI DAN RANGKAIAN PENSIJILAN KUSTODI

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ABSTRACT

Tectonic movement and climatic oscillations during the Cenozoic have had dramatic effect on the biota of the tropical rain forest. This study aims to reveal the phylogeography and evolutionary history of a Peninsular Malaysian endemic tropical timber, *Neobalanocarpus heimii* (Dipterocarpaceae), based on chloroplast DNA (cpDNA) variation. Fifteen haplotypes were identified from 10 intraspecific variable sites of five non-coding cpDNA regions: *trn*L intron, *trn*S-*trn*G spacer, *trn*G intron, *trn*K intron and *psb*K-*trn*S spacer. Two major genealogical cpDNA lineages of *N. heimii* were elucidated: a widespread southern and a northern region. The species is predicted to survive in multiple refugia during climatic oscillation: the northwestern region (R1: Sungkop), the northeastern region (R2: Gunung Basur), and the southern region (R3: Panti compartment 16). Recolonization of refugia R1 and R2 could have first expanded into the northern region and migrated both northeastwardly and northwestwardly. Meanwhile, recolonization of *N. heimii* throughout the southern region could have commenced from refugia R3, and migrated toward northeast and northwest respectively. The populations of Tersang, Pasir Raja and Rotan Tunggal exhibited remarkably high haplotype diversity, which could have been the contact zones that received an admixture of organisms from the northerly and also southerly regions. As a whole, understanding the past history of the extant populations is of the utmost importance when developing sound conservation policies or sustainable management strategies.

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 *N. heimii* as an example, a population identification database and a haplotype distribution map in Peninsular Malaysia were generated for authenticity testing based on four cpDNA 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 the 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 the wood lot was used to test whether a suspected timber conforms to a given regional origin. Overall, the observed types 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 the northern and southern regions of Peninsular Malaysia. In terms of application, this database could be applied to traceability in two different circumstances: (1) to verify the provenance of a wood lot in the context of forest certification and chain of custody certification and (2) to identify the potential population of origin of the suspected illegal harvested wood lot.

Wood can be a good source of DNA for various applications in forensic forestry and timber trade if high quality DNA can be retrieved from the dry wood. In order to provide a general guideline for DNA authenticity testing established for *N. heimii*, this study was designed to evaluate the potential for extracting DNA from the dry wood of *N. heimii* using the Qiagen kit, CTAB, and CTAB with PTB protocols. Overall, the efficacy of DNA extraction was higher for the cambium and sapwood than for the heartwood tissues. In terms of tissue types, the Qiagen kit yielded higher PCR amplification rates from the cambium tissue, while the CTAB with PTB protocol showed higher amplification rates in the sapwood and heartwood tissues. In order to safeguard the intactness of the DNA, it is recommended that DNA extraction from the wood should be carried out within six weeks after felling for logs and six months after felling for stumps. The results also showed that the amplicon size might not account for the PCR amplification success rate and chloroplast genome yielded higher amplification success rate compared with nuclear genome. Additionally, the PCR amplifications also showed that both the nuclear and chloroplast regions can be retrieved from lumber that was heat-treated at 40 °C to 100 °C, although the phenomena of allelic dropout and inconsistency of genotyping were noted for some of the nuclear regions. In short, the guideline obtained from this study are ready to be used together with the population and

individual identification databases developed for the timber tracking system of *N. heimii* in Peninsular Malaysia.

ABSTRAK

Pergerakan tektonik dan perubahan iklim semasa Senozoik memberi kesan yang dramatis ke atas biota dalam hutan hujan tropikal. Kajian ini bertujuan untuk mendedahkan filogeografi dan sejarah evolusi bagi salah satu balak tropikal yang endemis di Semenanjung Malaysia, iaitu *Neobalanocarpus heimii* (Dipterocarpaceae) dengan berdasarkan variasi DNA kloroplas (cpDNA). Daripada lima kawasan bukan pengekodan cpDNA: intron *trn*L, penjarak *trn*S-*trn*G, intron *trn*G, intron *trn*K dan penjarak *psb*K-*trn*S, 15 haplotip telah dikenalpasti daripada 10 kawasan jujukan berubah yang intraspesifik. Dua genealogi cpDNA yang utama bagi *N. heimii* telah ditemui di sebelah kawasan utara dan selatan. Spesies ini dijangka hidup dalam pelbagai refugia semasa perubahan iklim, iaitu di kawasan utara-barat (R1: Sungkop), kawasan utara-timur (R2: Gunung Basur) dan kawasan selatan (R3: Panti bahagian 16). Penghijrahan *N. heimii* dari refugia R1 dan R2 mungkin diperluaskan ke kawasan utara pada permulaan, dan kemudian berpindah secara beransur-ansur ke sebelah utara-timur dan utara-barat. Pada masa yang sama, penghijrahan *N. heimii* ke kawasan selatan mungkin berpunca daripada refugia R3, dan kemudian berhijrah ke kawasan utara-timur dan utara-barat. Antaranya, populasi Tersang, Pasir Raja dan Rotan Tunggal menunjukkan kepelbagaian haplotip yang tinggi. Ini mungkin disebabkan oleh populasi tersebut merupakan zon pertemuan yang menerima campuran organisma daripada kawasan utara dan selatan. Secara keseluruhan, memahami sejarah lepas untuk populasi yang masih wujud ini adalah amat penting apabila menyediakan polisi pemuliharaan atau strategi pengurusan secara berkekalan.

Dalam konteks pembalakan haram, pensijilan hutan dan rangkaian pensijilan kustodi, kandungan DNA yang unik dalam kayu balak boleh dijadikan sebagai alat pengesan dan pengawas untuk menguji kesahan kayu balak. Dengan menggunakan *N. heimii* sebagai contoh, pangkalan data untuk identifikasi populasi dan peta taburan haplotip di Semenanjung Malaysia telah dijana untuk ujian kesahan berdasarkan empat penanda cpDNA (intron *trn*L, intron *trn*G, intron *trn*K dan penjarak *psb*K-*trn*S).

Antaranya, 21 haplotip telah dikenalpasti daripada 10 kawasan jujukan berubah yang penting dari segi intraspesifik. Keputusan dengan jelasnya menunjukkan terdapat perbezaan antara kawasan utara dan selatan Semenanjung Malaysia. Maka, pangkalan data ini hanya dapat digunakan untuk menentusahkan punca sesuatu kayu balak sama ada berasal daripada kawasan utara atau selatan sahaja. Prosedur statistik yang berdasarkan komposisi kayu telah digunakan untuk menguji sama ada kayu balak yang dicurigai akur dengan kawasan asal yang diisytihar. Secara keseluruhan, ralat cerapan jenis I dan II untuk pangkalan data menunjukkan keselarasan yang baik dengan jangkaan ambang sebanyak 5%. Ini juga menunjukkan bahawa pangkalan data ini adalah sesuai digunapakai untuk menentukan asal-usul dan konformasi kayu balak sama ada berasal daripada kawasan utara atau selatan Semenanjung Malaysia. Pendek kata, pangkalan data ini boleh digunakan untuk sistem pengesanan balak dalam dua situasi, (1) menentusahkan asal-usul kayu balak dalam konteks pensijilan hutan dan rangkaian pensijilan kustodi, dan (2) mengenal pasti populasi asal untuk balak yang ditebang secara haram.

Kayu merupakan sumber DNA yang sesuai untuk pelbagai aplikasi forensic perhutanan dan perdagangan kayu balak jika DNA yang berkualiti tinggi dapat diekstrak daripada kayu. Dengan tujuan menghasilkan satu panduan untuk ujian kesahan DNA yang telah dijana bagi *N. heimii*, kajian ini direka bentuk untuk menilai kebolehan mengekstrak DNA daripada kayu dengan menggunakan protokol kit Qiagen, CTAB dan CTAB dengan PTB. Secara keseluruhan, keberkesanan pengekstrakkan DNA adalah lebih tinggi untuk tisu kambium dan sapwood berbanding dengan tisu heartwood. Dari segi jenis tisu, protokol kit Qiagen menghasilkan kadar amplifikasi PCR yang lebih tinggi untuk tisu kambium, manakala protokol CTAB dengan PTB menunjukkan kadar amplifikasi yang tinggi dalam tisu sapwood dan heartwood. Untuk memastikan kesempurnaan DNA, pengekstrakkan DNA daripada bahagian balak perlulah dilakukan dalam masa enam minggu selepas ditebang, manakala enam bulan untuk bahagian tunggul. Keputusan ini juga menunjukkan saiz amplikon tidak mempengaruhi kadar amplifikasi PCR dan secara keseluruhannya genom kloroplas menghasilkan kadar amplifikasi yang lebih tinggi berbanding dengan genom nukleus.

Selain itu, amplifikasi PCR juga menunjukkan kedua-dua DNA nukleus dan kloroplas dapat diekstrak daripada kayu yang dipanaskan daripada 40 °C ke 100 °C, walaupun berlakunya fenomena kecicir an alel dan ketidaktekalan pengenotipan untuk sesetengah lokus nukleus. Pendek kata, keputusan yang diperoleh daripada kajian ini boleh diguna bersama dengan pangkalan data identifikasi populasi dan individu yang dijana bagi sistem pengesanan balak *N. heimii* di Semenanjung Malaysia.

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AFP	Asia Forest Partnership
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
bp	Base pair
BSA	Bovine serum albumin
CAPS	Cleaved amplified polymorphic sequences (CAPS)
cpDNA	Chloroplast DNA
cpSSR	Chloroplast microsatellites
CSA	Canadian Standards Association
CTAB	Hexadecyltrimethyl-ammonium bromide
DIECA	Diethyldithiocarbamic acid
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	Diaminoethanetetra-acetic acid
FSC	Forest Stewardship Council
FLEGT	Forest Law Enforcement, Governance and Trade
FR	Forest Reserve
FRIM	Forest Research Institute Malaysia
HCl	Hydrochloric acid
Indel	Insertion and deletion
IUCN	International Union for Conservation of Nature and Natural Resources
kbp	Kilobase pair
KCl	Potassium chloride
LGM	Last glacial maximum
$MgCl_2$	Magnesium chloride
min	Minute
MTC	Malaysian Timber Council
MTCC	Malaysian Timber Certification Council
Ma	Million years ago

NaCl	Sodium chloride
NCPA	Nested Clade Phylogeographic Analysis
NH_4OAc	Ammonium acetate
nSTR	Nuclear Short Tandem Repeat
PCR	Polymerase chain reaction
PEFC	Programme for the Endorsement of Forest Certification Schemes
PTB	*N*-phenacylthiazolium bromide
PVP	Polyvinylpyrrolidone
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
s	Second
SFI	Sustainable Forestry Initiative
STR	Short tandem repeat
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
Tris	Trishydroxymethylaminomethane