# การวิเคราะห์อณูพันธุศาสตร์ของพืชสกุล *Phyllanthus* ที่พบในประเทศไทย โดยใช้วิธีอาร์เอพีดีร่วมกับการหาลำดับนิวคลีโอไทด์

นางสาวจุฑาทิพย์ มานิสสรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# MOLECULAR ANALYSIS OF *PHYLLANTHUS* SPP. IN THAILAND BASED ON RAPD AND DNA SEQUENCING

Miss Juthatip Manissorn

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmacognosy
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| Thesis Title | MOLECULAR ANALYSIS OF *PHYLLANTHUS* SPP. IN THAILAND BASED ON RAPD AND DNA SEQUENCING |
| By | Miss Juthatip Manissorn |
| Field of Study | Pharmacognosy |
| Thesis Advisor | Associate Professor Nijsiri Ruangrungsi, Ph.D. |
| Thesis Co-Advisor | Assistant Professor Suchada Sukrong, Ph.D. |
| Thesis Co-Advisor | Professor Hajime Mizukami, Ph.D. |

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Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

…….. Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Professor Kittisak Likhitwitayawuid, Ph.D.)

…….. Thesis Advisor

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

…….. Thesis Co-Advisor

(Assistant Professor Suchada Sukrong, Ph.D.)

…….. Thesis Co-Advisor

(Professor Hajime Mizukami, Ph.D.)

…….. Examiner

(Associate Professor Surattana Amnuoypol, Ph.D.)

…….. External Examiner

(Naowarat Kanchanakhan, Ph.D.)

…….. External Examiner

(Piyarat Chareonsap, Ph.D.)

จุฑาทิพย์ มานิสสรณ์ : การวิเคราะห์อณูพันธุศาสตร์ของพืชสกุล *Phyllanthus* ที่พบในประเทศไทยโดยใช้วิธีอาร์เอพีดีร่วมกับการหาลำดับนิวคลีโอไทด์ (MOLECULAR ANALYSIS OF *PHYLLANTHUS* SPP. IN THAILAND BASED ON RAPD AND DNA SEQUENCING) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. นิจศิริ เรืองรังษี, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร. สุชาดา สุขหร่อง, Professor Hajime Mizukami, Ph.D., 124 หน้า.

พืชสกุลมะขามป้อม (*Phyllanthus* spp.) วงศ์ Euphorbiaceae มีการกระจายพันธุ์อยู่ในบริเวณเขตร้อนและกึ่งร้อน พืชในสกุลนี้เป็นแหล่งของสารจากธรรมชาติและสารออกฤทธิ์ทางชีวภาพที่สำคัญจึงถูกนำมาใช้เป็นยาในหลายประเทศ ฤทธิ์ทางยาที่สำคัญของพืชสกุล *Phyllanthus* ได้แก่ ฤทธิ์ลดไข้ แก้ปวด ต้านการอักเสบ ต้านความเป็นพิษต่อตับ และต้านไวรัส ในการวิจัยนี้ ได้ศึกษาความผันแปรทางพันธุกรรมของพืชสกุล *Phyllanthus* จำนวน 23 ชนิด ที่พบในประเทศไทย โดยใช้วิธีอาร์เอพีดีในการจำแนกพืชสกุล *Phyllanthus* จากการคัดเลือกไพรเมอร์แบบสุ่มทั้งหมดจำนวน 80 ไพรเมอร์ พบว่ามี 9 ไพรเมอร์ ที่สามารถบอกความแตกต่างทางพันธุกรรม ได้แก่ OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, OPS-19, OPD-02, OPD-04, และ OPD-07 และสามารถสร้างเป็นลายพิมพ์ดีเอ็นเอที่แสดงเอกลักษณ์จำเพาะต่อ *Phyllanthus* ทั้ง 12 ชนิด จากการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ internal transcribed spacers (ITS) ซึ่งเป็นดีเอ็นเอในนิวเคลียส นำมาสร้างเป็นเครื่องหมายโมเลกุลพีซีอาร์-อาร์เอฟแอลพี สามารถใช้ในการจำแนกพืชสมุนไพร *Phyllanthus* ที่สำคัญทั้ง 3 ชนิด ออกจากกัน คือ *P. amarus*, *P. debilis*, และ *P. urinaria* และพบว่าเทคนิคนี้ยังประสบความสำเร็จในการนำไปประยุกต์ใช้กับตัวอย่างเครื่องยาสมุนไพรที่ได้มาจากตลาดเครื่องยาในประเทศไทยด้วย

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| ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ | ลายมือชื่อนิสิต ..... |
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| ปีการศึกษา 2553 | ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ..... |
| | ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ..... |

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JUTHATIP MANISSORN : MOLECULAR ANALYSIS OF *PHYLLANTHUS* SPP. IN THAILAND BASED ON RAPD AND DNA SEQUENCING. THESIS ADVISOR : ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., THESIS CO-ADVISOR : ASSIST. PROF. SUCHADA SUKRONG, Ph.D., PROF. HAJIME MIZUKAMI. Ph.D., 124 pp.

The genus *Phyllanthus* (Euphorbiaceae) is distributed in tropical and subtropical regions, and its members are widely used as medicinal plants in many countries. Plants in this genus are important source of natural products and bioactive compounds. The medicinal effects of *Phyllanthus* are diverse including antipyretic, analgesic, anti-inflammatory, antihepatotoxic and antiviral activity. In this study the genetic variation of twenty-three *Phyllanthus* species existing in Thailand were analyzed. Random amplified polymorphic DNA (RAPD) markers were used to discriminate between *Phyllanthus* species. Nine out of eighty arbitrary primers were screened for DNA polymorphism. Primer OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, OPS-19, OPD-02, OPD-04, and OPD-07 produced unique DNA fingerprints that individuated the twelve *Phyllanthus* species. In this study, we also analyzed the nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. Furthermore, a simple protocol to discriminate three important medicinal *Phyllanthus* species, *P. amarus*, *P. debilis*, and *P. urinaria*, was developed using a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method based on ITS sequences and successfully applied to the crude drug samples obtained in Thai markets.

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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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# CONTENTS

CHAPTER Page

CHAPTER Page

# LIST OF TABLES

# LIST OF FIGURES

Figure Page

Figure Page

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5.8s rDNA | 5.8s ribosomal DNA |
| 18s rDNA | 18s ribosomal DNA |
| 26s rDNA | 26s ribosomal DNA |
| A, T, C, G | nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively |
| AFLP | amplified fragment length polymorphism |
| bp | base pair |
| °C | degree celcius |
| CI | consistency index |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP) |
| EDTA | ethylenediaminetetraacetic acid |
| g | gram |
| h | hour |
| ITS | internal transcribed spacer |
| kb | kilobase |
| L | liter |
| M | molar |
| $MgCl_2$ | magnesium chloride |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| ng | nanogram |
| nrDNA | nuclear ribosomal DNA |
| nt | nucleotide |
| PAUP | phylogenetic analysis using parsimony |

| | |
|---|---|
| PCR-RFLP | polymerase chain reaction – restriction fragment length polymorphism |
| RC | rescaled consistency index |
| pH | the negative logarithm of the concentration of hydrogen ions |
| RAPD | random amplified polymorphic DNA |
| rDNA | ribosomal deoxyribonucleic acid |
| RFLP | restriction fragment length polymorphism |
| RI | retention index |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| RNase A | ribonuclease A |
| rpm | revolution per minute |
| SCAR | sequence characterized amplified regions |
| sp. | species |
| TAE buffer | tris-acetate and EDTA buffer |
| U | unit |
| UV | ultraviolet |
| µg | microgram |
| µl | microliter |
| µM | micromolar |

# CHAPTER I

# INTRODUCTION

*Phyllanthus* is a large genus of the family Euphorbiaceae. It comprises over 800 species, distributed in tropical and subtropical regions (Govaerts *et al*., 2000). We found 36 species of trees, shrubs, or herbs of this genus in Thailand (Chantaranothai, 2007). Plants of the genus *Phyllanthus* are important sources of medicines, exhibiting a wide array of pharmacological activities including diuretic, antipyretic, antioxidative, and anti-inflammatory effects. They are used as folk medicines in many countries such as India, China, and Thailand to treat jaundice, coughs, diabetes, fevers, and stomachaches (Chang *et al*., 2003; Rao *et al*., 2006; Unander *et al*., 1992; Unander *et al*., 1995; Zhang *et al*., 2004). Among medicinal *Phyllanthus* species, *P. amarus* has attracted attention because it shows effective hepatoprotective activity due to anti-hepatitis B virus activity (Ott *et al*., 1997; Rai *et al*., 2005; Unander *et al*., 1991). In Thailand, *P. amarus*, *P. debilis*, and *P. urinaria* are popular medicinal plants found in the herbal markets. *P. amarus* and *P. debilis* are known as “Luk Tai Bai (ลูกใต้ใบ)” while *P. urinaria* is called “Ya Tai Bai (หญ้าใต้ใบ)” in Thai. Furthermore, the dried aerial parts of these plants are well known as “Luk Tai Bai (ลูกใต้ใบ)” regardless of the original species, in the markets and used mostly as anti-fever treatments. These three species have very similar habitats and thus are difficult to identify morphologically, which leads to confusion and misuse of crude drugs.

In the past few decades, DNA-based techniques have been applied to authenticate important medicinal plants and crude drugs because they are less affected by age or environmental factors (Joshi *et al*., 2004) and a small sample from non-specific tissue is sufficient for analysis (Mihalov *et al*., 2000; Zhang *et al*., 2007). Random amplified polymorphic DNA (RAPD) is a robust and simple assay and one of the most commonly used DNA techniques for primary assay of genomic DNA (Bussell *et al*., 2005; Hon *et al*., 2003; Na *et al*., 2004). One of the most important applications of RAPD is the identification of plant species with no prior sequence data or clarification of the difference between species (Williams *et al*., 1990). Moreover, ribosomal RNA (rRNA) genes are an attractive target for molecular analysis, because (1) a large number of

copies of these genes are present in the plant genome, and (2) the regions encoding 18S-, 5.8S-, and 26S-rRNA are highly conserved. Variable internal transcribed spacers (ITS1 and ITS2) are useful as DNA markers for plant identification (Joshi *et al*., 2004). However, to reduce cost and time PCR-RFLP is the appropriate approach to use for detect an adulterant in herbal medicine (Cai *et al*., 1999; Guo *et al*., 2006; Hon *et al*., 2003). In this method the target DNA even known entire sequence is first amplified by PCR and then digested with a restriction endonuclease to generate a unique restriction profile.

The objectives of this dissertation were to discriminate *Phyllanthus* species in Thailand based on the RAPD technique and determined nucleotide sequences of their nuclear internal transcribed spacer (ITS) 1-5.8S ribosomal DNA (rDNA)-ITS2 of rRNA gene. Based on the sequence alignment of these plant samples together with the sequences retrieved from the DNA database, we analyzed the molecular phylogenetic relationship of *Phyllanthus* species distributed in Thailand for the first time. Furthermore, we established a polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) approach to discriminate between the three Thai medicinal plants, *P. amarus*, *P. debilis*, and *P. urinaria*, and to authenticate the source species of the important Thai crude drug “Luk Tai Bai”.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Genus *Phyllanthus*

The genus *Phyllanthus* was first described by Linnaeus (1753). It comprises 750-800 species (Radcliffe-Smith, 2001) mainly in the tropics and subtropics. The genus is often superficially similar to *Breynia*, *Glochidion* and *Sauropus*. Some studies have upheld the genus as separate entity (van Welzen, 2000), although there are new molecular data that suggest it is paraphyletic (Kathriarachchi *et al*., 2006). *Phyllanthus* is left in the Euphobiaceae to maintain consistency with the Flora of Thailand (Chantaranothai, 2005; Chayamarit and van Welzen, 2005) although recent research firmly establishes it in a separate family Phyllanthaceae (Wurdack *et al*., 2005)

### 2.1.1 Taxonomic study

The taxonomic description of *Phyllanthus* spp. was reported by Chantaranothai (2007) as following

"*Phyllanthus* spp. are trees, shrubs or herbs (or climbing), often with the stems and branches differentiated into orthotropic long shoots of unlimited growth and plagiotropic leafy and/or floriferous shoots of limited growth, resembling pinnate leaves. *Indumentum* simple hairs, rarely dendritic. *Leaves* often of 2 types: scale-like cataphylls on the orthotropic shoots, and foliage-leaves usually only on the plagiotropic shoots. *Foliage-leaves* alternate, often distichous, shortly petolate, stipulate, simple, entire, penninerved; nerves brochidodromous. *Flowers* small, axillary; staminate flower usually fasciculate in the lower axils; postulate flowers solitary in the upper leaf axils, rarely the fascicles bisexual. *Staminate flowers* pedicellate; pedicel often filiform; sepals (4)5 or 6, subequal, imbricate; petals absent; disc glands (4)5 or 6, free, altermisepalous, or rarely disc annular; stamens 2-6, filaments free or some or all partially or completely fused, anthers basifixed, extrorse; pistillode absent. *Pistillate flowers*: pedicel more robust than

in the staminate flower; sepals more or less as in the staminate flower but often larger; petals absent; disc annular or copular, entire or variously-lobate, rarely of distinct glands; ovary sessile or stipitate, 3(-10)-locular, ovules 2 per locule; styles 3(4), free or slightly connate; stigmas usually recurved. *Fruite* either a 3 (or more)-lobed capsule, dry, septicidally and loculicidally dehiscent into 3 or more 2-valved cocci or separate valves, or the fruit fleshy and more or less indehiscent. Seeds 2 per locule, segmentiform, trigonous, dorsally convex, rarely plano-convex, verruculose, tuberculate, ridged, lineate or smooth, ecarunculate".

There are 36 species in Thailand (Table 2.1), at least 700 species in the tropics (Chantaranothai, 2007). Plant morphology of *Phyllanthus* species is shown in Appendix A.

**Table 2.1** The list of *Phyllanthus* species in Thailand

| No. | Scientific name | Thai name |
|---|---|---|
| 1 | *Phyllanthus acidus* (L.) Skeels | Mayom (มะยม) (General); Star gooseberry |
| 2 | *P. acutissimus* Miq. | Chan tia (จันเตี้ย) (Loei); Pla lai phueak (ปลาไหลเผือก) (Nakhon Ratchasima); Phak wan chang khlong (ผักหวานช้างโขลง) (Nakhon Si Thammarat) |
| 3 | *P. airy-shawii* Brunel & J.P.Roux | - |
| 4 | *P. albidiscus* (Ridl.) Airy Shaw | Phak wan tai (ผักหวานใต้) (Peninsular) |
| 5 | *P. amarus* Schum. & Thonn. | Ma kham pom din (มะขามป้อมดิน) (Northern); Luk tai bai (ลูกใต้ใบ) (Central); Ya tai bai khao (หญ้าใต้ใบขาว) (Surat thani) |
| 6 | *P. ankorensis* Beille in Lecomte | Siao yai (เสียวใหญ่) (Udon Thani); Mayom khamen (มะยมเขมร) (Eastern) |
| 7 | *P. caroliniensis* Walter | - |

| No. | Scientific name | Thai name |
|---|---|---|
| 8 | *P. chamaepeuce* Ridl. | Ma kham khwak (มะขามขวัก); Makham nam (มะขามน้ำ) (Lampang) |
| 9 | *P. clarkei* Hook.f. | Mayom doi (มะยมดอย) (Chiang Mai) |
| 10 | *P. collinsiae* Craib | Kha naeng phroi (แขนงพร้อย), Khang ten (ค่างเต้น), Cha kham pom (ช้าขามป้อม) (Prachuap Khiri Khan); Siao (เสี่ยว) (Saraburi) |
| 11 | *P. columnaris* Müll.Arg. | Khao san (ข้าวสาร) (General); Ma kham pom din luang (มะขามป้อมดินหลวง) (Chiang Mai); San tui (สานตุ้ย) (Northern); Si siat cho (สีเสียดช่อ) (Peninsular); Hue-ni (ฮือนิ) (Karen-mae Hong Son) |
| 12 | *P. debilis* Klein ex Willd. | Luk tai bai (ลูกใต้ใบ) |
| 13 | *P. elegans* Wall. ex Müll.Arg. | Cha phak wan (จ๊าผักหวาน) (Lamphun); Ton tai bai (ต้นใต้ใบ), Tai bai thuean (ใต้ใบเถื่อน), Phak wan chang (ผักหวานช้าง) (Surat Thani); Phak wan (ผักหวาน) (Satun); Phak wan khwae (ผักหวานแขว ) (Chumphon); Phak wan song (ผักหวานดง) (Trat) |
| 14 | *P. emblica* L. | Kan-tot (กันโตด) (Khmer-Chanthaburi); Kam thuat (กำทวด) (Ratchavuri); Ma kham pom (มะขามป้อม) (General); Mang-luu (มั่งลู่), San-yaa-saa (สันยาสา) (Karen-Mae Hong Son); Emblic myrabolan, Malacca tree |
| 15 | *P. geoffrayi* Beille in Lecomte | Sa-phak-wan(ซ้าผักหวาน) (Laos); Phak wan (ผักหวาน) (Prachin Buri) |

| No. | Scientific name | Thai name |
|---|---|---|
| 16 | *P. gracilipes* (Miq.) Müll.Arg. | Chum racha (ชุมราชา), Phak wan raet (ผักหวานแรด) (Pattani) |
| 17 | *P. harmandii* Beille in Lecomte | - |
| 18 | *P. kerrii* Airy Shaw | Mayom don (มะยมดอน) (General) |
| 19 | *P. lingulatus* Beille | Mayom nuea (มะยมเหนือ) (Northern) |
| 20 | *P. microcarpus* (Benth.) Müll.Arg. | Mayom dong (มะยมดง) (General); Mat come (หมัดคำ) |
| 21 | *P. mirabilis* Müll.Arg. | Khi lek ruesi (ขี้เหล็กฤาษี) (Saraburi); Ai thao (ไอ้เทา) (Phetchaburi) |
| 22 | *P. myrtifolius* (Wight) Müll.Arg. | - |
| 23 | *P. orientalis* (Craib) Airy Shaw | Kang pla (ก้างปลา) (Lampang), Kang pla thet (ก้างปลาเทศ), Ya som kung (หญ้าส้มกุ้ง) (Northern); Khamao raet (เขม่าแรด) (Saraburi) |
| 24 | *P. oxyphyllus* Miq. | Yom hin (ยมหิน) (Surat Thani); Yai chung lan (ยายจูงหลาน) (Chumphon); Yai thip lan (ยายถีบหลาน) (Trang) |
| 25 | *P. pachyphyllus* Müll.Arg. | Mayom tai (มะยมใต้) (Peninsular) |
| 26 | *P. pulcher* Wall. ex Müll.Arg. | Kra thuep yop (กระทืบยอบ) (Chumphon); Kang pla (ก้างปลา) (Narathiwat); Kang pla din (ก้างปลาดิน), Dok tai bai (ดอกใต้ใบ) (Nakhon Si Thammarat); Kang pla daeng (ก้างปลาแดง), Khrip yot (ครีบยอด) (Surat Thani); Khot sai (Songkhla); Dee-ko-noh (เดอก้อเนาะ) (Malay-Narathiwat); Trueng baa daan (ตรึงบาดาล) (Prachuap Khiri Khan); Ru ri (รุรี) (Satun); Wan thorani san (ว่านธรณีสาร), Saniat (เสนียด) (Bangkok) |

| No. | Scientific name | Thai name |
|---|---|---|
| 27 | *P. pulcheroides* Beille in Lecomte | Mayom chat (มะยมฉัตร) (Loei) |
| 28 | *P. reticulatus* Poir. | Kraong (กระออง) (Prachuap Khiri Khan); Kang pla khao (ก้างปลาขาว) (Ang Thong, Chiang Mai); Kang pla khruea (ก้างปลาเครือ) (General); Kang pla daeng (ก้างปลาแดง) (Surat Thani); Kla khlong (ข่าคล่อง) (Suphan Buri); Ta-kha-kho-khuey (ต่าคะโคคึย), Sa-brae-thi (สะแบรที่) (Karen-Mae Hong Son); Mat kham (หมัดคำ) (Phrae); Ma yiao (หมาเยี่ยว) (Nakhon Pathom); Am ai (อำอ้าย) (Nakhon Ratchasima) |
| 29 | *P. ridleyanus* Airy Shaw | Mayom bai khaeng (มะยมใบแข็ง) (General) |
| 30 | *P. roseus* (Craib & Hutch.) Beille in Lecomte | Phak yot tong (ผักยอดทอง) (Chiang Mai); Ma yom pa (มะยมป่า) (Chanthaburi) |
| 31 | *P. sikkimensis* Müll.Arg. | Phak wan khao (ผักหวานเขา) (Chumphon) |
| 32 | *P. sootepensis* Craib | Plia luk phon (เปลี้ยลูกฟ้อน) (Lampang); Ma kham pom din (มะขามป้องดิน) (Chiang Mai) |
| 33 | *P. taxodiifolius* Beille | Khrai hang nak (ไคร้หางนาค), Siao nam (เสียวน้ำ), (Prachin Buri); Ta khrai hang sing (ตะไคร้หางสิงห์) (Suphan Buri); Siao noi (เสียวน้อย), Siao lek (เสียวเล็ก) (Khon Kaen) |

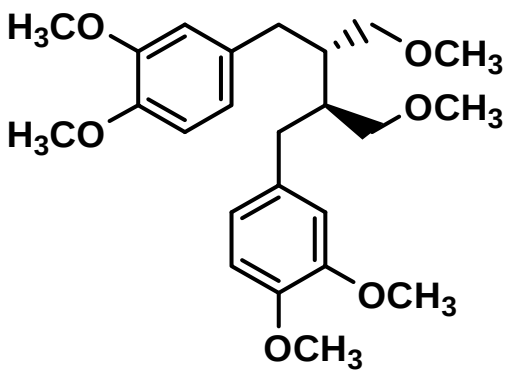
| No. | Scientific name | Thai name |
|---|---|---|
| 34 | *P. urinaria* L. | Fai duean ha (ไฟเดือนห้า) (Chon Buri); Ma kham pom din (มะขามป้อมดิน) (Northern); Ya tai bai (หญ้าใต้ใบ) (Ang Thong, Surat Thani); Mak khai lang (หมากไข่หลัง) (Loei) |
| 35 | *P. virgatus* G.Forst. | Khang amphai (ขางอำไพ) (Phrae); Phaeng kham hoi (แพงคำห้อย) (Si Sa Ket); Luk tai bai (ลูกใต้ใบ) (Central) |
| 36 | *P. welwitschianus* Müll.Arg. | Mayom thiam (มะยมเทียม) |

### 2.1.2 Phytochemical study and medicinal usage in *Phyllanthus* spp.

Genus *Phyllanthus* has been the subject of much phytochemical research to determine the active constituents and their pharmacological activities. This genus is a rich source of phytochemical compounds. Various groups of medicinally interesting compounds are present in *Phyllanthus*, including lignans, terpenoids, flavonoids, alkaloids, and ellagitannins. These compounds are found in the leaf, stem and root of the plants (de Padus *et al*., 1999; Youkwan *et al*., 2005; Zhang *et al*., 2004). Major lignans are isolated from *Phyllanthus* spp. such as phyllanthin (Figure 2.1A) and hypophyllanthin (Figure 2.1B) (Chang *et al*., 2003; Khatoon *et al*., 2006).

*Phyllanthus* plants have been shown to possess cytotoxic and antioxidant activities (Chang *et al*., 2003; Xu *et al*., 2007). Moreover, lignans from *Phyllanthus* plants are reported to reduce hepatotoxicity (Naaz *et al*., 2007; Padma and Setty, 1999; Pramyothin *et al*., 2007; Shyamsunder *et al*, 1985). In Thai folk medicine, many species of *Phyllanthus* have long been used such as *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. acidus*, *P. pulcher, P. debilis, P. virgatus, P. ankorensis, P. reticulatus, P. oxyphyllus, P. roseus, and P. welwitschianus* (คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล, 2539, 2541, 2543; ลีนา ผู้พัฒนพงศ์, 2530; วุฒิ วุฒิธรรมเวช, 2540; Rao *et al*., 2006; Unander *et al*., 1990; Unander *et al*., 1992). The medicinal effect of *Phyllanthus* species are treated jaundice, diabetes, cough, fevers, and stomachaches (Chang *et al*., 2003; Padma and Setty, 1999; Sousa *et al*., 2007; Unander *et al*., 1995; Zhang *et al*., 2004) The herbaceous plants *P. amarus* is an important herbal medicine due to its effectively antiviral activities especially anti-hepatitis B virus (Bhattacharyya *et al*., 2003; Ott *et al*., 1997; Rai *et al*., 2005; Unander *et al*., 1991). Other *Phyllanthus* species are also used for dye and tanning purposes (*P. emblica*, *P. reticulatus*), as edible fruits (*P. acidus, P. emblica*), and as ornamentals (*P. pulcher*) (de Padus *et al*., 1999).

(A)




(B)

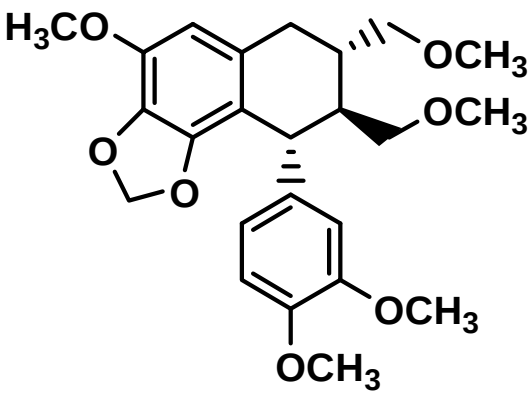



**Figure 2.1** Structure of (A) Phyllanthin and (B) Hypophyllanthin.

## 2.2 Molecular markers

### 2.2.1 PCR-based molecular markers

PCR-based molecular markers have now become a popular means for the identification and authentication of medicinal plants. Various types of PCR-based molecular techniques are utilized to evaluate DNA polymorphism. These techniques include RAPD, SCAR, PCR-RFLP.

**Random Amplified Polymorphic DNA (RAPD)**

PCR with random sequence primers offers a fast approach to genome profiling. The most popular technique of this type is called RAPD. RAPD were first developed in 1990 using PCR to randomly anonymous segments of nuclear DNA (Williams *et al*., 1990). RAPD marker has frequently been used for the detection of the genetic variability in plants. The advantage of the approach are its rapidity, simplicity, and the absence of any need for prior genetic information of the plant (Hon *et al*, 2003). In the reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template (Sharma *et al*., 2008). The primers used are normally 10 bases in length. This is short enough for annealing to occur at a number of positions in the genome. When annealing of the single primer in the reaction occurs on opposite strands of a strength of DNA which is short enough to form a template for PCR, a product will be formed. It is probable that this situation will occur at several points in the genome, giving rise to several PCR products which can be resolved by gel electrophoresis to give a characteristic pattern. Small sequence differences at a point of primer annealing will prevent annealing and amplification from that part of the genome. Additionally, insertions or deletions between primer annealing positions will lead to a change in the length of the PCR product (Hammond and Spanswick, 1997). RAPD marker was applied to differentiate various kinds of medicinal plants. For instance, it was used to differentiate Thai *Piper* from Japanese *Piper* (Chaveerach *et al*., 2002). This marker has been used for detecting the origin between Korean and Chinese Astragali radix (Na, *et. al*., 2004) and used to confirm the relationships among *Boesenbergia*

species (Vanijajiva *et al*., 2005). However, the limitation of RAPD is poor reproducibility of PCR product.

**Sequence Characterized Amplified Region (SCAR)**

SCAR marker can be used for detection or differentiation of samples by using specific primers designed from polymorphic RAPD. The main steps of this method are first identification of a polymorphic band using RAPD, then nucleotide sequencing of the polymorphic band, and finally PCR reproduction of the polymorphic band as template DNA using a pair of long primer (25-30 bp) that are specific to the band. The conversion of a RAPD to a SCAR improves reproducibility of PCR product and enhances the discriminatory power and reliability of the identification methods (Hui *et al*., 2000). For instance, this method has been used to discriminate *Artemisia* Herbs (Lee *et al*., 2006) and used to authenticate of *Pueraria tuberosa* (Roxb. ex. Willd.) DC. in Indian medicinal herbs (Devaiah and Venkatasubramanian, 2008).

**Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP)**

The concept of PCR-RFLP is amplification of DNA fragments from know primers and digested the product with restriction enzymes. This technique uses endonucleases to digest PCR products of regions with sequence polymorphisms. By using an endonuclease which recognizes and cleaves at the polymorphic sites, the digestion of a longer PCR fragment into smaller fragments will change the banding pattern (Yip *et al*, 2007). PCR-RFLP has been use for authentication of various medicinal plants, for instance, authenticate of *Panax* species (Um *et al*., 2001), *Atractylodes* species (Mizukami *et al*., 2000), *Rheum* species (Yang *et al*., 2004) and differentiate of *Codonopsis* from their adulterants (Fu *et al*., 1999). Screening PCR products by using various restriction enzymes can be an alternative of sequencing to find out polymorphic regions among samples. This method is more reproducible than random priming methods, but it is limited by the degree of polymorphism among individuals within a species.

### 2.2.2 DNA sequencing-based markers

DNA polymorphisms can be studies by several methods but the most direct approach is determination of nucleotide sequences of a defined region. For this technique, the primer is specifically designed based on a defined region of gene sequences. Variations due to transitions, transversion, insertion or deletion can be assessed directly and information on a defined locus can be obtained (Joshi *et al*., 2004). This approach provides a highly reproducible and informative data that can be used for identifying species. Currently, DNA sequencing is applied to distinguish species and study phylogenetic relationship, population genetics, systematics and evolution (Zhang *et al*., 2007). There are many reports concerning the application of DNA sequencing-based markers to differentiate medicinal plants from its substitutes or adulterants. Most of them involves the sequencing of internal transcribed spacer (ITS) of ribosomal DNA (rDNA) (Mihalov *et al*., 2000), *trn*K genes (Mizukami *et al*., 1998), 5S-rRNA gene (Ma *et al*., 2000), 18S rRNA gene (Zhu *et al*., 2003), *mat*K (Liu *et al*., 2009) and *trn*H-*psb*A Chloroplast DNA (Vangsak *et al*., 2008).

## 2.3 Phylogenetic analysis

Phylogenetic analysis of DNA or protein sequences has become an important tool for studying the evolutionary history of organisms. Since the rate of sequence evolution varies extensively with gene or DNA segment, one can study the evolutionary relationships of virtually all levels of classification of organisms such as families, genera, species, and intra-specific populations by using different genes or DNA segments (Li, 1997).

Traditionally, phylogenies have been constructed from morphological data but following the growth of genetic information it has become common practice to construct phylogenies based on molecular data. The data is most commonly in the form of DNA or protein sequences but can also be in the form of molecular marker such as RAPD, AFLP or microsatellite. The most important method for inferring phylogenetic relationships of

life is that of acquiring DNA sequences. Comparisons of homologous regions of DNA among the taxa under study yield the characters and character states that are used to infer relationships in phylogenetic analyses (Simpson, 2006). Each molecular marker provides different type of data. Nevertheless, these can be gathered to two categories, distance based method and character based method.

### 2.3.1 Distance based method

#### Unweighted Pair-Group Method with Arithmetic Mean (UPGMA)

UPGMA is a clustering method. The program first finds the pair of taxa with the smallest distance between them and defines the branching between them as half of that distance in effect placing a node at the midpoint of the branch. It then combines the two taxa into a “cluster” and rewrites the matrix with the distance from the cluster to each of the remaining taxa. Since the “cluster” serves as a substitute for two taxa, the number of entries in the matrix is now reduce by one. That process is repeated on the new matrix and reiterated until the matrix consists of a single entry. That set of matrices is then used to build up the tree by starting at the root and moving out to the first two nodes represented by the last two clusters (Hall, 2004). UPGMA is intended to reconstruct a species tree, although topological errors often occur when the rate of gene substitution is not constant or when the number of genes or nucleotides used is small (Nei and Kumar, 2000).

#### Neighbor Joining (NJ)

NJ is similar to UPGMA in that it manipulates a distance matrix, reducing it in size at each step, then reconstructs the tree from that series of matrices. It differs from UPGMA in that it does not construct clusters but directly calculates distances to internal nodes. (Hall, 2004; Saitou and Nei, 1987). One of the important concepts in the NJ method is neighbors, which are defined as two taxa that are connected by a single node in an unrooted tree (Nei and Kumar, 2000). NJ is, like parsimony, a minimum-change

method, but it does not guarantee finding the tree with the smallest overall distance (Hall, 2004).

### 2.3.2 Character based method

#### Maximum Parsimony (MP)

MP is one of the most commonly use for phylogenetic analysis. Parsimony is based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment. The algorithm is used to evaluate a possible tree at each informative site (Hall, 2004). MP is a computationally hard problem so heuristics are used to find good solutions. Since there can be equally good solutions, the majority consensus tree of the set of optimal solution is returned.

#### Maximum Likelihood (ML)

ML is currently considered to be one of the most reliable criteria for phylogenetic inference from nucleotide or amino acid sequence data. In the ML method of the phylogenetic inferences the likelihood of observing a given set of sequence data for a specific substitution model is maximized for each topology, and the topology that gives the highest maximum likelihood is chosen as the final tree (Nei and Kumar, 2000). Construction of ML trees is extremely time-consuming, especially when large data and complex substitution models are used (Piontkivska, 2004).

#### Bootstrap values

A popular way of evaluating the reliability of an inferred phylogenetic tree is bootstrap analysis. The concept of bootstrap analysis is to re-sample the alignment columns with replacement. A high bootstrap score is a sign of greater reliability.

## 2.4 Molecular study of nuclear Internal Transcribed Spacer (ITS)

Nuclear DNA data provide valuable information in phylogenetic study of plants, and the internal transcribed spacer (ITS) of the nrDNA have been used in numerous systematic studies at the generic and specific levels of a wide array of plant taxa (Baldwin, 1992, 1993; Baldwin *et al*, 1995, Sang *et al*, 1995). The internal transcribed spacers, ITS1 and ITS2, are located between genes encoding the 5.8S, 18S and 26S nuclear ribosomal RNA (nrRNA) subunits (Figure 2.2). The ITS1 and ITS2 spacers, in addition to the 5.8 nrRNA gene are referred to as the ITS region.


**Figure 2.2** Schematic diagram of the nuclear rDNA internal transcribed spacer region. The three rDNA subunits: 18S, 5.8S and 26S are separated by internal transcribed spacers (ITS1 and ITS2).

Several factors make the ITS region valuable for use in phylogenetic analyses at interspecific-level and intergeneric-level among angiosperms and other eukaryotes (Baldwin *et al*., 1995). First, the ITS region is highly repeated in plant nuclear genomes, along with the other components of the nrDNA multigene family including a highly variable region between the ribosomal repeat, the intergenic spacer. The high copy number of the nrDNA repeat facilitates the amplification and sequencing of the nrDNA. Second, the nrDNA multigene family undergoes rapid concerted evolution. This property of the ITS region is most important from a phylogenetic standpoint and promotes accurate reconstruction of species relationships from sequencing. However, non-

homologous copies are occasionally present with point mutations and/or insertion/ deletion events, causing small variation among the copies within a species. Third, the small size of the ITS region (<700 bp in angiosperms) and the presence of highly conserved sequences flanking each of the two spacers make this region easy to amplify, even from herbarium material, using universal eukaryotic primers.

The ITS1 sequences from different families are virtually too diverse to unambiguously align. In spite of this variability, Liu and Schardl (1994) reported that a conserved sequence, GGCRY-(4 to 7n)-GYGYCAAGGAA (Y = C or T; R = G or A; n = A or G or C or T), is present in ITS1 of many flowering plant species. Conservation of the AAGGAA motif suggests that it may be such a recognition factor.

In recent literature, the ITS region is frequently used for authentication of medicinal plants such as *Dendrobium* (Lau *et al*., 2000; Ding *et al*., 2002), *Atractylodes* (Guo *et al*., 2006; Shiba *et al*., 2006) *Angelica* (Feng *et al*., 2010). Another example of the utility of ITS is detection of narcotic species: *Mitragyna speciosa* (Sukrong *et al*., 2007).

For plant systematic, the ITS region was able been used in reconstructing a phylogeny such as *Panax* (Araliaceae) (Wen and Zimmer, 1996), and *Clerodendrum* (Lamiacea) (Steane *et al*., 1999).

## 2.5 Molecular markers in *Phyllanthus* spp.

Classification of the genus *Phyllanthus* has long been controversial as this genus shares many overlapping vegetative and floral characters with other members of the Phyllanthaceae and Euphorbiaceae (APG, 2003), and very few morphological characters can be designated as representative indicators for identification purposes.

In Thailand, there are several *Phyllanthus* species have been used in traditional medicine and some species are subjected for phytochemical analysis. However, there have been a few molecular reported in this genus. By using RAPD markers, Jain *et al*. (2003), have studied the molecular diversity in *Phyllanthus amarus* and develop SCAR markers for identification of *P. amarus*, *P. fraternus*, *P. debilis* and *P. urinaria* in Indian herbal drug (Jain *et al*., 2008). Moreover, RAPD-SCAR marker has developed for identification of *Phyllanthus emblica* Linn. (Dnyaneshwar *et al*., 2006) and identification of *P. amarus*, *P. debilis*, and *P. urinaria* (Theerakulpisut *et al*., 2008). However, there have not been reported for authentication in Thai herbal drug.

As for molecular phylogenetic analysis by DNA sequencing, there have been several studied in Family Phyllanthaceae. Wurdack *et al*. (2004) have studied plastid *rbc*L and Samuel *et al*. (2005) studied platid *mat*K and nuclear *phy*C sequences. In addition, Kathriarachchi *et al*. (2005) have analysed molecular phylogenetics of Phyllanthaceae family inferred from five genes (plastid *atp*B, *mat*K, 3'*ndh*F, *rbc*L and nuclear *phy*C) and analysed nuclear ITS and plastid *mat*K sequence data in Tribe Phyllantheae (Kathriarachchi *et al*., 2006). Lee *et al*. (2006) have investigated phylogeny of medicinal *Phyllanthus* species in China based on nuclear ITS and chloroplast *atp*B and *rbc*L sequences. They also developed multiplex PCR for detection of *P. amaurs*, *P. niruri* and *P. urinaria*.

As previous reports, there have been many molecular studies of family Phyllanthaceae. However, there have been a few reports in Thailand. Due to the medicinal usage of *Phyllanthus* spp. and questionable classification based on morphological characters, a preliminary molecular marker was analyzed using RAPD

analysis. Furthermore, a phylogenetic study was conducted using a highly variable region of nuclear ribosomal DNA and the simple technique, PCR-RFLP was applied to generated the DNA fingerprinting for the important medicinal *Phyllanthus* species.

# CHAPTER III

# RAPD ANALYSIS OF *PHYLLANTHUS* SPP.

## 3.1 Materials and methods

### 3.1.1 Plant materials

In this present study, 51 individuals from twelve *Phyllanthus* species, *Phyllanthus amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius, P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. ankorensis*, and *P. reticulatus*, were collected from various locations in Thailand. Plant specimens were identified by Assoc. Prof. Dr. Nijsiri Ruangrungsi and Assoc. Prof. Thatree Phadungcharoen. Voucher specimens are deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Plant materials are presented in Table 3.1.

**Table 3.1** List of *Phyllanthus* species used for RAPD analysis.

| Species | Sample Codes | Provinces | Date of Collection | Voucher ID |
|---|---|---|---|---|
| *P. amarus* Schum. & Thonn. | P1B1 | Bangkok | 10.2005 | MJT-4810101 |
| | P1K1 | Kanchanaburi | 10.2005 | MJT-4810102 |
| | P1S1 | Sakon Nakhon | 10.2006 | MJT-4910103 |
| | P1PM1 | Nakhon Phanom | 10.2006 | MJT-4910106 |
| | P1NK1 | Nong Khai | 10.2006 | MJT-4910107 |
| | P1UT1 | Udon Thani | 10.2006 | MJT-4910108 |
| | P1KK1 | Khon Kaen | 10.2006 | MJT-4910109 |
| | P1CM1 | Chiang Mai | 11.2006 | MJT-4911110 |
| *P. urinaria* L. | P2B1 | Bangkok | 10.2005 | MJT-4810201 |
| | P2HK1 | Nong Khai | 10.2006 | MJT-4910204 |
| | P2NP1 | Nakhon Pathom | 03.2007 | MJT-5003205 |
| | P2RT1 | Ratchaburi | 09.2007 | NSR-5009206 |
| *P. emblica* L. | P3B1 | Bangkok | 10.2005 | MJT-4810301 |
| | P3S1 | Sakon Nakhon | 06.2006 | MJT-4906302 |
| | P3PM1 | Nakhon Phanom | 10.2006 | MJT-4910303 |
| | P3HK1 | Nong Khai | 10.2006 | MJT-4910304 |
| | P3UT1 | Udon Thani | 10.2006 | MJT-4910305 |
| | P3KS1 | Kalasin | 10.2006 | MJT-4910306 |
| | P3KK1 | Khon Kaen | 10.2006 | MJT-4910307 |
| | P3CM1 | Chiang Mai | 11.2006 | MJT-4911309 |
| *P. taxodiifolius* Beille | P4B1 | Bangkok | 10.2005 | MJT-4810401 |
| | P4NP1 | Nakhon Pathom | 01.2006 | MJT-4901402 |
| | P4PR1 | Prachinburi | 06.2007 | TRP-5006403 |
| *P. collinsiae* Craib | P5B1 | Bangkok | 10.2005 | MJT-4810501 |
| | P5R1 | Rayong | 10.2005 | TRP-4810503 |

| Species | Sample Codes | Provinces | Date of Collection | Voucher ID |
|---|---|---|---|---|
| *P. acidus* L. | P6B1 | Bangkok | 10.2005 | MJT-4810601 |
| | P6S1 | Sakon Nakhon | 06.2006 | MJT-4906602 |
| | P6NN1 | Nakhon Nayok | 10.2006 | TT-4910603 |
| | P6CR1 | Chiang Rai | 10.2006 | NR-4910604 |
| | P6KK1 | Khon Kaen | 10.2006 | MJT-4910611 |
| | P6NS1 | Nakhon Si Thammarat | 10.2006 | TT-4910612 |
| | P6ST1 | Satun | 10.2006 | TT-4910613 |
| | P6CM1 | Chiang Mai | 11.2006 | MJT-4911615 |
| *P. pulcher* Wall. ex Müll.Arg. | P7B1 | Bangkok | 10.2005 | MJT-4810701 |
| | P7K1 | Kanchanaburi | 10.2005 | MJT-4810702 |
| | P7CM1 | Chiang Mai | 11.2006 | MJT-4911703 |
| | P7NP1 | Nakhon Pathom | 03.2007 | MJT-5003704 |
| | P7R1 | Rayong | 08.2007 | NSR-5008705 |
| *P. debilis* Klein ex Willd. | P9B1 | Bangkok | 10.2005 | NSR-4810801 |
| | P9NP1 | Nakhon Pathom | 01.2006 | MJT-4901802 |
| *P. virgatus* G.Forst. | P12B1 | Bangkok | 10.2005 | TRP-4810901 |
| | P12PR1 | Prachinburi | 01.2006 | TRP-4901902 |
| | P12PL | Pattalung | 04.2007 | TRP-5004903 |
| | P12C1 | Chachoengsao | 08.2007 | TRP-5008904 |
| | P12R1 | Ratchaburi | 09.2007 | NSR-5009905 |
| *P. acutissimus* Miq. | P13C1 | Chachoengsao | 01.2006 | TRP-4901101 |
| | P13UB1 | Ubon Ratchathani | 04.2007 | TRP-5004102 |
| *P. ankorensis* Beille in Lecomte | P14NP1 | Nakhon Pathom | 01.2006 | MJT-4901111 |
| | P14NP2 | Nakhon Pathom | 03.2007 | NSR-5008112 |
| *P. reticulatus* Poir. | P17B1 | Bangkok | 02.2006 | MJT-4902121 |
| | P17NP1 | Nakhon Pathom | 03.2007 | MJT-5003124 |
| Total | 12 *Phyllanthus* species, 51 samples | | | |

### 3.1.2 DNA extraction from fresh specimens

Fresh and silica gel-dried leaves were ground in liquid nitrogen for DNA extraction. Total genomic DNA was extracted by DNeasy™ Plant Mini Kit (QIAGEN, Germany), follow the manufacturer's protocol. Then, genomic DNA was purified using GENECLEAN® II Kit (Qbiogene, Germany). Total genomic DNA was performed on 0.8% agarose gel electrophoresis stained by ethidium bromide and visualized under UV light to determine quality and quantity. 2-Log DNA Ladder® (New England BioLabs, USA) was used as standard molecular size. The extracted DNA was kept at -20°C for further use as template in PCR amplification.

### 3.1.3 Primer screening and PCR condition

PCR amplification were carried out using eighty arbitrary deca-oligonucleotide primers, kit D, N, O, and S, obtained from Operon DNA Technologies (Alameda, USA) (Table 3.2). Screening and selecting the proper primers was the prior step in PCR experiment for this study. The PCR reaction mixture in 25 µl contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100, 3.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM random primers, 0.6 U of *Taq* Polymerase (Promega, USA) and 5-40 ng of template DNA. RAPD amplification was carried out with the Eppendorf Mastercycler® (Perkin-Elmer, USA). Initial denaturation was at 95°C for 3 min, followed by 45 cycles of 95°C for 1 min, 36°C for 1 min, 72°C for 2 min and a final extension for 4 min. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. RAPD fragments were analyzed using Gel Doc™ XR System PC/Mac (Bio-Rad Laboratories, USA). The selected primers should give high enough polymorphism and a constant pattern was chosen for further study.

**Table 3.2** List of deca-oligonucleotide primers used for RAPD screening (Kit D, N, O, S).

| Primer code | Sequence (5′-3′) | Primer code | Sequence (5′-3′) |
|---|---|---|---|
| OPD-01 | ACCGCGAAGG | OPN-11 | TCGCCGCAAA |
| OPD-02 | GGACCCAACC | OPN-12 | CACAGACACC |
| OPD-03 | GTCGCCGTCA | OPN-13 | AGCGTCACTC |
| OPD-04 | TCTGGTGAGG | OPN-14 | TCGTGCGGGT |
| OPD-05 | TGAGCGGACA | OPN-15 | CAGCGACTGT |
| OPD-06 | ACCTGAACGG | OPN-16 | AAGCGACCTG |
| OPD-07 | TTGGCACGGG | OPN-17 | CATTGGGGAG |
| OPD-08 | GTGTGCCCCA | OPN-18 | GGTGAGGTCA |
| OPD-09 | CTCTGGAGAC | OPN-19 | GTCCGTACTG |
| OPD-10 | GGTCTACACC | OPN-20 | GGTGCTCCGT |
| OPD-11 | AGCGCCATTG | OPO-01 | GGCACGTAAG |
| OPD-12 | CACCGTATCC | OPO-02 | ACGTAGCGTC |
| OPD-13 | GGGGTGACGA | OPO-03 | CTGTTGCTAC |
| OPD-14 | CTTCCCCAAG | OPO-04 | AAGTCCGCTC |
| OPD-15 | CATCCGTGCT | OPO-05 | CCCAGTCACT |
| OPD-16 | AGGGCGTAAG | OPO-06 | CCACGGGAAG |
| OPD-17 | TTTCCCACGG | OPO-07 | CAGCACTGAC |
| OPD-18 | GAGAGCCAAC | OPO-08 | CCTCCAGTGT |
| OPD-19 | CTGGGGACTT | OPO-09 | TCCCACGCAA |
| OPD-20 | ACCCGGTCAC | OPO-10 | TCAGAGCGCC |
| OPN-01 | CTCACGTTGG | OPO-11 | GACAGGAGGT |
| OPN-02 | ACCAGGGGCA | OPO-12 | CAGTGCTGTG |
| OPN-03 | GGTACTCCCC | OPO-13 | GTCAGAGTCC |
| OPN-04 | GACCGACCCA | OPO-14 | AGCATGGCTC |
| OPN-05 | ACTGAACGCC | OPO-15 | TGGCGTCCTT |
| OPN-06 | GAGACGCACA | OPO-16 | TCGGCGGTTC |
| OPN-07 | CAGCCCAGAG | OPO-17 | GGCTTATGCC |
| OPN-08 | ACCTCAGCTC | OPO-18 | CTCGCTATCC |
| OPN-09 | TGCCGGCTTG | OPO-19 | GGTGCACGTT |
| OPN-10 | ACAACTGGGG | OPO-20 | ACACACGCTG |

| Primer code | Sequence (5′-3′) | Primer code | Sequence (5′-3′) |
|---|---|---|---|
| OPS-01 | CTACTGCGCT | OPS-11 | AGTCGGGTGG |
| OPS-02 | CCTCTGACTG | OPS-12 | CTGGGTGAGT |
| OPS-03 | CAGAGGTCCC | OPS-13 | GTCGTTCCTG |
| OPS-04 | CACCCCCTTG | OPS-14 | AAAGGGGTCC |
| OPS-05 | TTTGGGGCCT | OPS-15 | CAGTTCACGG |
| OPS-06 | GATACCTCGG | OPS-16 | AGGGGGTTCC |
| OPS-07 | TCCGATGCTG | OPS-17 | TGGGGACCAC |
| OPS-08 | TTCAGGGTGG | OPS-18 | CTGGCGAACT |
| OPS-09 | TCCTGGTCCC | OPS-19 | GAGTCAGCAG |
| OPS-10 | ACCGTTCCAG | OPS-20 | TCTGGACGGA |

### 3.1.4 Phylogenetic tree reconstruction

Standard DNA marker (Hyper Ladder I, Bioline, USA) was used to assign the size of each RAPD fragment. Only fragments that could be accurately scored (0.3–2.5 kb) were chosen. RAPD fragments were assigned a DNA length and recorded in a binary matrix for each individual as presence (1) or absence (0) of a given band. Phylogenetic relationship of *Phyllanthus* species was constructed using Dice clustering of NTSYS-pc version 2.11T (Exeter Software, Setauket, N.Y.) based on a UPGMA method (Nei and Li, 1979).

## 3.2 Results

### 3.2.1 DNA determination

Total genomic DNA was isolated from fresh and silica-gel dried leaves using a DNeasy Plant Mini Kit (Qiagen, Germany) purified using GENECLEAN® II Kit (Qbiogene, Germany) follow the manufacturer's protocol. From 1.0% agarose gel as compare with standard DNA markers, 2-Log DNA Ladder® (New England BioLabs, USA) and Hyper Ladder I (Bioline, USA), the extracted DNA show good quality and consisted of high molecular weight DNA greater than 10.0 kb (Figure 3.1). The amount of DNA that was extracted from fresh leaves of *Phyllanthus* was approximately 5-40 ng/µl, which is adequate for RAPD analysis. In addition, we employed a DNA purification step to remove a large amount of polysaccharides and alkaloids, which from some plant species, insures a high DNA quality that is considered the major factor affecting the reproducibility of RAPD. The PCR reaction step of RAPD was first investigated and optimized since it could affect the pattern of PCR products and held constant throughout the experiment. The concentration of magnesium chloride varied from 2 to 5 mM. The optimum magnesium concentration was 3.5 mM since it could produce clear and reproducible RAPD bands (Figure 3.2). Gradient PCR was used to investigate the suitable annealing temperature that was set up at 36°C in this study.

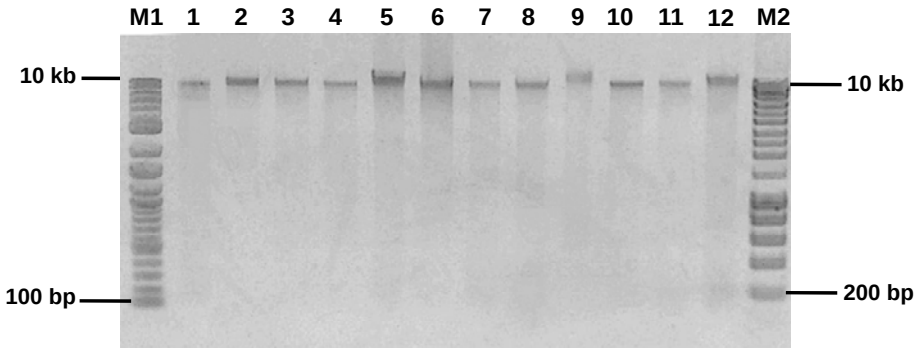



**Figure 3.1** Agarose gel electrophoresis of genomic DNA from twelve *Phyllanthus* species.

Lane M1: 2-Log DNA Ladder

Lane M2: HyperLadder I

Lane 1-12: typical genomic DNA of *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*.

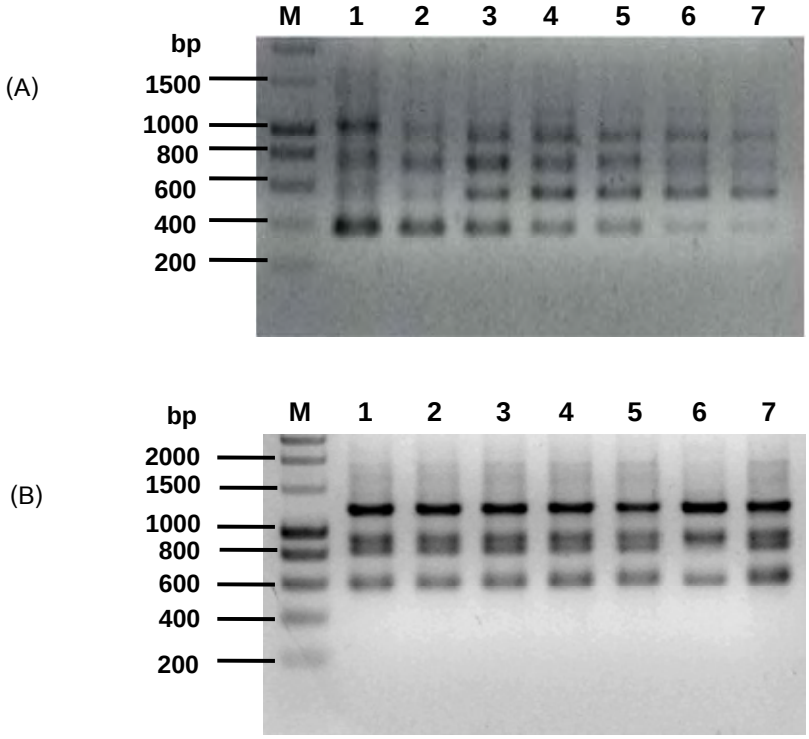



**Figure 3.2** Variation of $MgCl_2$ concentration (A) RAPD patterns of *P. taxodiifolus* using primer OPS-03 (B) RAPD patterns of *P. reticulatus* using primer OPS-19.

Lane M: HyperLadder I DNA ladder

Lane 1-7: 2.0 mM, 2.5 mM, 3mM, 3.5 mM, 4 mM, 4.5 mM, and 5 mM $MgCl_2$

**Primer screening**

PCR amplification were carried out using eighty arbitrary deca-oligonucleotide primers, kit D, N, O, and S, obtained from Operon DNA Technologies (Alameda, USA) (Table 3.2). In preliminary screening, eighteen primers could be used to amplified DNA from *Phyllanthus* species (Table 3.3). Only nine, OPD-02, OPD-04, OPD-07, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19, out of eighteen primers produced clear and reproducible polymorphic RAPD band and used as the markers for investigating the genetic variation across all twelve *Phyllanthus* species (Figure 3.3-3.11). The RAPD patterns of individuals between intra-species were compared using primer OPD-02, OPD-04, OPD-07, OPD-11, OPN-04, OPN-06, OPN-09, OPN-10, OPN12, OPN-15, OPN-18, OPO-18, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19 (Figure 3.12-3.29)

**Table 3.3** Sequence list of deca-oligonucleotide primers used for RAPD analysis in inter- and intra- species.

| Primer code | Sequence (5′-3′) | Primer code | Sequence (5′-3′) |
|---|---|---|---|
| OPD-02 | GGACCCAACC | OPN-15 | CAGCGACTGT |
| OPD-04 | TCTGGTGAGG | OPN-18 | GGTGAGGTCA |
| OPD-07 | TTGGCACGGG | OPO-18 | CTCGCTATCC |
| OPD-11 | AGCGCCATTG | OPS-01 | CTACTGCGCT |
| OPN-04 | GACCGACCCA | OPS-03 | CAGAGGTCCC |
| OPN-06 | GAGACGCACA | OPS-07 | TCCGATGCTG |
| OPN-09 | TGCCGGCTTG | OPS-08 | TTCAGGGTGG |
| OPN-10 | ACAACTGGGG | OPS-12 | CTGGGTGAGT |
| OPN-12 | CACAGACACC | OPS-19 | GAGTCAGCAG |

### 3.2.2 RAPD fingerprint analysis

**Analysis of genetic variation in inter-species**

Genetic variation within inter-species were produced by OPD-02, OPD-04, OPD-07, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19 that were totally 363 scorable bands ranging from 0.3 to 2.5 kb in size (Table 3.4). The largest number of RAPD bands was detected for primer OPS-03 (52 bands, Figure 3.7), while the lowest was scored for OPS-08 (24 bands, Figure 3.9).

**Table 3.4** Summary of PCR products generated by RAPD primer from twelve *Phyllanthus* species

| Primer | Number of scorable band | Size range (bp) | No. of monomorphic/polymorphic bands | % of polymorphic bands |
|---|---|---|---|---|
| OPD-02 | 49 | 500-1800 | 0/49 | 100 |
| OPD-04 | 34 | 300-1500 | 0/34 | 100 |
| OPD-07 | 47 | 400-2300 | 0/47 | 100 |
| OPS-01 | 45 | 300-2500 | 0/45 | 100 |
| OPS-03 | 52 | 300-1800 | 0/52 | 100 |
| OPS-07 | 50 | 400-2500 | 0/50 | 100 |
| OPS-08 | 24 | 300-1500 | 0/24 | 100 |
| OPS-12 | 37 | 400-1800 | 0/37 | 100 |
| OPS-19 | 26 | 500-1500 | 0/26 | 100 |

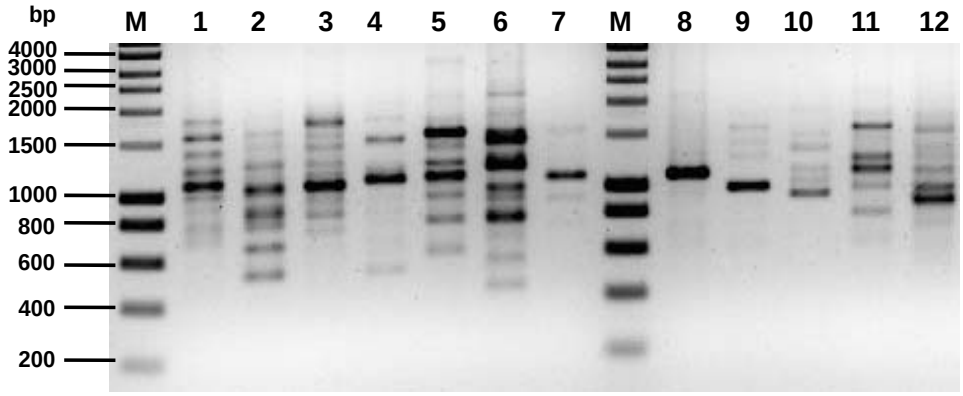



**Figure 3.3** RAPD patterns of twelve *Phyllanthus* species generated by primer OPD-02.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

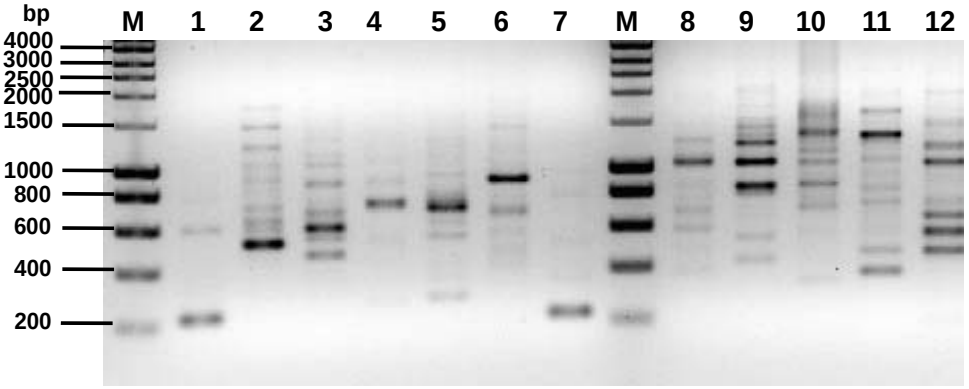



**Figure 3.4** RAPD patterns of twelve *Phyllanthus* species generated by primer OPD-04.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

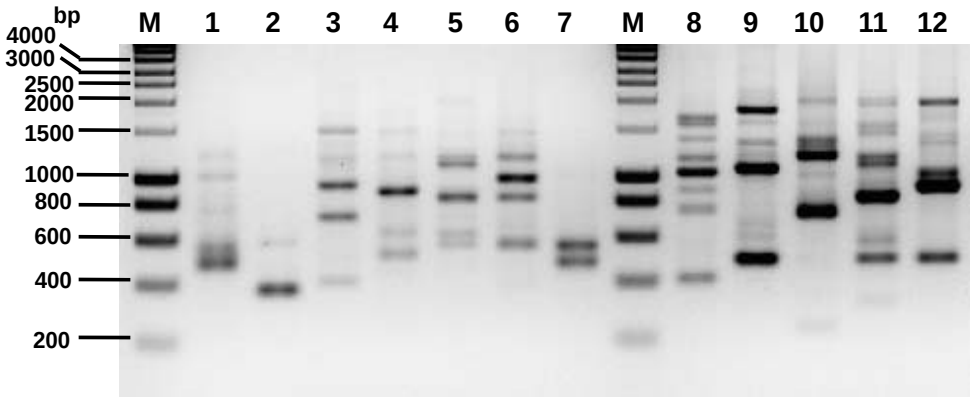



**Figure 3.5** RAPD patterns of twelve *Phyllanthus* species generated by primer OPD-07.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

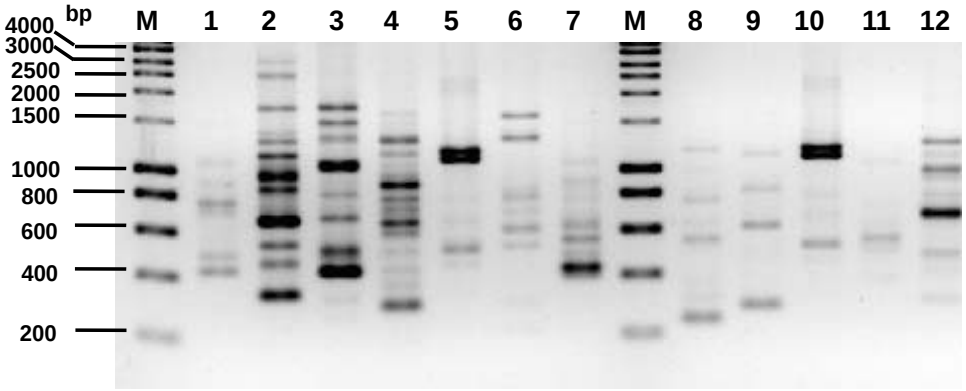



**Figure 3.6** RAPD patterns of twelve *Phyllanthus* species generated by primer OPS-01.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

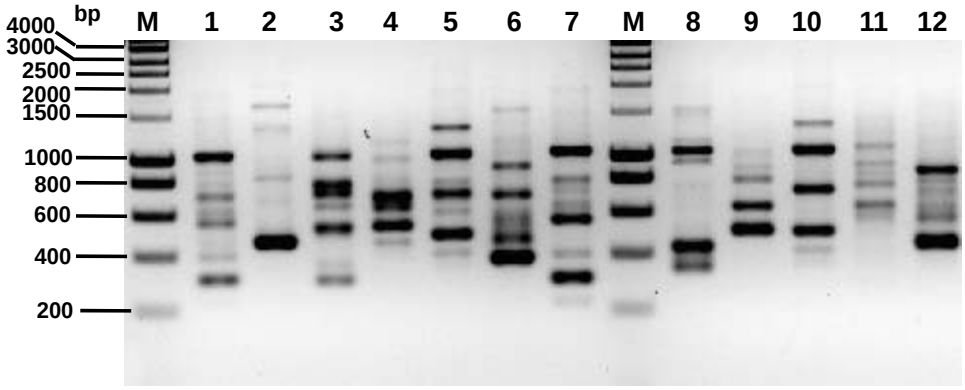



**Figure 3.7** RAPD patterns of twelve *Phyllanthus* species generated by primer OPS-03.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

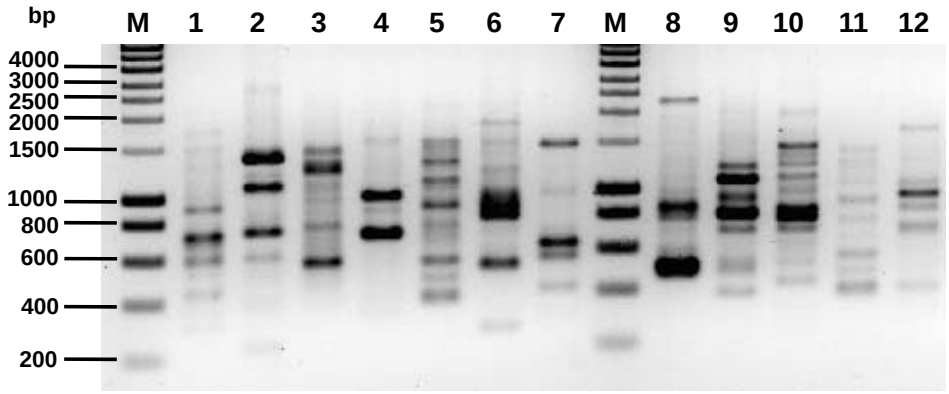



**Figure 3.8** RAPD patterns of twelve *Phyllanthus* species generated by primer OPS-07.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

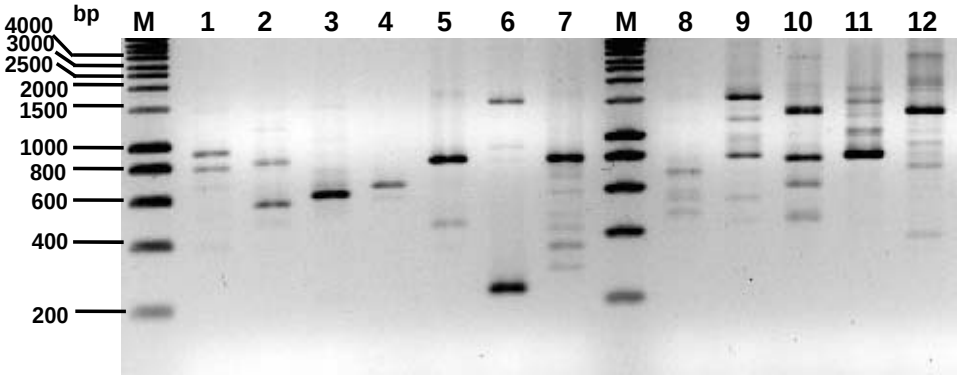



**Figure 3.9** RAPD patterns of twelve *Phyllanthus* species generated by primer OPS-08.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

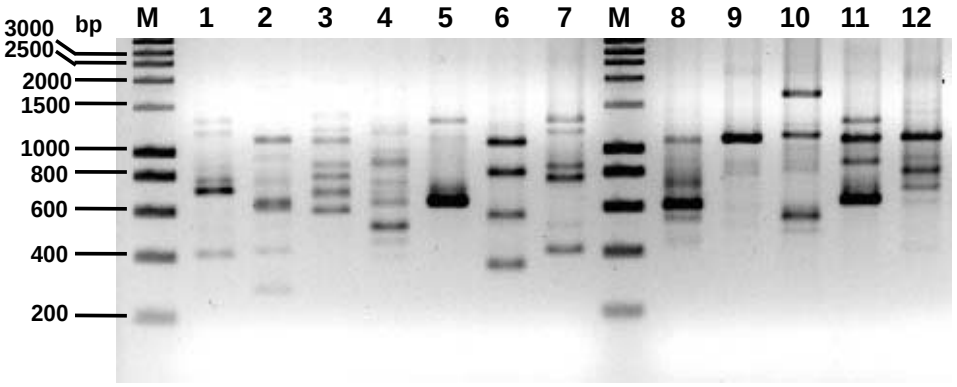



**Figure 3.10** RAPD patterns of twelve *Phyllanthus* species generated by primer OPS-12.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

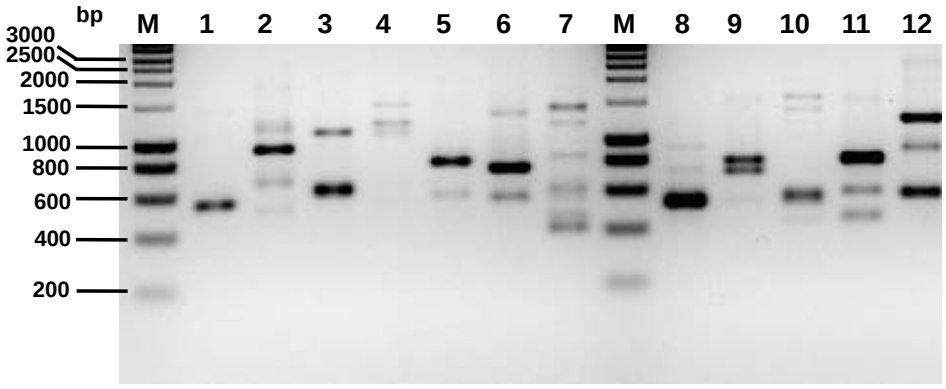



**Figure 3.11** RAPD patterns of twelve *Phyllanthus* species generated by primer OPS-19.

Lane M: HyperLadder I DNA ladder

Lane 1-12: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

**Analysis of genetic variation in intra-species**

*P. amarus*, *P. emblica*, and *P. acidus* were chosen to study of genetic variation within intra-species since they were most generally found in various parts of Thailand. RAPD fingerprints were produced by OPD-02, OPD-04, OPD-07, OPD-11, OPN-04, OPN-06, OPN-09, OPN-10, OPN12, OPN-15, OPN-18, OPO-18, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19. The PCR products were totally 693 scorable bands ranging from 350 to 2500 bp in size (Table 3.5). The largest number of RAPD bands was detected for OPN-04 in *P. amaus* (66 bands) (Figure 3.14), while the lowest was scored for OPN-10 in *P. acidus* (8 bands, Figure 3.27).

**Table 3.5** Summary of PCR products within intra-species of selected *Phyllanthus* species generated by RAPD primer.

| Species | Primer | Size range (bp) | Number of scorable band | No. of monomorphic/polymorphic band | % of polymorphic band |
|---|---|---|---|---|---|
| *P. amarus* | OPD-02 | 1000-1600 | 40 | 40/0 | 0.00 |
| | OPD-11 | 500-1500 | 49 | 32/17 | 34.69 |
| | OPN-04 | 400-2300 | 66 | 32/34 | 51.51 |
| | OPN-06 | 500-1200 | 57 | 56/1 | 1.75 |
| | OPN-09 | 500-1600 | 46 | 32/14 | 30.43 |
| | OPN-15 | 800-900 | 10 | 8/2 | 20.00 |
| | OPO-18 | 600-1500 | 42 | 32/10 | 2.38 |
| | OPS-03 | 350-1200 | 55 | 24/31 | 56.36 |
| | OPS-19 | 590-2300 | 37 | 24/13 | 35.14 |
| *P. emblica* | OPS-03 | 350-1000 | 47 | 8/39 | 82.98 |
| | OPS-12 | 400-1300 | 21 | 8/13 | 61.90 |
| | OPS-19 | 580-1200 | 20 | 8/12 | 60.00 |
| *P. acidus* | OPN-10 | 800 | 8 | 8/0 | 0.00 |
| | OPN-12 | 550-1400 | 30 | 24/6 | 20.00 |
| | OPN-18 | 500-1000 | 30 | 8/22 | 73.33 |
| | OPS-03 | 400-1500 | 49 | 24/25 | 51.02 |
| | OPS-12 | 390-2500 | 38 | 32/6 | 15.79 |
| | OPS-19 | 490-1400 | 40 | 8/36 | 90.00 |

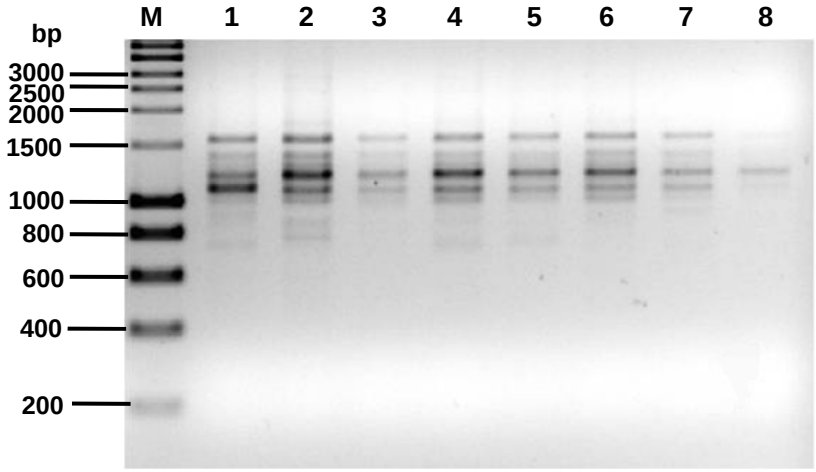



**Figure 3.12** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPD-02.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Bangkok, Kanchanaburi, Nong Khai, Nakhon Phanom, Udon Thani, Khon Kaen, Sakon Nakhon, and Chiang Mai, respectively.

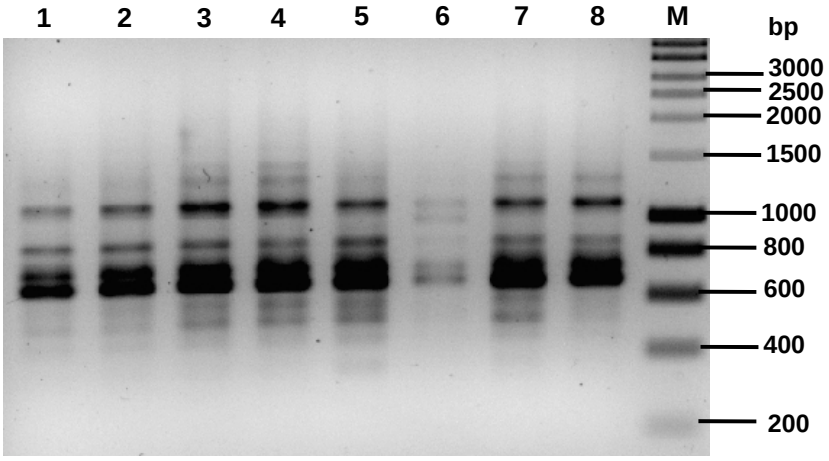



**Figure 3.13** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPD-11.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Nong Khai, Nakhon Phanom, Khon Kaen, Udon Thani, Sakon Nakhon, Bangkok, Chiang Mai and Kanchanaburi, respectively.

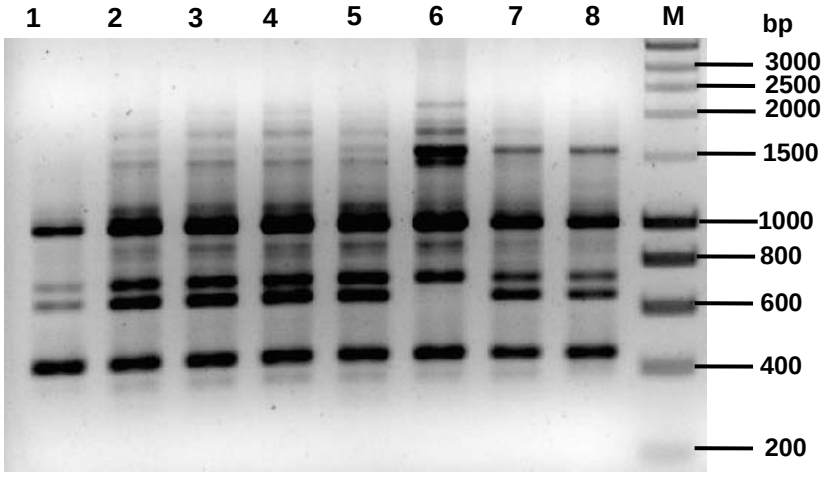



**Figure 3.14** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPN-04

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Nong Khai, Nakhon Phanom, Khon Kaen, Udon Thani, Sakon Nakhon, Bangkok, Chiang Mai and Kanchanaburi, respectively.

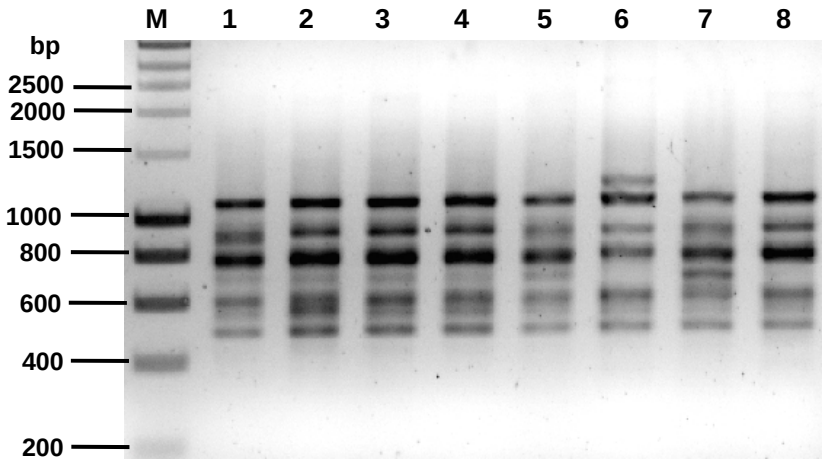



**Figure 3.15** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPN-06

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Nong Khai, Nakhon Phanom, Khon Kaen, Udon Thani, Sakon Nakhon, Bangkok, Chiang Mai and Kanchanaburi, respectively.

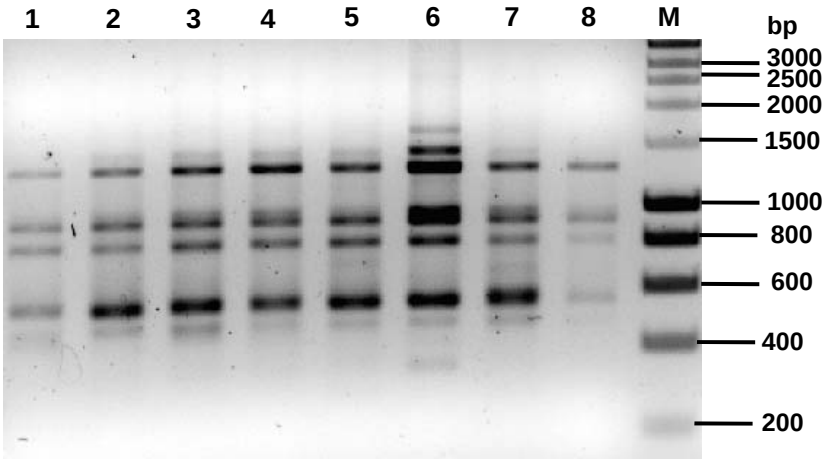



**Figure 3.16** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPN-09

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Nong Khai, Nakhon Phanom, Khon Kaen, Udon Thani, Sakon Nakhon, Bangkok, Chiang Mai and Kanchanaburi, respectively.

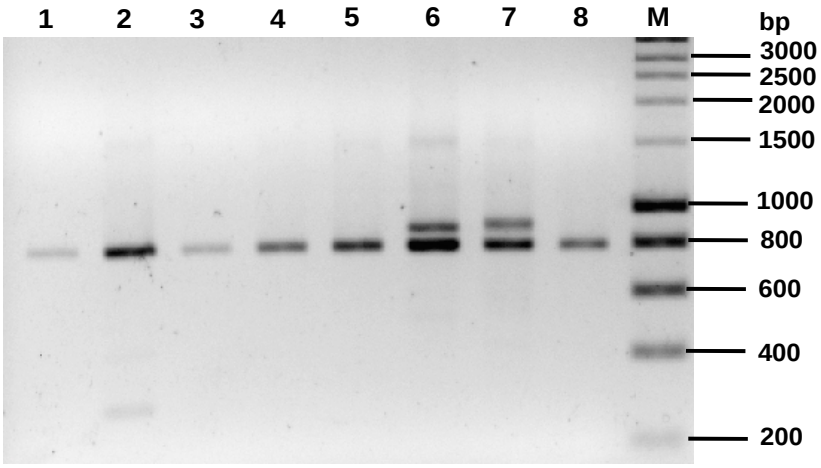



**Figure 3.17** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPN-15

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Nong Khai, Nakhon Phanom, Khon Kaen, Udon Thani, Sakon Nakhon, Bangkok, Chiang Mai and Kanchanaburi, respectively.

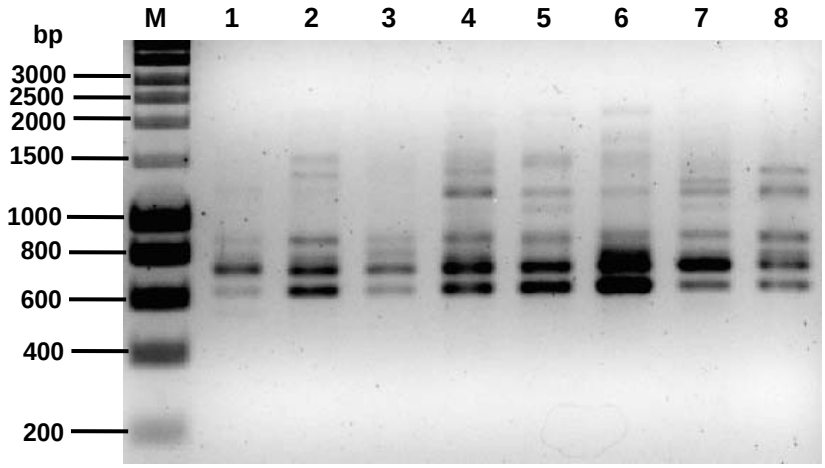



**Figure 3.18** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPO-18

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Nong Khai, Nakhon Phanom, Khon Kaen, Udon Thani, Sakon Nakhon, Bangkok, Chiang Mai and Kanchanaburi, respectively.

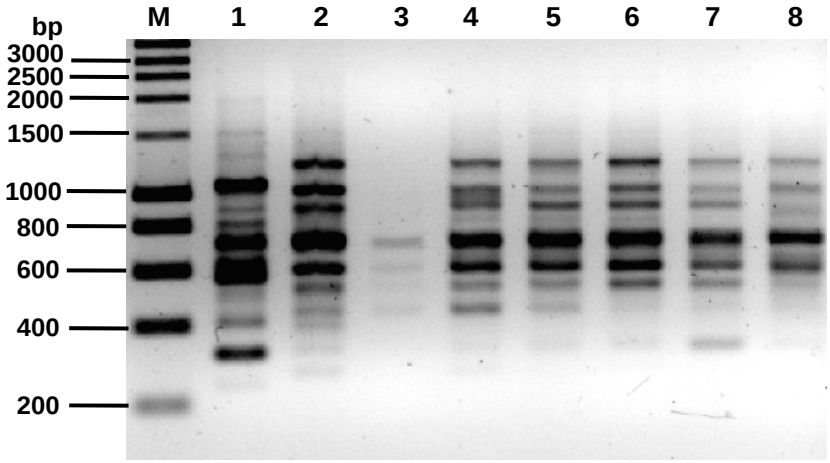



**Figure 3.19** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPS-03.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Bangkok, Kanchanaburi, Nong Khai, Nakhon Phanom, Udon Thani, Khon Kaen, Sakon Nakhon, and Chiang Mai, respectively.

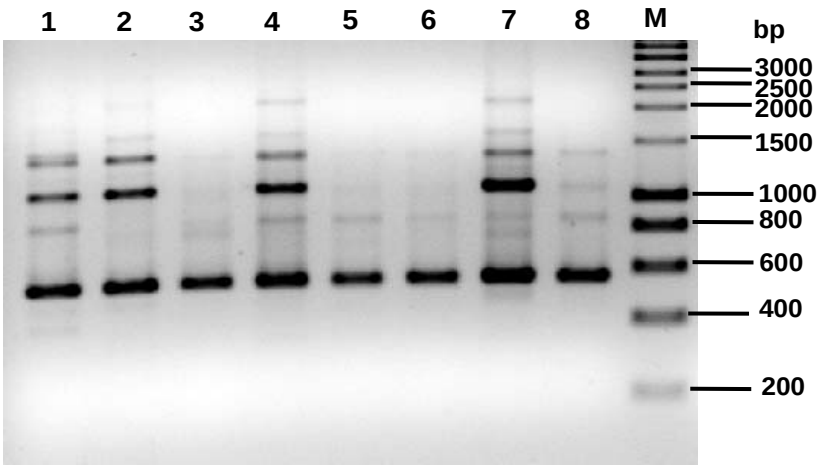



**Figure 3.20** RAPD patterns from intra-species variation of *P. amarus* generated by primer OPS-19

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. amarus* obtained from Bangkok, Kanchanaburi, Nong Khai, Nakhon Phanom, Udon Thani, Khon Kaen, Sakon Nakhon, and Chiang Mai, respectively.

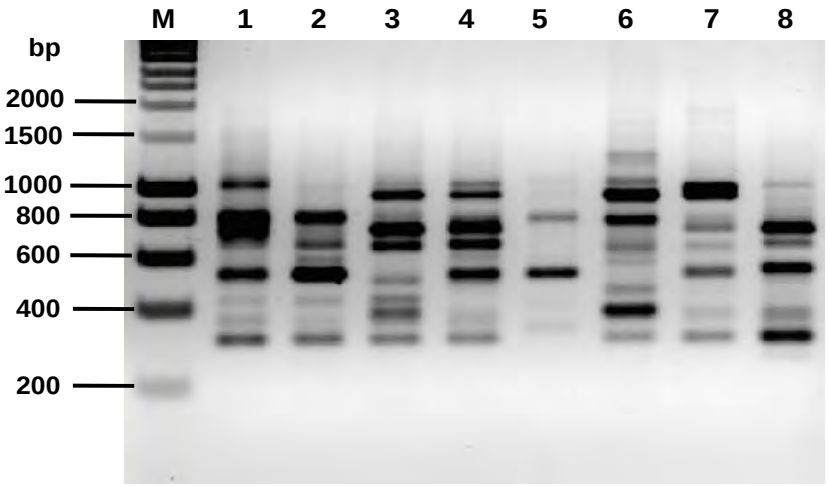



**Figure 3.21** RAPD patterns from intra-species variation of *P. emblica* generated by primer OPS-03.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. emblica* obtained from Bangkok, Sakon Nakhon, Kalasin, Khon Kaen, Udon Thani, Nakhon Phanom, Nong Khai, and Chiang Mai, respectively.

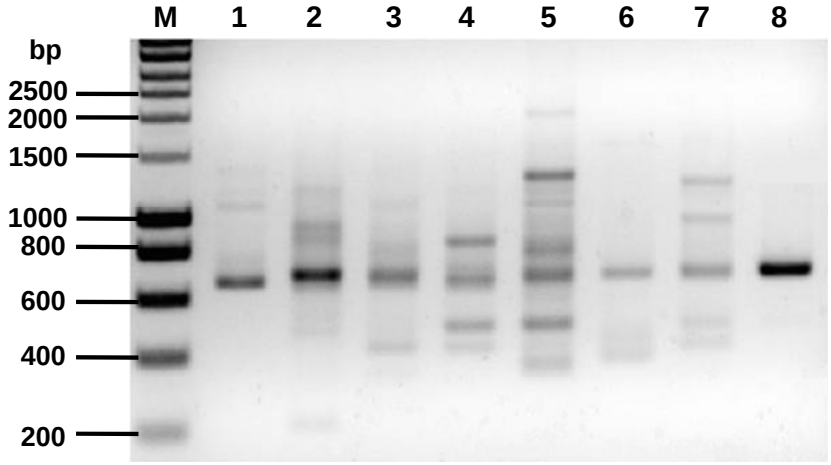



**Figure 3.22** RAPD patterns from intra-species variation of *P. emblica* generated by primer OPS-12

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. emblica* obtained from Bangkok, Sakon Nakhon, Kalasin, Khon Kaen, Udon Thani, Nakhon Phanom, Nong Khai, and Chiang Mai, respectively.

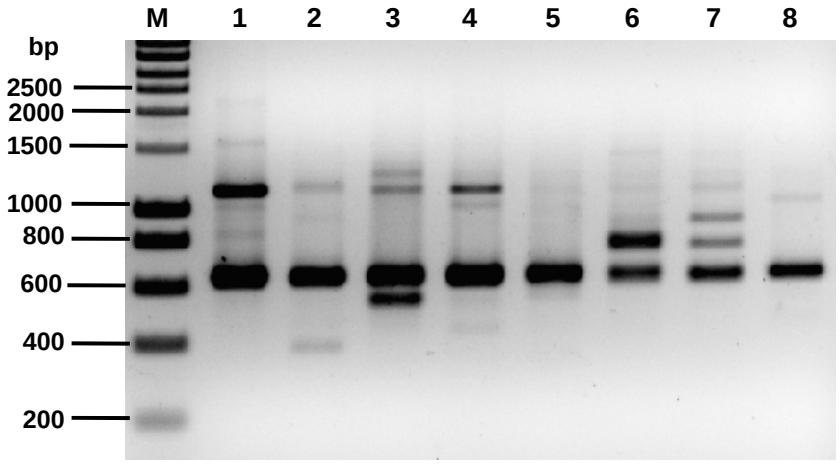



**Figure 3.23** RAPD patterns from intra-species variation of *P. emblica* generated by primer OPS19.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. emblica* obtained from Bangkok, Sakon Nakhon, Kalasin, Khon Kaen, Udon Thani, Nakhon Phanom, Nong Khai, and Chiang Mai, respectively.

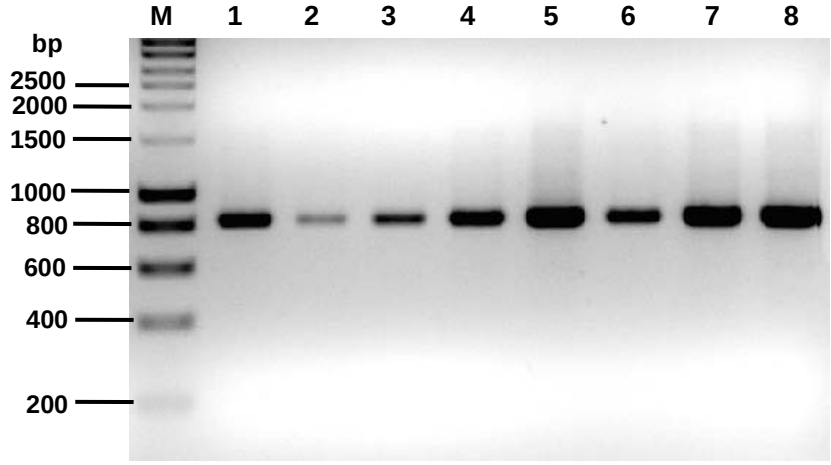



**Figure 3.24** RAPD patterns from intra-species variation of *P. acidus* generated by primer OPN-10.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. acidus* obtained from Nakhon Nayok, Bangkok, Chiang Rai, Khon Kaen, Nakhon Si Thammarat, Sakon Nakhon, Satun and Chiang Mai, respectively.

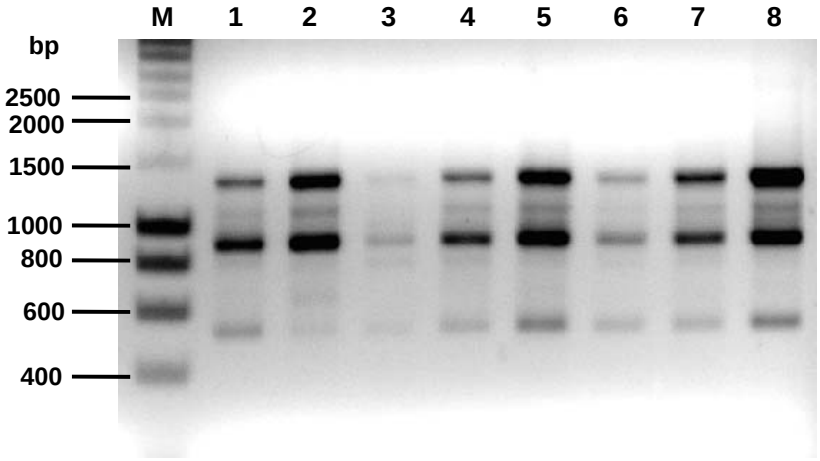



**Figure 3.25** RAPD patterns from intra-species variation of *P. acidus* generated by primer OPN-12.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. acidus* obtained from Nakhon Nayok, Bangkok, Chiang Rai, Khon Kaen, Nakhon Si Thammarat, Sakon Nakhon, Satun and Chiang Mai, respectively.

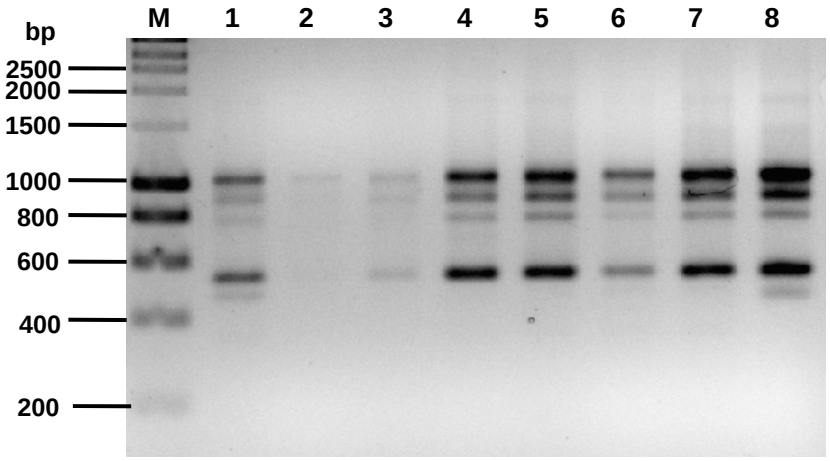



**Figure 3.26** RAPD patterns from intra-species variation of *P. acidus* generated by primer OPN-18.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. acidus* obtained from Nakhon Nayok, Bangkok, Chiang Rai, Khon Kaen, Nakhon Si Thammarat, Sakon Nakhon, Satun and Chiang Mai, respectively.

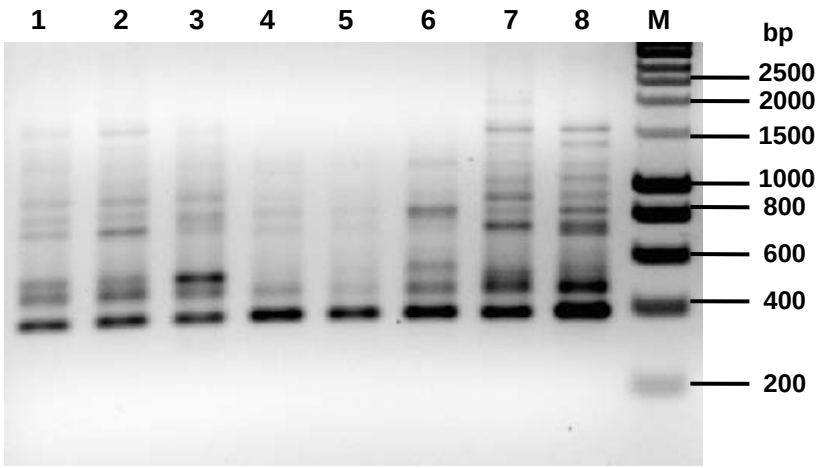



**Figure 3.27** RAPD patterns from intra-species variation of *P. acidus* generated by primer OPS-03.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. acidus* obtained from Nakhon Nayok, Bangkok, Chiang Rai, Khon Kaen, Nakhon Si Thammarat, Sakon Nakhon, Satun and Chiang Mai, respectively.

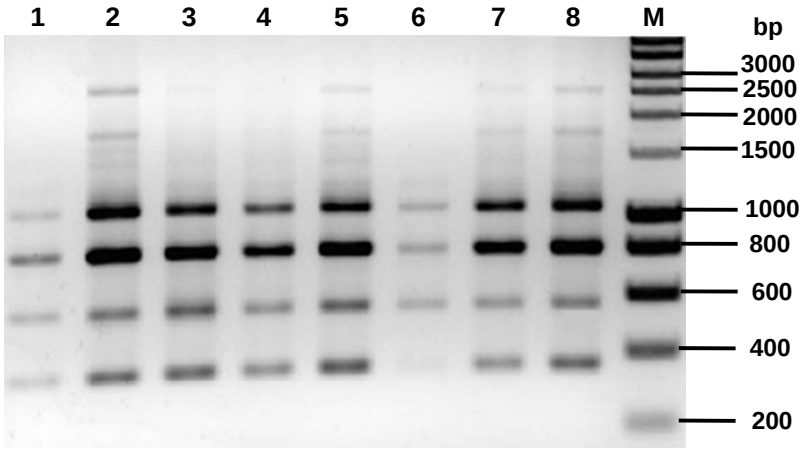



**Figure 3.28** RAPD patterns from intra-species variation of *P. acidus* generated by primer OPS-12

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. acidus* obtained from Nakhon Nayok, Bangkok, Chiang Rai, Khon Kaen, Nakhon Si Thammarat, Sakon Nakhon, Satun and Chiang Mai, respectively.

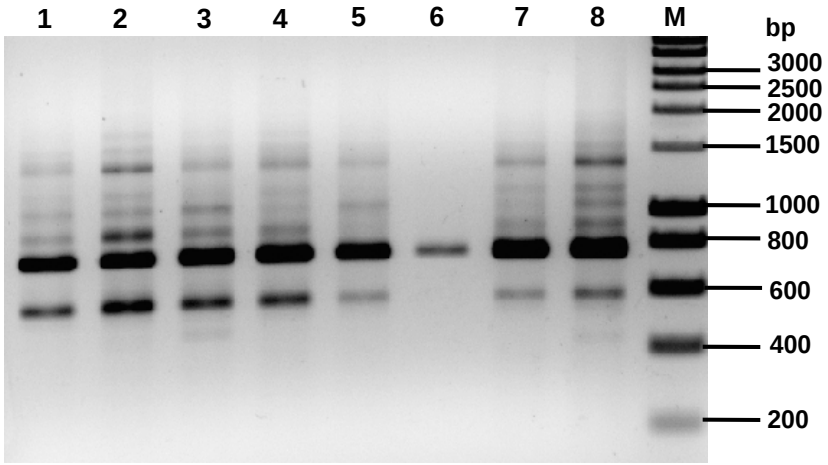



**Figure 3.29** RAPD patterns from intra-species variation of *P. acidus* generated by primer OPS-19.

Lane M: HyperLadder I DNA ladder

Lane 1-8: *P. acidus* obtained from Nakhon Nayok, Bangkok, Chiang Rai, Khon Kaen, Nakhon Si Thammarat, Sakon Nakhon, Satun and Chiang Mai, respectively.

### 3.2.3 Phylogenetic relationship of *Phyllanthus* spp. by RAPD analysis

One individual representative of each species was selected for construction of a phylogenetic tree since it showed the same pattern of the major RAPD band in RAPD analysis. To evaluate the genetic relationship between *Phyllanthus* species, RAPD bands of twelve *Phyllanthus* species produced by nine primers (OPD-02, OPD-04, OPD-07, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19), were scored and constructed as a phylogenetic tree. Standard DNA marker (Hyper Ladder I, Bioline, USA) was used to assign the size of each RAPD fragment. Only fragments that could be accurately scored (0.3–2.5 kb) were chosen. RAPD fragments were assigned a DNA length and recorded in a binary matrix for each individual as presence (1) or absence (0) of a given band. Phylogenetic relationship of *Phyllanthus* species was constructed using Dice (Nei and Li, 1979) clustering of NTSYS-pc version 2.11T (Exeter Software, Setauket, N.Y.) based on a UPGMA method (Figure 3.30). Among the twelve species of *Phyllanthus*, Dice similarity index (S.I.) ranged from 0.125 to 0.500 (Table 3.4) and could be divided into four clusters. Cluster I includes four species showing 0.375-0.500 similarity index (*P. amarus*, *P. pulcher*, *P. taxodiifolius*, and *P. reticulatus*). Cluster II includes two species showing similarity index 0.462 (*P. virgatus* and *P. ankorensis*) and cluster III includes five species with 0.258-0.500 similarity index (*P. urinaria*, *P. emblica*, *P. collinsiae*, *P. acidus*, and *P. acutissimus*). *P. debilis* is the distinct species in cluster IV. It was only 12% genetically similar to the rest of the 11 species. In addition, the four most closely related species were *P. pulcher* and *P. amarus* (cluster I) and *P. collinsiae* and *P. emblica* (cluster III) with the highest similarity index (0.500).

**Table 3.6** Nei and Li's genetic similarity index of twelve species based on RAPD markers.

| Species | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. amarus* | 1 | 1.000 | | | | | | | | | | | |
| *P. urinaria* | 2 | 0.237 | 1.000 | | | | | | | | | | |
| *P. emblica* | 3 | 0.419 | 0.479 | 1.000 | | | | | | | | | |
| *P. taxodiifolius* | 4 | 0.298 | 0.321 | 0.373 | 1.000 | | | | | | | | |
| *P. collinsiae* | 5 | 0.393 | 0.400 | 0.500 | 0.302 | 1.000 | | | | | | | |
| *P. acidus* | 6 | 0.305 | 0.412 | 0.451 | 0.321 | 0.462 | 1.000 | | | | | | |
| *P. pulcher* | 7 | 0.500 | 0.230 | 0.344 | 0.449 | 0.345 | 0.230 | 1.000 | | | | | |
| *P. debilis* | 8 | 0.227 | 0.151 | 0.286 | 0.146 | 0.240 | 0.340 | 0.174 | 1.000 | | | | |
| *P. virgatus* | 9 | 0.296 | 0.286 | 0.212 | 0.196 | 0.333 | 0.381 | 0.286 | 0.125 | 1.000 | | | |
| *P. acutissimus* | 10 | 0.189 | 0.387 | 0.338 | 0.240 | 0.407 | 0.258 | 0.145 | 0.298 | 0.351 | 1.000 | | |
| *P. ankorensis* | 11 | 0.426 | 0.171 | 0.329 | 0.207 | 0.358 | 0.400 | 0.381 | 0.255 | 0.462 | 0.344 | 1.000 | |
| *P. reticulatus* | 12 | 0.375 | 0.356 | 0.447 | 0.426 | 0.314 | 0.411 | 0.333 | 0.276 | 0.353 | 0.418 | 0.400 | 1.000 |

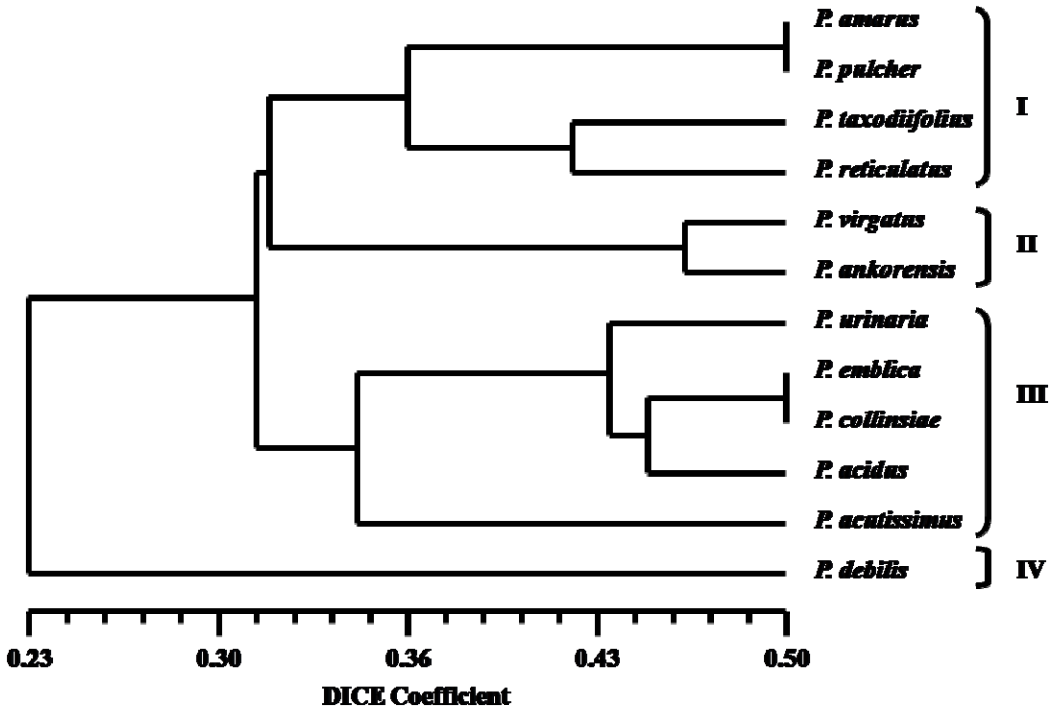



**Figure 3.30** Unweighted pair group method with arithmetic average (UPGMA) tree showing the genetic relationships between twelve *Phyllanthus* species as determined by RAPD marker.

### 3.3 Discussions

In this present study, we discriminated twelve species in the genus *Phyllanthus* by RAPD with nine primers, OPD-02, OPD-04, OPD-07, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19, since they generated the unique DNA profiles. RAPD is a simple, rapid and inexpensive method for detecting DNA polymorphism in various organisms because it has the advantage no need prior knowledge of the genome of the plants (Hon *et al*, 2003). However, one obvious disadvantage of RAPD is its sensitivity to slight changes in reaction conditions. The amount and purity of DNA template can affect to RAPD profile. The impurity in DNA templates such as polysaccharide and phenolic compound might inhibit PCR amplification. The optimization of each PCR component is also very essential. From the results, optimization concentration of magnesium chloride, *Taq* DNA polymerase, DNA template and annealing temperature were found to be important for RAPD analysis. The annealing temperature was suggested to be the first choice variable to optimize for PCR amplification condition. The optimize magnesium concentration used in this study was most suitable for 3.5 mM. High concentration of magnesium causes nonspecific amplification whereas low concentration of magnesium causes low activity of *Taq* DNA polymerase (Weising *et al*, 2005). Therefore, the reaction conditions and PCR cycling conditions should be stringently controlled to obtain high reproducibility (Cheng *et al*, 1997).

RAPD analysis revealed high genetic diversity with 100% of polymorphic bands among twelve *Phyllanthus* species in Thailand. To consider genetic diversity within intra-species that collected from various parts of Thailand, the genetic diversity varied from 0.00% – 90.00% of polymorphic bands. Regarding the percent of polymorphic bands in Table 3.5, it indicated that *P. emblica* showed greater intra-species variation than *P. amarus* and *P. acidus*. The minor bands were observed within intra-species but the major bands were still showed the same RAPD pattern. Thus, intra-species variation was not affected by geographic locations.

In addition, there is a risk of misinterpretation in a genetic analysis from different RAPD patterns with similar size. This can be minimized by the use of several RAPD

primers (Ambak *et al*, 2006). For example, PCR amplification using primer OPS-19 (Figure 3.11) showed a unique fragment approximately 590 bp in both *P. amarus* (Lane 1) and *P. debilis* (Lane 8). It was difficult to identify those two species by OPS-19. Meanwhile, amplification using OPS-03 (Figure 3.7) produced four major fragments, 300, 580, 700 and 1000 bp, in *P. amarus* and three major fragments, 390, 420, and 1000 bp, in *P. debilis*, so it could be identified by using this primer. Sequencing characterized amplified region (SCAR) markers could be further developed as a tool to prevent misuse among *Phyllanthus* species.

To evaluate the genetic relationship between *Phyllanthus* species, RAPD bands of twelve *Phyllanthus* species produced by nine primers (OPD-02, OPD-04, OPD-07, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19), were scored and constructed as a phylogenetic tree. A genetic similarity index was derived from Dice (Nei and Li, 1979) and UPGMA tree was constructed by using NTSYS-pc version 2.11T (Exeter Software, Setauket, N.Y.) (Figure 3.30). From RAPD based dendrogram, it clearly showed that the twelve *Phyllanthus* species could be divided into 4 clusters. The three frequently misidentify herbaceous *Phyllanthus* species, *P. amarus*, *P. urinaria*, and *P. debilis* were separated into different clusters. Among these three species, the similarity index of the pair *P. urinaria* and *P. debilis*, *P. amaurs* and *P. debilis*, *P. urinaria* and *P. amarus* were 0.151, 0.227, and 0.237, respectively. It indicated that *P. amaurs* and *P. urinaria* is more closely related than *P. debilis*. In Thailand, *P. amarus*, *P. debilis* and *P. urinaria* are common weed species which are very similar and often found growing together in the same habitat (Chantaranothai, 2007). They were usually harvested for production of medicinal products (Jain *et al*., 2008, Theerakulpisut *et al*., 2008). In addition, the two other well-known medicinal species *P. urinaria* and *P. emblica* were grouped in the same cluster (Cluster III) with 0.479 similarity index that corresponded to the previous report of Theerakulpisut *et al.* (2008). However, phylogenetic relationship based on RAPD analysis showed that the morphology variation of twelve *Phyllanthus* species is not reflected a DNA level. In this experiment, we can use nine primers to discriminate all twelve *Phyllanthus* species and generated the unique RAPD profiles.

# CHAPTER IV

# SEQUENCE ANALYSIS OF NUCLEAR ITS IN *PHYLLANTHUS* SPP.

## 4.1 Material and methods

### 4.1.1 Plant materials

Samples of 23 *Phyllanthus* species were collected from various habitats in Thailand. Fresh plant samples were identified by Assoc. Prof. Dr. Nijsiri Ruangrungsi and Assoc. Prof. Thatree Phadungcharoen of Chulalongkorn University, and voucher samples were deposited in the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. In addition, herbarium specimens for DNA extraction were collected from Bangkok Herbarium (BK) and Forest Herbarium (BKF), Bangkok, Thailand (Table 4.1).

**Table 4.1** List of *Phyllanthus* species used for DNA sequencing in this study.

| Species | Sample Codes | Provinces | Date of Collection | Voucher ID | GenBank accession |
|---|---|---|---|---|---|
| *P. amarus* Schum. & Thonn. | P1K1 | Kanchanaburi | 10.2005 | MJT-4810102 | AB550080 |
| | P1B2 | Bangkok | 10.2005 | MJT-4810101 | |
| | P1CM1 | Chiang Mai | 11.2006 | MJT-4911110 | |
| | P1KK1 | Khon Kaen | 10.2006 | MJT-4910109 | |
| | P1NP1 | Nakhon Pathom | 03.2007 | MJT-5003111 | |
| | P1PM1 | Nakhon Phanom | 10.2006 | MJT-4910106 | |
| | P1UT1 | Udon Thani | 10.2006 | MJT-4910108 | |
| *P. urinaria* L. | P2B1 | Bangkok | 10.2005 | MJT-4810201 | AB550081 |
| | P2B2 | Bangkok | 02.2006 | MJT-4810202 | |
| | P2B3 | Bangkok | 06.2007 | MJT-4810203 | |
| | P2NP1 | Nakhon Pathom | 03.2007 | MJT-5003205 | |
| *P. emblica* L. | P3B1 | Bangkok | 10.2005 | MJT-4810301 | AB550082 |
| | P3CM1 | Chiang Mai | 11.2006 | MJT-4911309 | |
| | P3HK1 | Nong Khai | 10.2006 | MJT-4910304 | |
| | P3KK1 | Khon Kaen | 10.2006 | MJT-4910307 | |
| | P3KS1 | Kalasin | 10.2006 | MJT-4910306 | |
| | P3PL1 | Pattalung | 04.2007 | MJT-5004311 | |
| | P3S1 | Sakon Nakhon | 06.2006 | MJT-4906302 | |

| Species | Sample Codes | Provinces | Date of Collection | Voucher ID | GenBank accession |
|---|---|---|---|---|---|
| *P. taxodiifolius* Beille | P4NP1 | Nakhon Pathom | 01.2006 | MJT-4901402 | AB550083 |
| | P4B1 | Bangkok | 10.2005 | MJT-4810401 | |
| | P4PR1 | Prachinburi | 06.2007 | TRP-5006403 | |
| *P. collinsiae* Craib | P5B1 | Bangkok | 10.2005 | MJT-4810501 | AB550084 |
| | P5B2 | Bangkok | 10.2005 | MJT-4810502 | |
| *P. acidus* L. | P6NN1 | Nakhon Nayok | 10.2006 | TT-4910603 | AB550085 |
| | P6CM1 | Chiang Mai | 11.2006 | MJT-4911615 | |
| | P6CR1 | Chiang Rai | 10.2006 | NR-4910604 | |
| | P6NN1 | Nakhon Nayok | 10.2006 | TT-4910603 | |
| | P6NS1 | Nakhon Si Thammarat | 10.2006 | TT-4910612 | |
| | P6S1 | Sakon Nakhon | 06.2006 | MJT-4906602 | |
| | P6ST1 | Satun | 10.2006 | TT-4910613 | |
| *P. pulcher* Wall. ex Müll.Arg. | P7CM1 | Chiang Mai | 11.2006 | MJT-4911703 | AB550086 |
| | P7K1 | Kanchanaburi | 10.2005 | MJT-4810702 | |
| | P7NP1 | Nakhon Pathom | 03.2007 | MJT-5003704 | |
| | P7R1 | Rayong | 08.2007 | NSR-5008705 | |
| | P7B1 | Bangkok | 10.2005 | MJT-4810701 | |
| *P. debilis* Klein ex Willd. | P9B1 | Bangkok | 10.2005 | NSR-4810801 | AB550087 |
| | P9NP1 | Nakhon Pathom | 01.2006 | MJT-4901802 | |

| Species | Sample Codes | Provinces | Date of Collection | Voucher ID | GenBank accession |
|---|---|---|---|---|---|
| *P. virgatus* G.Forst. | P12C1 | Chachoengsao | 08.2007 | TRP-5008904 | AB550088 |
| | P12RT1 | Ratchaburi | 09.2007 | NSR-5009905 | AB550089 |
| | P12B1 | Bangkok | 10.2005 | TRP-4810901 | |
| *P. acutissimus* Miq. | P13UB1 | Ubon Ratchathani | 04.2007 | TRP-5004102 | AB550090 |
| | P13C1 | Chachoengsao | 01.2006 | TRP-4901101 | |
| *P. angkorensis* Beille in Lecomte | P14NP1 | Nakhon Pathom | 01.2006 | MJT-4901111 | AB550091 |
| | P14NP2 | Nakhon Pathom | 03.2007 | MJT-4901112 | |
| *P. reticulatus* Poir. | P17B1 | Bangkok | 02.2006 | MJT-4902121 | AB550092 |
| *P. microcarpus* (Benth.) Müll.Arg. | P22H2 | Narathiwat | 11.2000 | BKF136348 | AB550093 |
| *P. columnaris* Müll.Arg. | P24H1 | Chiang Mai | 12.1985 | BK59507 | AB550094 |
| *P. gracilipes* (Miq.) Müll.Arg. | P27H2 | Trat | 12.1972 | BKF57742 | AB550095 |
| *P. harmandii* Beille in Lecomte | P28H1 | Si Sa Ket | 10.1984 | BKF111044 | AB550096 |
| *P. lingulatus* Beille | P30H1 | Chiang Mai | 03.1967 | BK39827 | AB550097 |
| *P. myrtifolius* (Wight) Müll.Arg. | P32H1 | Bangkok | 06.1976 | BK55987 | AB550098 |
| *P. orientalis* (Craib) Airy Shaw | P33H1 | Lampang | 02.1983 | BK21398 | AB550099 |
| *P. oxyphyllus* Miq. | P34H3 | Phuket | 04.2007 | BKF149043 | AB550100 |
| *P. roseus* (Craib & Hutch.) Beille in Lecomte | P37H1 | Si Sa Ket | 08.1976 | BK21430 | AB550101 |
| *P. sikkimensis* Müll.Arg. | P38H1 | Prachuap Khiri Khan | 10.1973 | BK50143 | AB550102 |
| *P. welwitschianus* Müll.Arg. | P40H1 | Saraburi | 06.1974 | BK51174 | AB550103 |
| Total | 23 *Phyllanthus* species, 56 samples | | | | |

### 4.1.2 DNA extraction and purification form herbarium specimens

Total genomic DNA of fresh and herbarium specimens were extracted using a DNeasy Plant Mini Kit (Qiagen, Germany) and purified using a High Pure PCR Product Purification Kit (Roche Diagnostics, Japan) according to the manufacturer's protocol. DNA content was estimated using a DyNA Quant 200 Fluorometer (GE Healthcare, Sweden) with calf thymus DNA (Sigma, USA) as a standard.

### 4.1.3 Primer design

PCR primers were designed for *Phyllanthus* species based on the rRNA gene sequence of *P. amarus* retrieved from the Genbank Nucleotide Sequence Database (accession no. AY725467). The forward primer (Phyll-ITS-1F) and the reverse primer (Phyll-ITS-1R) were used to amplify the entire ITS region (ITS1-5.8S rDNA-ITS2) from fresh plants. For herbarium specimens, it was difficult to amplify the whole ITS region, so nested PCR was performed. In such case, the inner primers, In-18S-26S-F, In-18S-26S-R, and In2R were designed. Annealing positions of these PCR primers were shown in Figure 4.1 and detail of these primers are presented in Table 4.2.

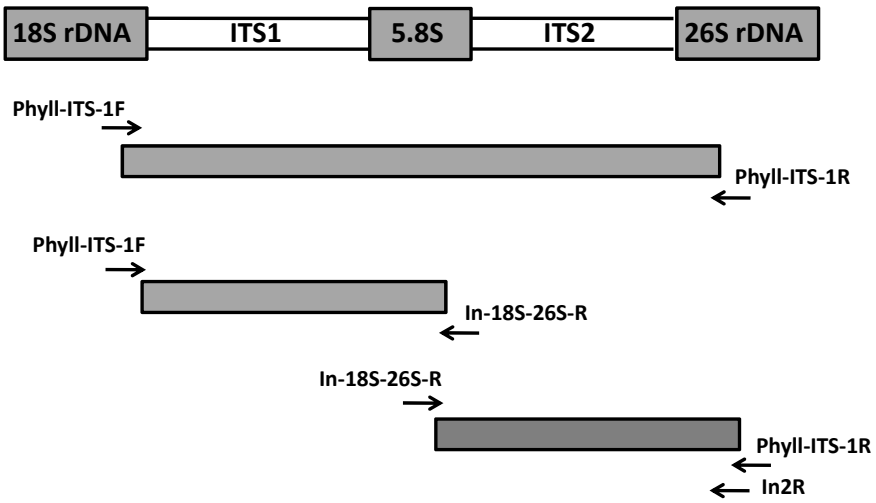



**Figure 4.1** Structure of ITS region and localization of primers used for the amplification and sequencing of the ITS region.

**Table 4.2** PCR amplification primers and sequencing primers of ITS region used in this study

| Direction | Primer | Sequence (5′-3′) | Length (bp) | Tm (°C) |
|---|---|---|---|---|
| Forward | Phyll-ITS-1F | GGA GAA GTC GTA ACA AGG TT | 20 | 54.3 |
| | In-18S-26S-F | AAT TGC AGA ATC CCG TGA AC | 20 | 54.3 |
| Reverse | Phyll-ITS-1R | GTA ATC CCG CCT GAC CTG | 18 | 58.1 |
| | In-18S-26S-R | TTG CGT TCA AAG ACT CGA TG | 20 | 54.3 |
| | In2R | CGT CGA ATA CAT AGA GGG TC | 20 | 56.3 |

### 4.1.4 PCR amplification from nuclear ITS region

The PCR mixture (25 µl) used to amplify the region from fresh samples contained 10 mM Tris-HCl (pH 8.8), 2 mM potassium chloride, 0.2 mM dNTP, 0.4 µM each primer, 0.02 units *Taq* DNA Polymerase (Roche Diagnostics, Japan), and 4–40 ng of DNA template. Amplification was carried out under the following conditions: a preliminary denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min, with a final extension at 72 °C for 5 min. When the DNA preparations from the herbarium specimens were used as PCR templates, the reaction mixture (20 µl) contained 25 mM TAPS (tris-hydroxymethyl-methyl-amino-propanesulfonic acid, sodium salt), pH 9.3, 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.2 mM dNTP, 0.4 µM each primer, 0.02 unit Nova*Taq* Hot Start DNA Polymerase (Novagen, USA), and 0.1 ng DNA template. The amplification was performed with 35 cycles consisting of precycling at 95 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products were separated by 1.2% agarose gel electrophoresis and detected by staining with ethidium bromide.

### 4.1.5 PCR product purification and DNA sequencing

PCR products were incubated with ExoSAP-IT solution (GE Healthcare, Sweden) according to the manufacturer's protocol, purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA.), and applied to sequencing reactions. The nucleotide sequences were determined using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, USA) or DSQ-2000L (Shimadzu, Japan). For each sample, forward and reverse sequencing reactions were performed to confirm the results and assembled using BioEdit (Hall, 1999). The nucleotide additive polymorphic sites (Aguilar and Feliner, 2003) were confirmed by comparing their intensity ratio of forward and reverse sequencing electropherograms. The nucleotide additive sequences were assigned according to the IUPAC codes, only species showing no additivity at the variable sites in ITS sequences were included in the phylogenetic analysis (Sang *et al*, 1995).

### 4.1.6 Phylogenetic tree reconstruction.

DNA sequences of the ITS1-5.8S rDNA-ITS2 were aligned using Clustal X version 2.0.7 (Larkin *et al*., 2007) and Muscle version 3.6 (Edgar, 2004), and then the alignment was manually adjusted. Maximum parsimony (MP) was conducted using PAUP version 4.0b 10 (Swofford, 2003). Strict and semi-strict 50% consensus trees were calculated from all parsimonious trees. The consistency (CI) and retention indices (RI) were calculated. Bootstrap analyses were performed using 1000 replicates and the heuristic search algorithm. The same sequence data were also analyzed using maximum likelihood (ML) and neighbor-joining (NJ) method.

## 4.2 Results

### Genomic DNA

Total genomic DNA was isolated from leaves of herbarium specimens using a DNeasy Plant Mini Kit (Qiagen, Germany). Herbarium specimens need the incubation period more than the fresh specimens and must be purified before the PCR amplification. Purified DNA was examined on 1.0% agarose gel electrophoresis. Genomic DNA from herbarium specimens was shown in Figure 4.2 and genomic DNA from fresh samples was shown in Figure 3.1, Chapter III. The EZ Load 1kb Molecular Ruler (Bio-Rad Laboratories, USA) was used as DNA marker.

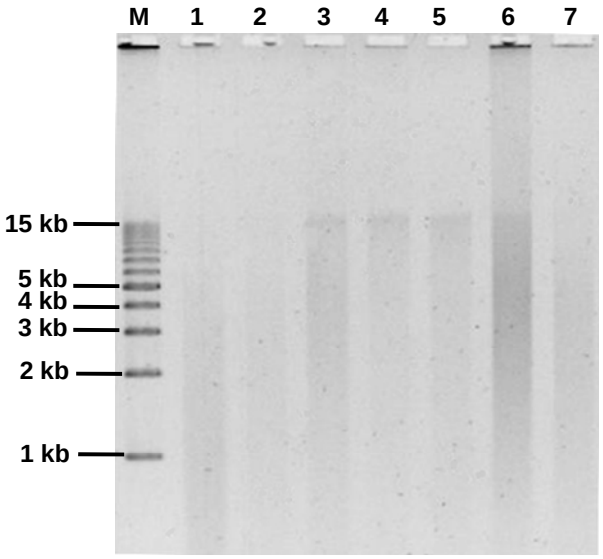



**Figure 4.2** Genomic DNA extracted from herbarium specimens.

M: EZ Load 1kb Molecular Ruler

Lane 1-7: DNA patterns of herbarium specimens extracted from *P. microcarpus*, *P. columnaris*, *P. gracilipes*, *P. harmandii*, *P. lingulatus*, *P. myrtifolius*, *P. orientalis,* respectively.

### 4.2.1 PCR product from nuclear ITS region

Forty-five of total genomic DNA were extracted from the fresh samples. The PCR products were amplified in the entire ITS region using two primers, Phyll-ITS-1F and Phyll-ITS-1R, which designed on the rDNA sequence of *P. amarus* retrieved from the DNA database. All fresh samples were successfully amplified (Table 4.1) and the size of the obtained PCR product was about 700 bp long (Figure 4.3).

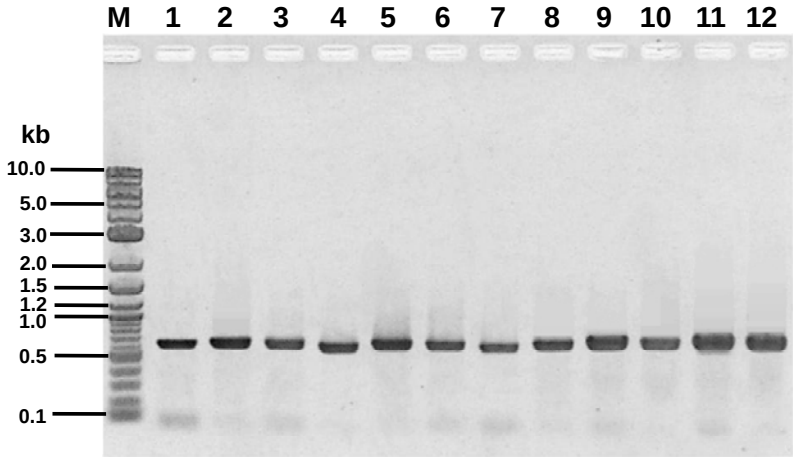



**Figure 4.3** The PCR products of ITS region amplified from fresh samples.

Lane M: 2-Log DNA Ladder

Lane 1-7: *P. amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*

For eleven of herbarium specimens, the DNA was highly degraded into small fragments and showed low DNA concentration (Figure 4.2). It did not yield a PCR product when DNA preparations from these samples were used as PCR templates. Therefore, the two strategies were used for amplified these herbarium specimens. 1) A pair of primer Phyll-ITS-1F and Phyll-Its-1R were used to amplified the entire ITS region using nested PCR technique (Figure 4.4). 2) ITS1 and ITS2 were separately amplified using inner primers annealed to the 5′- and 3′-end of the 5.8S rDNA (Figure 4.1). Then, the PCR product was sliced and purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) before sequencing (Figure 4.5). The PCR products were

directly applied to sequence reactions following treatment with endonuclease/alkaline phosphatase, and their nucleotide sequences were determined. The nucleotide sequences have been submitted to the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the accession numbers shown in Table 4.1.

The amplified ITS regions of herbarium specimens showed the same size of PCR product with fresh samples about 700 bp long (Figure 4.5). In addition, The inner primer, In-18S-26S-F, In-18S-26S-R, and In2R, were separately amplified ITS1 and ITS2 fragments, giving PCR product size about 400 and 320 bp long, respectively. The full nucleotide sequence of the ITS region was determined by combination of two separate products.

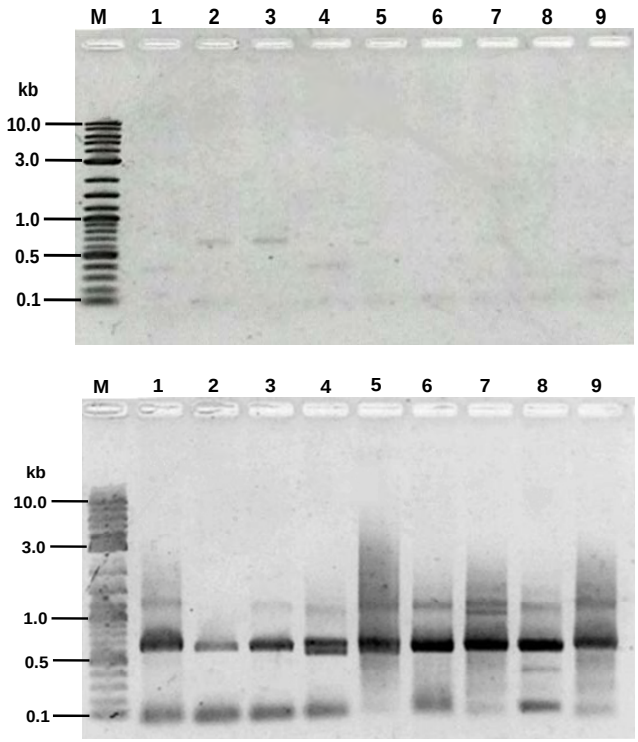



**Figure 4.4** The PCR products of ITS region amplified from herbarium specimens. (A) The first PCR amplification, (B) Nested PCR amplification

Lane M: 2-Log DNA Ladder

Lane 1-9: ITS patterns of herbarium specimens amplified from *P. microcarpus*, *P. columnaris*, *P. gracilipes*, *P. harmandii*, *P. lingulatus*, *P. myrtifolius*, *P. orientalis, P. oxyphyllus*, and *P. roseus*, respectively.

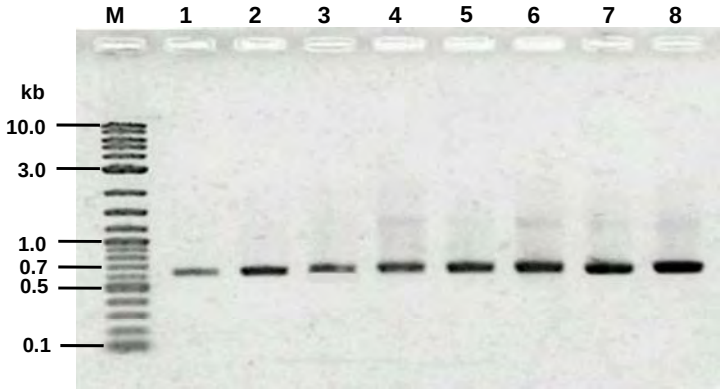



**Figure 4.5** The purified PCR products of ITS region amplified from herbarium specimens.

Lane M: 2-Log DNA Ladder

Lane 1-8: Nested PCR of herbarium specimens from amplified from *P. microcarpus*, *P. columnaris*, *P. gracilipes*, *P. harmandii*, *P. lingulatus*, *P. myrtifolius*, *P. orientalis, P. oxyphyllus*, and *P. roseus*, respectively.

### 4.2.2 Sequence analysis of nuclear ITS region

PCR products encompassing ITS1, 5.8S rDNA and ITS2 were amplified from 56 plant samples covering 23 *Phyllanthus* species collected from various habitats in Thailand (Table 4.1) using a PCR primer sets and their nucleotide sequences were determined. The boundaries of ITS1, ITS2, and adjacent coding regions were determined by comparing the published sequences from GenBank.

The size of the ITS1 and ITS2 regions were 197–222 bp and 196–210 bp, respectively, varying according to species. The 5.8S rDNA size was 163 bp, irrespective of species. The alignment of the entire ITS region is shown in Appendix B. The GC contents were in range of 51-60%. Details of the entire ITS nucleotide sequences of *Phyllanthus* species are listed in Table 4.5. Within the entire region, ITS2 provides a greater number of variable sites, 174, than ITS1, 169 sites but ITS1 contributes a greater number of informative sites, 145, than ITS2 (137 sites). In 5.8S, 30 variable sites were found with 20 being parsimony informative (12.3%). Of the variable nucleotide sites in the entire region, 302 (44.6%), are phylogenetically informative. A summary of the variation is shown in Table 4.3. However, some intra-species variation was found in the nucleotide sequence of ITS1 and ITS2, the numbers of intra-specifically varied nucleotides were within the range of one (*P. taxodiifolius*) to 27 (*P. virgatus*), and much smaller than those between species (Table 4.4). Within a species, pairwise difference ranges from 0.00% in most species to high of 7.00% in *P. virgatus* (Appendix C). Moreover, a conserved sequence, GGCRC-(NNNNN) -GYGCCAAGGAA (Y = C or T; R = G or A; N = A or G or C or T), was found at position 124–144 in the ITS1 of all plant samples of 23 *Phyllanthus* species examined.

**Table 4.3** Summary of variation within the ITS region.

| Region | Length (bp) | Number of Variable sites | Phylogenetically Informative sites | %Phylogenetically Informative |
|---|---|---|---|---|
| ITS1 | 197-222 | 169 | 145 | 61.2 |
| 5.8S nrDNA | 163 | 30 | 20 | 12.3 |
| ITS2 | 196-210 | 174 | 137 | 56.4 |
| Entire Region | 566-595 | 373 | 302 | 44.6 |

**Table 4.4** Nucleotide variation of the ITS sequence between intra-species spp.

| Species | No. of Sample | No. of nucleotide variation | % Intra-species variation |
|---|---|---|---|
| *P. urinaria* | 4 | 20 | 3.40 |
| *P. emblica* | 7 | 2 | 0.34 |
| *P. taxodiifolius* | 3 | 1 | 0.18 |
| *P. virgatus* | 3 | 27 | 4.54 |

**Table 4.5** Detail of the ITS nucleotide sequences of *Phyllanthus* species.

| Species | Sample codes | ITS1 (bp) | 5.8S (bp) | ITS2 (bp) | Entire region (bp) | % GC content |
|---|---|---|---|---|---|---|
| *P. amarus* | P1K1 | 219 | 163 | 202 | 584 | 56 |
| | P1B2 | 219 | 163 | 202 | 584 | 56 |
| | P1CM1 | 219 | 163 | 202 | 584 | 56 |
| | P1KK1 | 219 | 163 | 202 | 584 | 56 |
| | P1NP1 | 219 | 163 | 202 | 584 | 56 |
| | P1PM1 | 219 | 163 | 202 | 584 | 56 |
| | P1UT1 | 219 | 163 | 202 | 584 | 56 |
| *P. urinaria* | P2B1 | 222 | 163 | 202 | 587 | 50 |
| | P2B2 | 222 | 163 | 202 | 587 | 50 |
| | P2B3 | 222 | 163 | 202 | 587 | 50 |
| | P2NP1 | 222 | 163 | 202 | 587 | 50 |
| *P. emblica* | P3B1 | 220 | 163 | 204 | 587 | 53 |
| | P3CM1 | 220 | 163 | 204 | 587 | 53 |
| | P3HK1 | 220 | 163 | 204 | 587 | 53 |
| | P3KK1 | 220 | 163 | 204 | 587 | 53 |
| | P3KS1 | 220 | 163 | 204 | 587 | 53 |
| | P3PL1 | 220 | 163 | 204 | 587 | 53 |
| | P3S1 | 220 | 163 | 204 | 587 | 53 |
| *P. taxodiifolius* | P4NP1 | 199 | 163 | 202 | 564 | 52 |
| | P4B1 | 199 | 163 | 202 | 564 | 52 |
| | P4PR1 | 199 | 163 | 202 | 564 | 52 |
| *P. collinsiae* | P5B1 | 221 | 163 | 205 | 589 | 52 |
| | P5B2 | 221 | 163 | 205 | 589 | 52 |
| *P. acidus* | P6NN1 | 222 | 163 | 204 | 589 | 56 |
| | P6CM1 | 222 | 163 | 204 | 589 | 56 |
| | P6CR1 | 222 | 163 | 204 | 589 | 56 |
| | P6NN1 | 222 | 163 | 204 | 589 | 56 |
| | P6NS1 | 222 | 163 | 204 | 589 | 56 |
| | P6S1 | 222 | 163 | 204 | 589 | 56 |
| | P6ST1 | 222 | 163 | 204 | 589 | 56 |

| *Phyllanthus* Species | Sample codes | ITS1 (bp) | 5.8S (bp) | ITS2 (bp) | Entire region (bp) | % GC content |
|---|---|---|---|---|---|---|
| *P. pulcher* | P7CM1 | 198 | 163 | 207 | 568 | 54 |
| | P7K1 | 198 | 163 | 207 | 568 | 54 |
| | P7NP1 | 198 | 163 | 207 | 568 | 54 |
| | P7R1 | 198 | 163 | 207 | 568 | 54 |
| | P7B1 | 198 | 163 | 207 | 568 | 54 |
| *P. debilis* | P9B1 | 220 | 163 | 197 | 580 | 56 |
| | P9NP1 | 220 | 163 | 197 | 580 | 56 |
| *P. virgatus* | P12C1 | 222 | 163 | 210 | 595 | 51 |
| | P12RT1 | 222 | 163 | 209 | 594 | 51 |
| | P12B1 | 222 | 163 | 210 | 595 | 51 |
| *P. acutissimus* | P13UB1 | 197 | 163 | 206 | 566 | 54 |
| | P13C1 | 197 | 163 | 206 | 566 | 54 |
| *P. angkorensis* | P14NP1 | 221 | 163 | 205 | 589 | 52 |
| | P14NP2 | 221 | 163 | 205 | 589 | 52 |
| *P. reticulatus* | P17B1 | 221 | 163 | 204 | 588 | 60 |
| *P. microcarpus* | P22H2 | 221 | 163 | 201 | 585 | 51 |
| *P. columnaris* | P24H1 | 221 | 163 | 204 | 588 | 51 |
| *P. gracilipes* | P27H2 | 221 | 163 | 205 | 589 | 52 |
| *P. harmandii* | P28H1 | 221 | 163 | 208 | 592 | 52 |
| *P. lingulatus* | P30H1 | 220 | 163 | 207 | 590 | 51 |
| *P. myrtifolius* | P32H1 | 222 | 163 | 206 | 591 | 53 |
| *P. orientalis* | P33H1 | 221 | 163 | 205 | 589 | 51 |
| *P. oxyphyllus* | P34H3 | 221 | 163 | 204 | 588 | 51 |
| *P. roseus* | P37H1 | 218 | 163 | 202 | 583 | 56 |
| *P. sikkimensis* | P38H1 | 198 | 163 | 206 | 567 | 52 |
| *P. welwitschianus* | P40H1 | 222 | 163 | 196 | 581 | 55 |

The obtained ITS sequences were all aligned as shown in Appendix B. The results revealed that genetic variations of ITS region were mostly caused from nucleotide substitutions. Nucleotide deletion was found in ITS1 mostly in *P. taxodiifolius*, *P. pulcher*, *P. acutissimus* and *P. sikkimensis* at position 192 to 210, and 219 to 224. In addition, based on the direct sequencing technique, the electropherograms of some samples appeared to have double signaling peaks at one nucleotide position. From a single PCR product sequencing, the obtained electropherogram showed similarity in both forward and reverse sequencing. This phenomenon has been called nucleotide additive sites (Sang *et al*., 1995).

The obtained entire ITS sequences (Appendix B) indicated that *P. urinaria*, *P. emblica*, *P. taxodiifolius*, and *P. debilis* appeared to have nucleotide additive sites. Whereas nineteen *Phyllanthus* species, including *P. amarus*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. virgatus*, *P. acutissimus*, *P. angkorensis*, *P. reticulatus*, *P. microcarpus*, *P. columnaris*, *P. gracilipes*, *P. harmandii*, *P. lingulatus*, *P. myrtifolius*, *P.orientalis*, *P. oxyphyllus*, *P. roseus*, *P. sikkimensis*, and *P. welwitchianus* have no nucleotide additive sites. The number of the nucleotide additives was in the range of 1 to 13 sites. *P. urinaria* was found to contain several nucleotide additive sites. The nucleotide additive positions were assigned following the IUPAC codes (Y=C/T, R=A/G, W=A/T, K=G/T, M=A/C, S=C/G).

### 4.2.3 Phylogenetic relationship of *Phyllanthus* spp. by nuclear ITS sequencing

The obtained sequences of entire ITS region were used to manipulate the data matrices. The ITS sequence data matrices were analyzed for the phylogenetic raltionship by using PAUP version 4.0b 10 (Swofford, 2003) program. All of possible phylogentic trees were consensus with semi-strict method. Bootstraping analysis was performed with 1000 replications. The entire ITS of *Margaritaria cyanosperma*, *Savia bahamensis*, *Flueggea leucopyrus*, and *Lingelsheimia* sp. were used as the outgroups.

The entire ITS sequences of twenty-four taxa representing twenty-three *Phyllanthus* species were used to produce the data matrix. The obtained ITS data matrix was 677 characters, 373 variable characters and 302 informative characters. Based on these data matrix, maximum parsimony method could be simulated for possible parsimony trees as 198 equally parsimonious trees. Maximum Parsimony analysis produced one parsimonious tree of 1397 steps, with a CI of 0.467, RI of 0.771, and RC of 0.360. The semi-strict consensus tree with bootstrap percentages is shown in Figure 4.6. The Maximum Likelihood algorithm was applied to the same dataset, resulting in one ML tree which was identical with the MP tree (Figure 4.7). Furthermore, the NJ analysis gave an essentially identical topology to the parsimony tree (Figure 4.8). It could be concluded that the phylogentic relationship of the twenty-three *Phyllanthus* species was the non-monophyletic embedded with *Sauropus* and *Breynia* (Clade M).

Previously, Kathriarachchi *et al*. analyzed the ITS sequences of 79 species belonging to *Phyllanthus* sensu lato, and classified these species into 15 clades (clade A–O) (Kathriarachchi *et al*., 2006). In the present investigation, *Phyllanthus* species in Thailand were found to be classified to 8 of the 15 clades of Kathariarachchi. Of the 12 species whose nucleotide sequences were determined in the present investigation for the first time, 8 belonged to clade N, 3 to clade C, and 1 to clade E. Moreover, the phylogram showed that *P. virgatus, P. myrtifolius, P. welwitschianus, P. reticulatus, P. pulcher, P. amarus, P. debilis, P. urinaria, P. emblica, P. oxyphyllus*, and *P. acidus*

were very close to those previously described with bootstrap percentages from 50% to 100%, which confirmed the identification of these species (Figure 4.6).

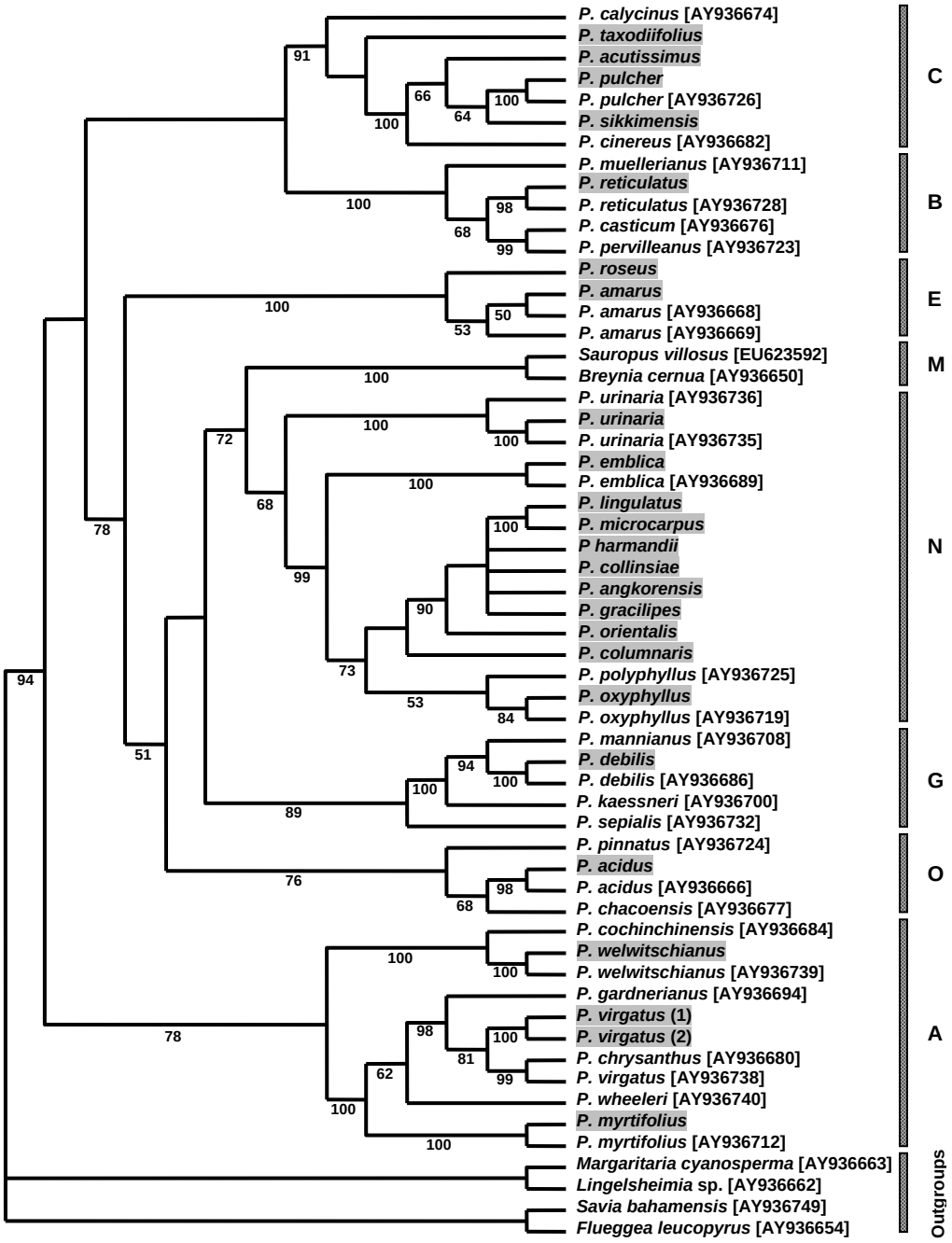



**Figure 4.6** Molecular phylogenetic tree of *Phyllanthus* species distributed in Thailand based on the ITS sequences using a maximum parsimony algorithm. Semi-strict consensus tree of 198 equally parsimonious trees (1397 steps, CI = 0.467, RI = 0.771, RC = 0.360) was determined by heuristic analysis. Bootstrap percentages >50 are shown below branches. Sequences from this study are highlighted in gray. Clade names follow Kathriarachchi *et al*. (2006). The sequence data of the species followed by accession numbers in brackets were retrieved from the GenBank DNA Database.

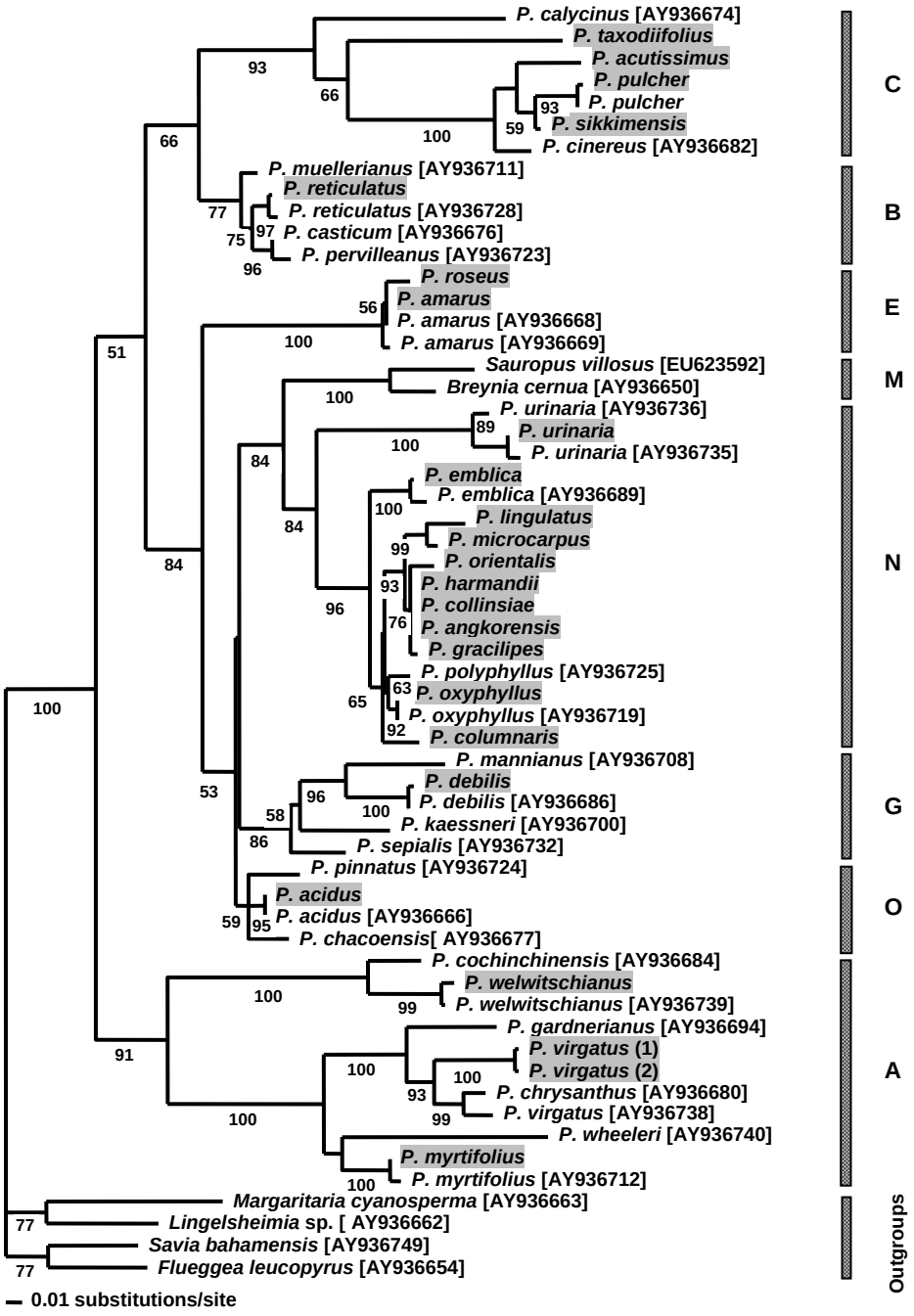



**Figure 4.7** Molecular phylogenetic tree of *Phyllanthus* species distributed in Thailand based on the ITS sequences using a Maximum Likelihood algorithm. Substitution model is HKY+G. Bootstrap percentages >50 are shown below branches. Sequences from this study are highlighted in gray. Clade names follow Kathriarachchi *et al*. (2006). The sequence data of the species followed by accession numbers in brackets were retrieved from the GenBank DNA Database.

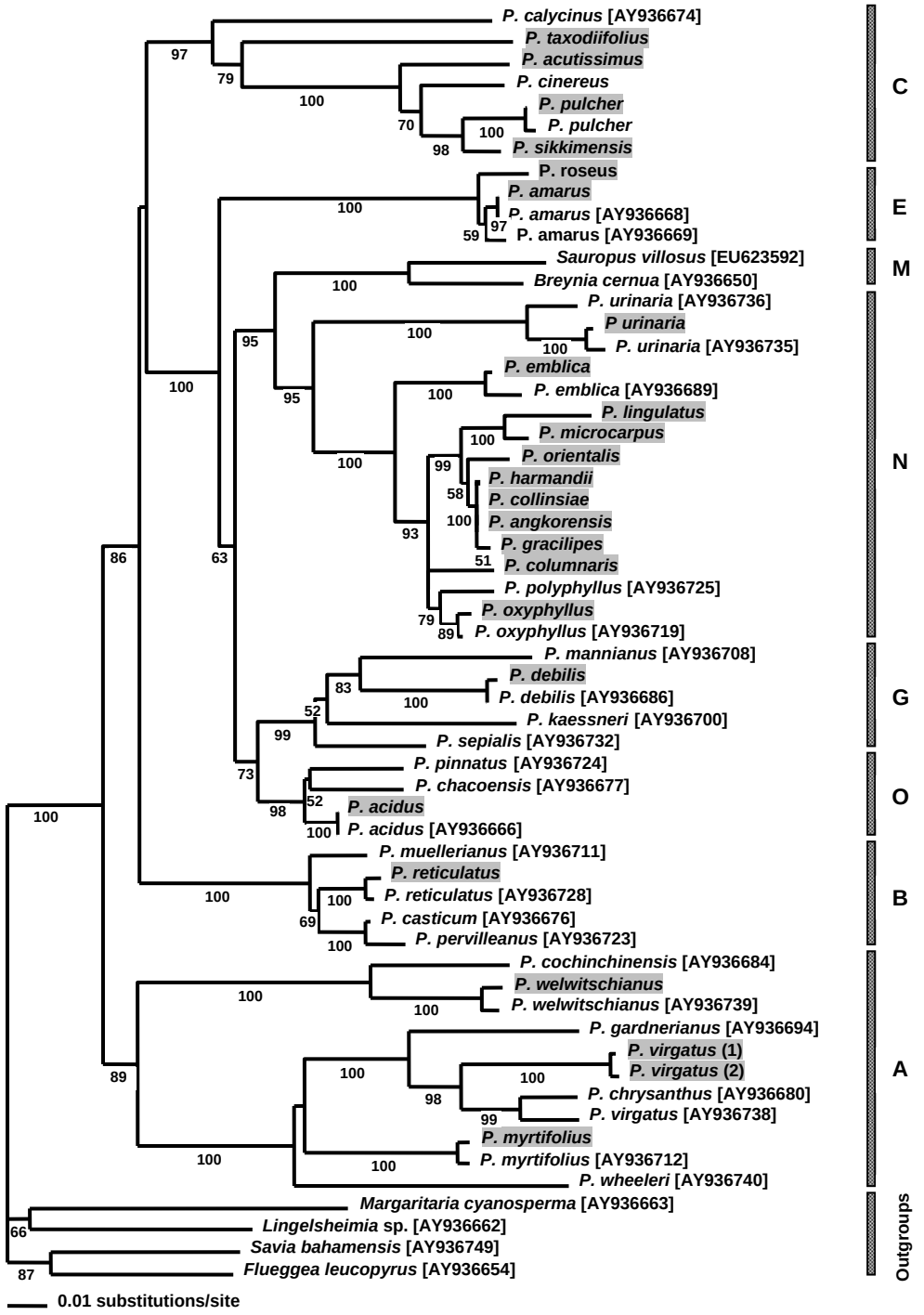



**Figure 4.8** Molecular phylogenetic tree of *Phyllanthus* species distributed in Thailand based on the ITS sequences using a Neighbor-joining algorithm. Bootstrap percentages >50 are shown below branches. Sequences from this study are highlighted in gray. Clade names follow Kathriarachchi *et al*. (2006). The sequence data of the species followed by accession numbers in brackets were retrieved from the GenBank DNA Database.

## 4.3 Discussions

### Genetic Variation of *Phyllanthus* species

This study has revealed the genetic variation of twenty-three *Phyllanthus* species in Thailand by determining their entire ITS nucleotide sequence. Based on all the obtained ITS sequences, the length of ITS1 regions are in range of 197-222 bp while that of ITS regions are in range of 196-210 bp. The ITS1 region of most of all *Phyllanthus* species is consistently longer than the ITS2 region, excepted in *P. taxodiifolius*, *P. pulcher*, *P. acutissimus* and *P. sikkimensis.* These four species appeared nucleotide deletion in the ITS1. Length variation between species is due to insertion-deletion events (indels) in the ITS1 and ITS2. Baldwin *et al*., (1995) reported the ITS1 region is longer than ITS2 in most angiosperms. As a result in Table 4.3, the percentage of potentially informative is greater in ITS1.

Their 5.8S coding regions are all 163 bp in length. This region is an evolutionary highly conserved sequence in angiosperms (Baldwin *et al*., 1995). The apparent entire ITS sequences of all 23 *Phyllanthus* species are in the range from 566-595 bp long. The GC contents of their ITS region are 51%-60%. In the ITS1 region, Liu and Schardl (1994) reported a conserved sequence that can be aligned across many flowering plant families. The conserved motif is 21 bp long, was found at position 124–144 in all plant samples of 23 *Phyllanthus* species examined. This position places the motif in a similar location to that in other angiosperms Liu and Schardl (1994). The highly conserved sequence in ITS1 suggests that this region may have a key function in processing rRNA gene transcripts.

From RAPD analysis in Chapter III, RAPD pattern of *P. collinsiae* and *P. ankorensis* were different that implied those two species should be different species. However, the resulting of sequence alignment of *P. collinsiae* and *P. ankorensis* showed the identical ITS sequences. The result indicated those two sequences were from the same species as *P. ankorensis* and suggested those two species was misidentified. There seems to be some confusion of plant habit and morphology between young plant

and adult plant of *P. ankorensis*. The result emphasizes that the sequencing method is more accurate than RAPD method since RAPD pattern may be changed by many factors.

In this experiment, we found that the high purity of genomic DNA or PCR products could be critical for successful ITS sequencing. DNA template purity appears to be important for ITS sequencing. This problem can be solved by further purification of genomic DNA before PCR amplification step.

**Phylogenetic Analysis of *Phyllanthus* Species Based on the Nucleotide Sequence of the ITS Region**

The molecular phylogenetic relationship among the *Phyllanthus* species distributed in Thailand was analyzed based on approximately 0.4 kb sequences of the ITS locus obtained in the present investigation together with those retrieved from the DNA database. The data set contained 54 ingroups and 4 outgroups, namely *Margaritaria cyanosperma*, *Savia bahamensis*, *Flueggea leucopyrus*, and *Lingelsheimia* sp. Maximum Parsimony (MP), Maximum Likelihood (ML), and Neighbor joining (NJ) methods were used to construct the phylogentic tree with the same data set, resulting an identical topology tree.

Kathriarachchi *et al*. (2006) analyzed ITS sequences of 79 species belonging to *Phyllanthus* sensu lato, and revealed these species were classified into 15 clades (clade A to clade O). *Phyllanthus* species in Thailand were found to belong to eight clades of these 15 clades. Of 12 species newly analyzed in the present investigation, eight belonged to clade N, three to clade C, and one to clade E. Taxonomy of *Phyllanthus* in Thailand are rather limited and not well studied. Based on Kathriarachchi *et al*. reported, we can classify twenty tree *Phyllanthus* in Thailand into five subgenera as follows; Clade A belonged to *Isocladus*, Clade B and O belonged to *Kirganelia*, Clade C belonged to *Eriococus* and *Isocladus*, Clade E and G belonged to *Phyllanthus,* and Clade N

belonged to *Phyllanthus* and *emblica*. Phylogenetic tree confirmed the previous finding that *Phyllanthus* species are non-monophyletic but embedded with *Sauropus* and *Breynia*, and suggested that these three taxa may be changed at the generic rank in the future.

According to phylogenetic analysis from ITS and RAPD data, the results showed that *P. pulcher* and *P. taxodiifolius* were grouped in the same clade (Clade I of RAPD and Clade C of ITS) and *P. urinaria*, *P. emblica*, and *P. collinsiae* were grouped in Clade III of RAPD and Clade N of ITS whereas the other members fell in different clades. Topological incongruence between ITS and RAPD may be caused by the limit of plant samples and/or the interpretation from RAPD data.

# CHAPTER V

# ITS SEQUENCE-BASED AUTHENTICATION OF *PHYLLANTHUS AMARUS*, *P. DEBILIS* AND *P. URINARIA* BY A PCR-RFLP METHOD

## 5.1 Material and methods

### 5.1.1 Plant materials

The aerial part of three *Phyllanthus* species, *P. amarus*, *P. urinaria* and *P. debilis* were used in this study. All of the collected plant materials and their localities are listed in Table 3.1 in Chapter III. The crude drug samples of "Luk Tai Bai (ลูกใต้ใบ)" were obtained from local Thai markets are listed in Table 5.1.

**Table 5.1** List of crude drug samples obtained in Thai local markets.

| Sample no. | Crude drug name | Type of crude drug | Year of collection | Drug market |
|---|---|---|---|---|
| 1 | ลูกใต้ใบ (Luk Tai Bai) | Dried Segments | 2006 | Bangkok |
| 2 | ลูกใต้ใบ (Luk Tai Bai) | Dried Segments | 2007 | Songkhla |
| 3 | หญ้าใต้ใบ (Ya Tai Bai) | Dried Segments | 2006 | Bangkok |
| 4 | ลูกใต้ใบ (Luk Tai Bai) | Dried Segments | 2006 | Bangkok |
| 5 | ลูกใต้ใบ (Luk Tai Bai) | Powder | 2006 | Bangkok |
| 6 | ลูกใต้ใบ (Luk Tai Bai) | Capsule | 2010 | Bangkok |
| 7 | ลูกใต้ใบ (Luk Tai Bai) | Capsule | 2006 | Nakhon Phathom |
| 8 | ลูกใต้ใบ (Luk Tai Bai) | Dried Segments | 2009 | Bangkok |
| 9 | ลูกใต้ใบ (Luk Tai Bai) | Capsule | 2010 | Suphanburi |
| 10 | ลูกใต้ใบ (Luk Tai Bai) | Capsule | 2010 | Phetchaburi |
| 11 | ลูกใต้ใบ (Luk Tai Bai) | Capsule | 2010 | Samutprakan |

### 5.1.2 PCR amplification

The ITS region was amplified using PhyII-ITS-1F and PhyII-ITS-1R as forward and reverse primers, respectively. For fresh plants, PCR amplification was carry out using *Taq* DNA Polymerase (Roche Diagnostics, Japan) as previously described in Chapter IV. For crude drugs, Nova*Taq* Hot Start DNA Polymerase (Novagen, USA) was used for PCR amplification.

### 5.1.3 Restriction enzyme digestion

The restriction sites of ITS region were analyzed by using NEBcutter program (http://tools.neb.com/NEBcutter2/). *Dde* I restriction enzyme was selected for identification and the prediction sites are shown in Figure 5.1. The PCR products of ITS region approximately 700 bp were digested with 2.5 units of *Dde* I restriction enzyme (Roche Diagnostics, Japan) at 37°C for 3 h, separated by 2.5% agarose gel electrophoresis, and visualized by ethidium bromide staining under UV light. The 100 bp plus DNA Ladder (Vivantis, USA) was used as a DNA marker.

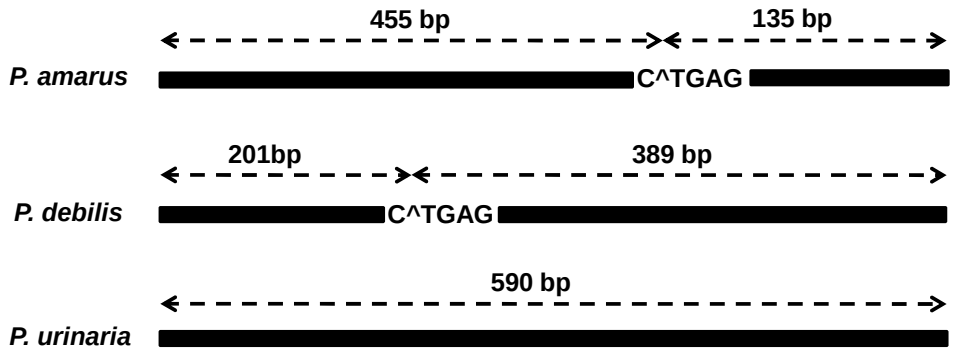



**Figure 5.1** *Dde I* restriction sites prediction of ITS region in *P. amarus*, *P. urinaria*, and *P. debilis*. ITS nucleotide position start from the first position of this region.

## 5.2 Results

### Genomic DNA from crude drug samples

Genomic DNA isolated from 11 crude drug samples (no.1-11) that were obtained from local Thai markets are shown in Figure 5.2.

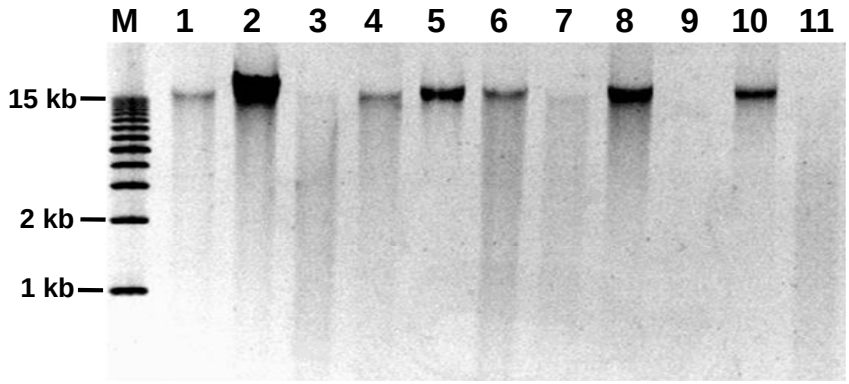



**Figure 5.2** Genomic DNA from 11 crude drugs of *Phyllanthus* species.

Lane M: EZ Load 1kb Molecular Ruler

Lane 1-11: Crude drug sample no. 1 to 11

### 5.2.1 PCR-RFLP analysis

Sequence alignment of the ITS regions from three most popular medicinal *Phyllanthus* species, *P. amarus*, *P. urinaria*, and *P. debilis* revealed the presence of a *Dde* I recognition site (C^TNAG) at nt 450–454 (from 5′-end) of the ITS region of *P. amarus* and at nt 198–202 (from 5′-end) of *P. debilis*. In contrast, *Dde* I restriction site was absent in the ITS region of *P. urinaria* (Figure 5.3).

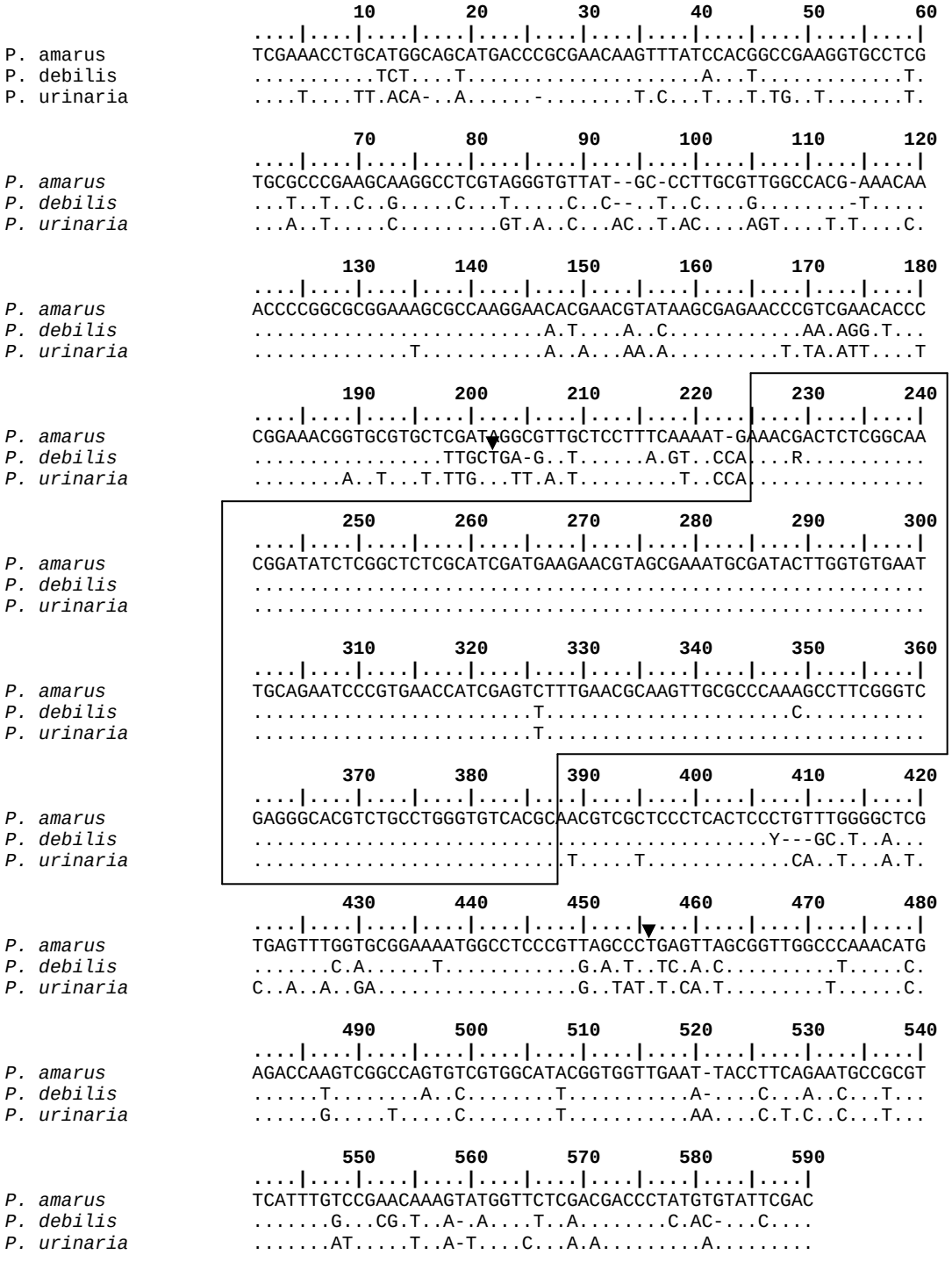



**Figure 5.3** Sequence alignment of the ITS1-5.8S rDNA-ITS2 region of the nuclear ribosomal gene of *Phyllanthus amarus, P. urinaria*, and *P. debilis*. The boxed sequences correspond to 5.8S rDNA. Arrows indicate *Dde* I restriction sites.

The ITS regions were amplified from *P. amarus, P. debilis*, and *P. urinaria* using a pair of primers, Phyll-ITS-1F and Phyll-ITS-1R, and the PCR products were incubated with *Dde* I. As shown in Figure 5.4, the PCR product from *P. amarus* was digested into two fragments of 500 and 190 bp, and that from *P. debilis* into 440 and 250 bp; however, the product from *P. urinaria* remained undigested.

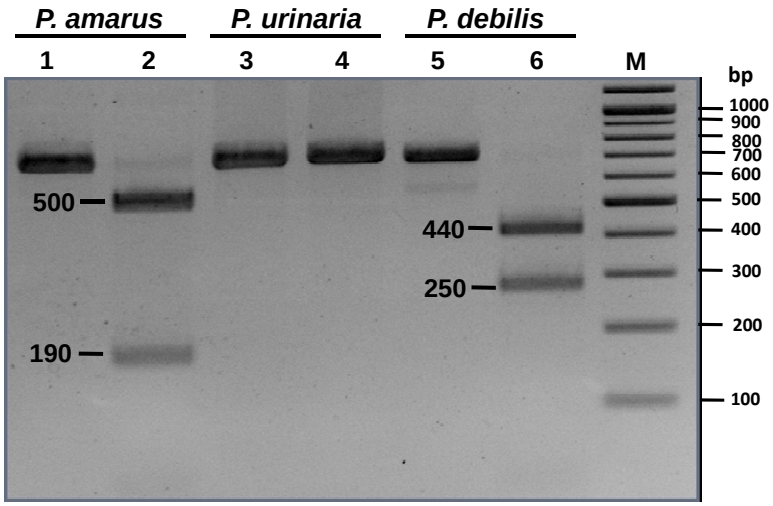



**Figure 5.4** PCR-RFLP analysis of the ITS1-5.8S rDNA-ITS2 region amplified from *Phyllanthus amarus*, *P. urinaria*, and *P. debilis*. The PCR products from the samples (lanes 1, 3, and 5) were digested by *Dde* I (lanes 2, 4, and 6). Lane M: VC 100bp Plus DNA Ladder

The *Dde* I restriction sites also were examined whether in the ITS regions of the 36 *Phyllanthus* species in Thailand. *P. roseus* has a *Dde* I site at the same position as *P. amarus*, and 13 species (*P. emblica*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. angkorensis*, *P. reticulatus*, *P. columnaris*, *P. harmandii*, *P. lingulatus*, *P. oxyphyllus*, *P. orientalis*, *P. gracilipes*, *P. microcarpus*) besides *P. urinaria* has no *Dde* I site.

PCR-RFLP protocol was applied to crude drug samples of “Luk Tai Bai (ลูกใต้ใบ)” obtained from the markets. The ITS fragments from nine samples (No. 1–9) were digested into two fragments of 500 and 200 bp with *Dde* I, while the fragments from two crude drug samples (No. 10 and No. 11) exhibited three fragments of 700, 500, and 200 bp after *Dde* I digestion (Figure 5.5). The result indicated that samples No. 1–9 were crude drugs from *P. amarus*, while samples No. 10 and 11 were mixtures of *P. amarus* and presumably *P. urinaria*.

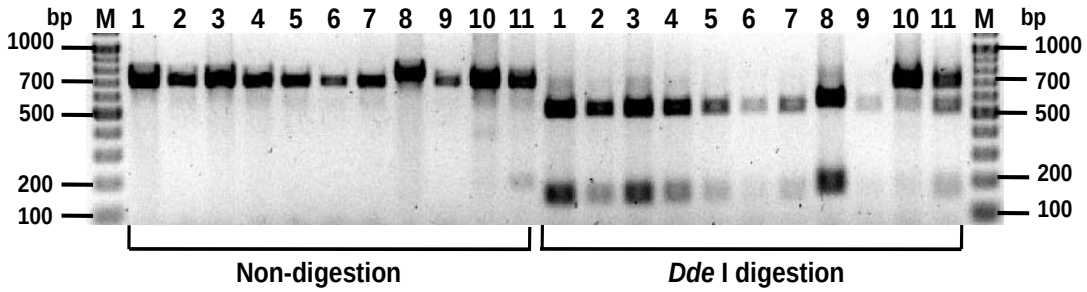



**Figure 5.5** DNA-based authentication of Thai crude drugs “Luk Tai Bai (ลูกใต้ใบ)” and “Ya Tai Bai (หญ้าใต้ใบ)” by PCR-RFLP analysis of ITS locus. The ITS-5.8S rDNA-ITS2 fragments were amplified from 11 crude drug samples obtained in the market and separated by agarose gel electrophoresis either without Dde I digestion (left 11 lanes) or after Dde I treatment (right 11 lanes).

Lane M: VC 100bp Plus DNA Ladder

Lane 1-11: Crude drug sample no. 1-11

## 5.3 Discussion

In the present study, PCR-RFLP method was performed for identification of three most popular medicinal *Phyllanthus* species (*P. amarus, P. debilis*, and *P. urinaria)*. We used Phyll-ITS-1F and Phyll-ITS-1R as forward and reverse primers for amplification of fresh and crude drug samples. The patterns of *Dde* I digestion were analyzed by using NEB cutter program. The PCR product from *P. amarus* was digested into two fragments of 500 and 190 bp, and that from *P. debilis* into 440 and 250 bp; however, the product from *P. urinaria* remained undigested. From 36 *Phyllanthus* species in Thailand, We found that *P. roseus* has a *Dde* I site at the same position as *P. amarus*. However, these two species are morphologically different and are found in different habitats. *P. amarus*, *P. debilis* and *P. urinaria* are most popular species in the Thai herbal markets, and this protocol may provide us with a convenient method to identify and/or discriminate *P. amarus, P. debilis*, and *P. urinaria* obtained from markets, without sequencing.

We applied the PCR-RFLP protocol to crude drug samples of “Luk Tai Bai (ลูกใต้ใบ)” obtained from the various crude drug markets in Thailand. The ITS fragments from nine samples (No. 1–9) were digested into two fragments of 500 and 200 bp with *Dde* I, while the fragments from two crude drug samples (No. 10 and No. 11) exhibited three fragments of 700, 500, and 200 bp after *Dde* I digestion. It was indicated that samples No. 1–9 were crude drugs from *P. amarus*, while samples No. 10 and 11 were mixtures of *P. amarus* and presumably *P. urinaria*. It is interesting to note that No. 3 is derived from *P. amarus* although it was sold as “Ya Tai Bai (หญ้าใต้ใบ)” which is the common plant name of *P. urinaria*. The amount of the PCR products (the intensities of the product bands) varied among the samples. However, this may not reflect the amounts of the particular crude drugs in the samples because the yield of the PCR products are affected by many factors including the amount of target nuclear DNA and/or impurities in the total DNA preparation from each sample.

*Phyllanthus amarus* has effective antiviral activities especially with respect to the hepatitis B virus. In Thai folk medicine, it is usually confused with other herbs such as *P. urinaria* and *P. debilis* because they are morphologically similar and share the same

drug name in the market. Although these three species can be discriminated based on the difference in nucleotide sequence of their respective ITS region, sequencing is time-consuming and relatively costly. The present study revealed that *P. amarus* as well as the crude drug derived from it can be easily discriminated from *P. debilis* and *P. urinaria* based on the PCR-RFLP profile after *Dde* I digestion of the amplified ITS regions.

# CHAPTER VI

# CONCLUSIONS

1. We discriminated twelve *Phyllanthus* species in Thailand by RAPD marker. Screening of eighty random deca-nucleotide primers, only nine primers (OPD-02, OPD-04, OPD-07, OPS-01-, OPS-03, OPS-07, OPS-08, OPS-12, and OPS-19) can be used to amplify all twelve *Phyllanthus* species and generated the unique RAPD profiles.
2. We successfully amplified the ITS region of twenty-three *Phyllanthus* in Thailand by using direct sequencing method. The results revealed that genetic variations of the ITS region were mostly from nucleotide substitutions. Nucleotide deletion was found in ITS1 mostly in *P. taxodiifolius*, *P. pulcher*, *P. acutissimus* and *P. sikkimensis*.
3. A conserved sequence, GGCRC-(NNNNN)-GYGCCAAGGAA (Y = C or T; R = G or A; N = A or G or C or T) was found in ITS1 of all twenty-three *Phyllanthus* species. The highly conserved sequence in ITS1 suggests that this region may have a key function in processing rRNA gene transcripts.
4. Phylogentic tree was reconstructed from ITS region. The results showed that topologies of the obtained parsimony tree, maximum likelihood tree, and neighbor joining tree were identical.
5. Phylogenetic relationship of all twenty-three *Phyllanthus* species was shown to non-monophyletic type with embedded *Sauropus* and *Breynia*. The results suggested that these three taxa may be changed at the generic rank in the future.
6. We applied PCR-RFLP to discriminate three medicinal *Phyllanthus* species. The results showed that this protocol was rapid and accurate to identify *P. amarus* from *P. debillis* and *P. urinaria*.

In conclusion, RAPD marker is a rapid and useful technique to discriminate *Phyllanthus* species since this technique could be generate polymorphic DNA patterns.

However, this technique shows low reproducibility. The present study indicates that DNA authentication using the ITS sequence is a reliable method to identify and/or discriminate between *Phyllanthus* species distributed in Thailand. The established PCR-RFLP protocol is a rapid and accurate method to identify *P. amarus*-derived “Luk-Tai-Bai (ลูกใต้ใบ)” in Thai herbal markets.

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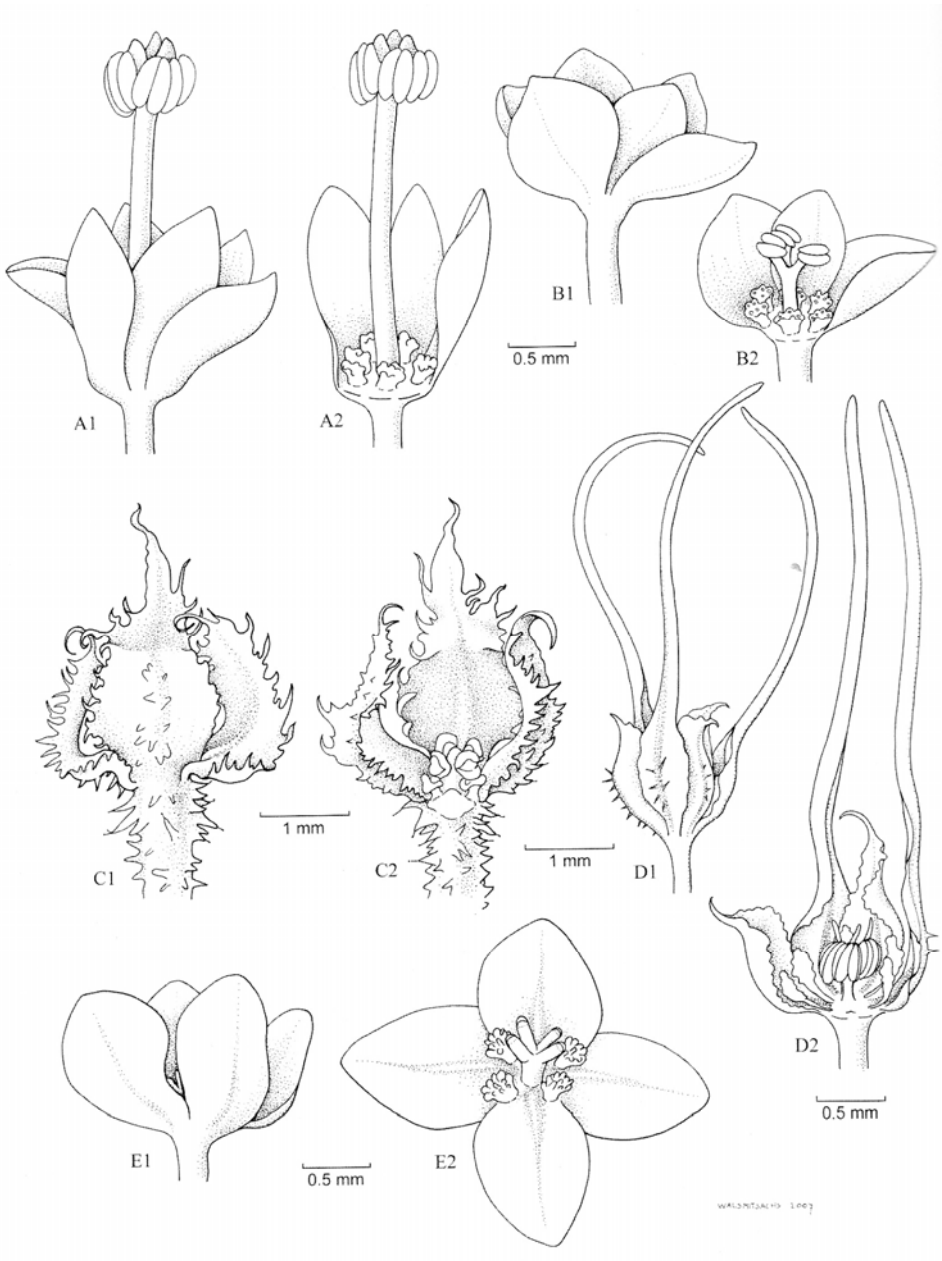
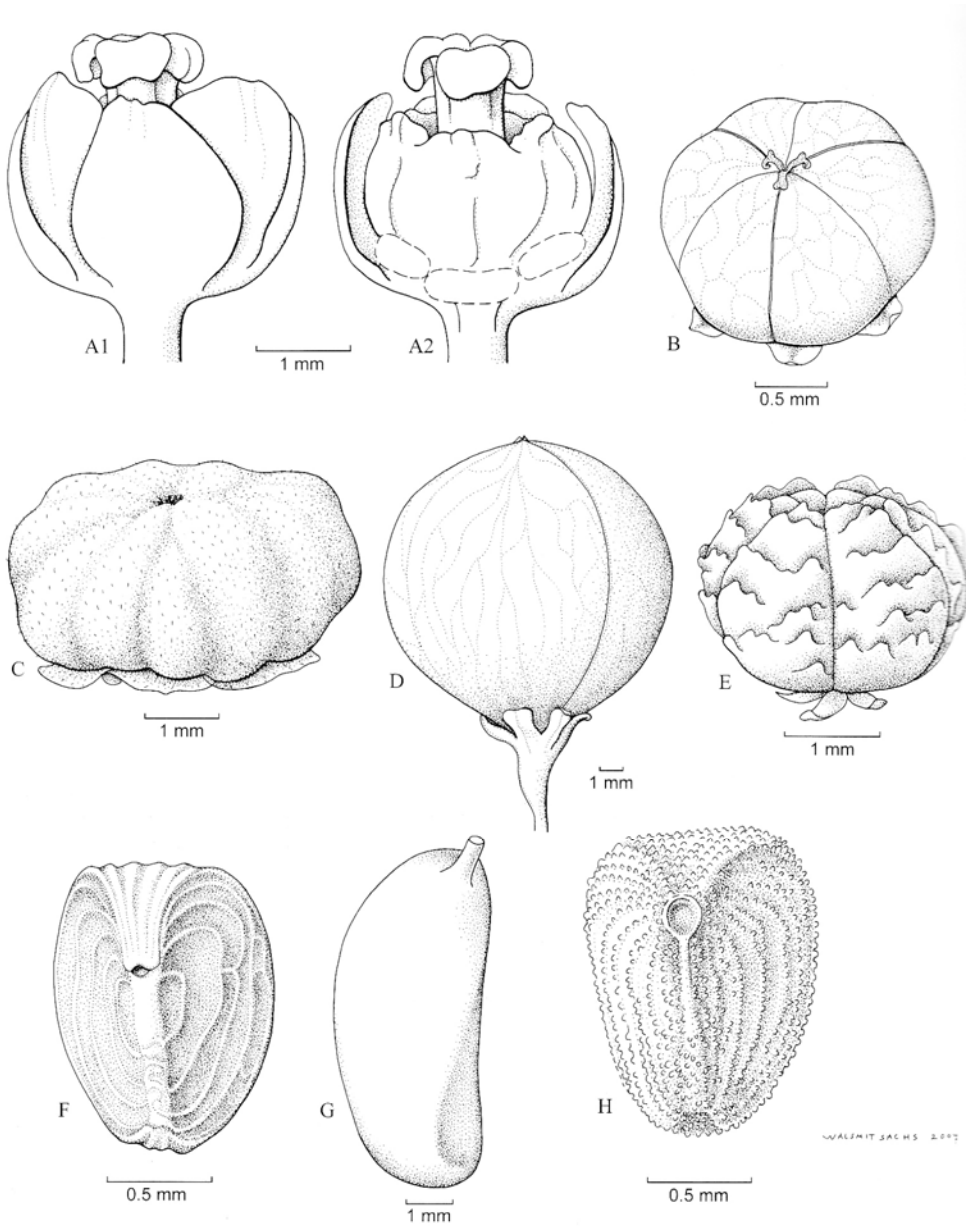
# APPENDICES

# APPENDIX A

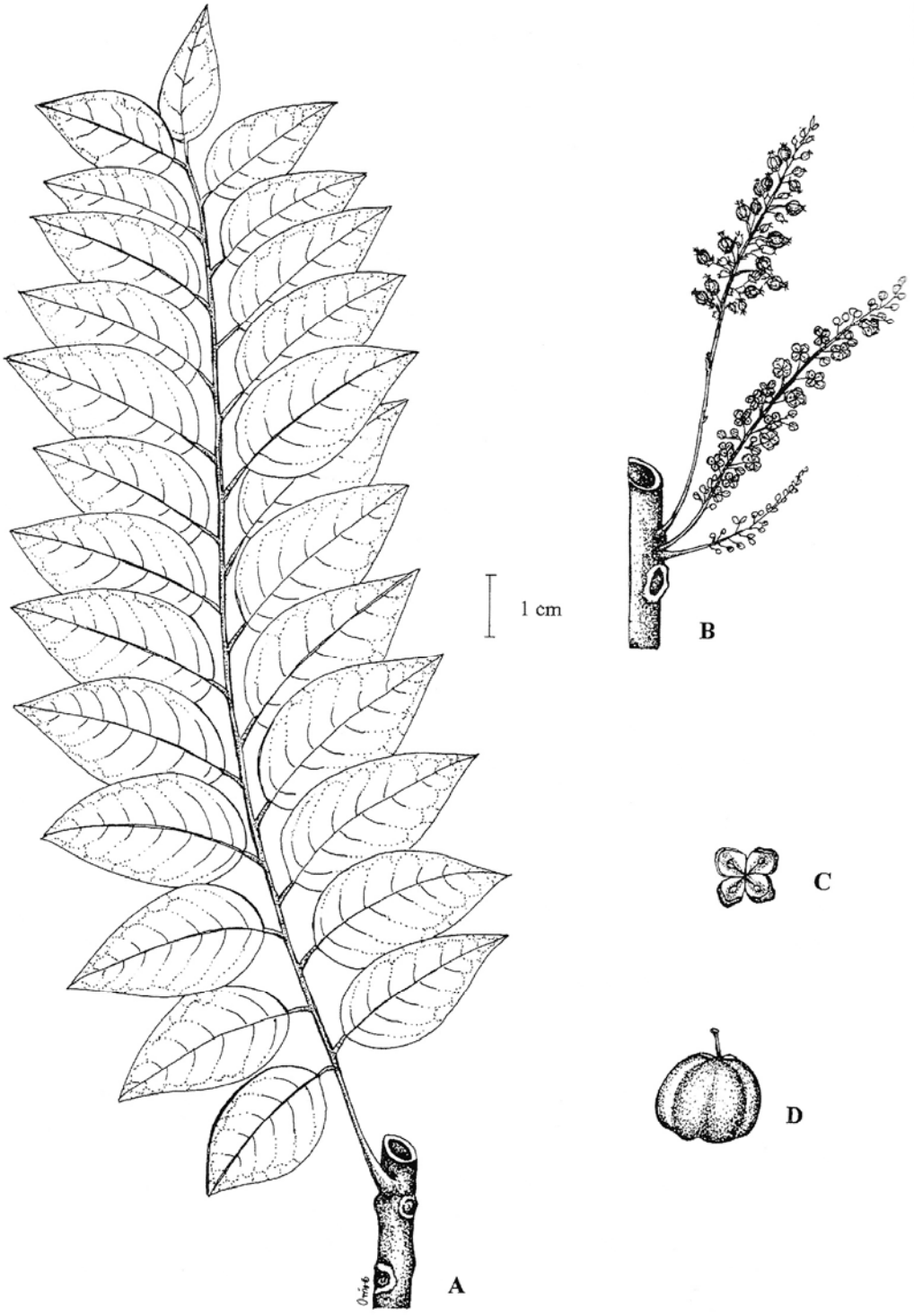
Plant morphology of *Phyllanthus* spp.




**A1**. *Phyllanthus* L.: Staminate flowers (1 = flower from outside, 2 = with few sepals removed to show disc glands and androecium). A: *P. columnaris* with long androphore; B. small flower of *P. debilis*; C. *P. gracilipes* with fimbriate sepal margins and small stamens; D. *P. ridleyanus* with long fimbriate sepals; E. *P. taxodiifolius* with four sepals (Chantaranothai, 2007).



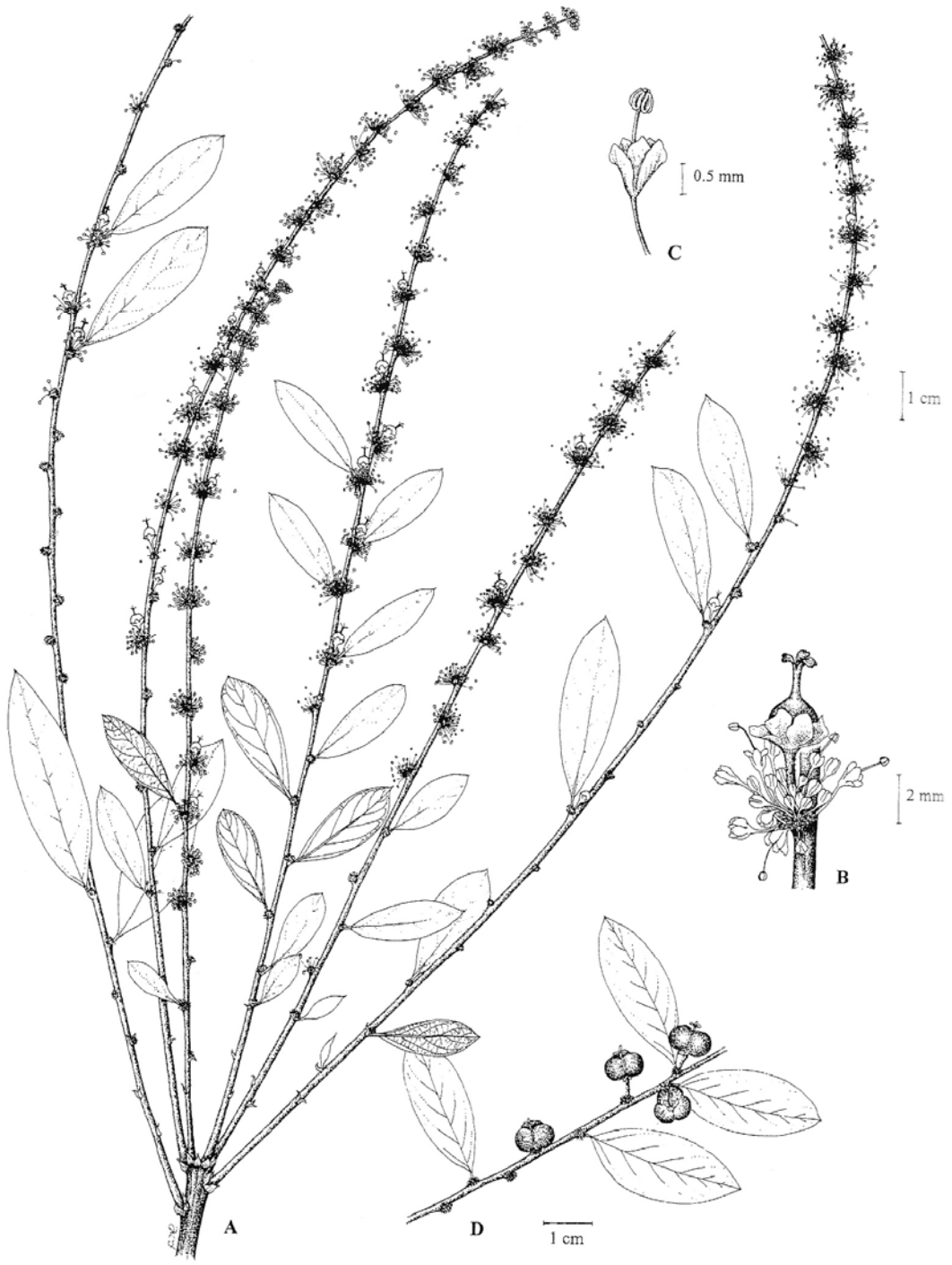

**A2**. *Phyllanthus* L.: Pistellate flowers, fruits and seeds. A: Postillate flower of *P. albidiscus*, 1 = with sepals; 2 = part sepals removed showing high disc; B. dehiscent small fruit of *P. amarus*; C. indehiscent, somewhat fleshy fruit of *P. reticulatus*; D. inflated, dehiscing fruit of *P. roseus*; E. scaly fruit of *P. urinaria*; F. ribbed seed of *P. debilis*; G. smooth seed of *P. roseus*; H. knobbly and ribbed seed of *P. virgatus* (Chantaranothai, 2007).



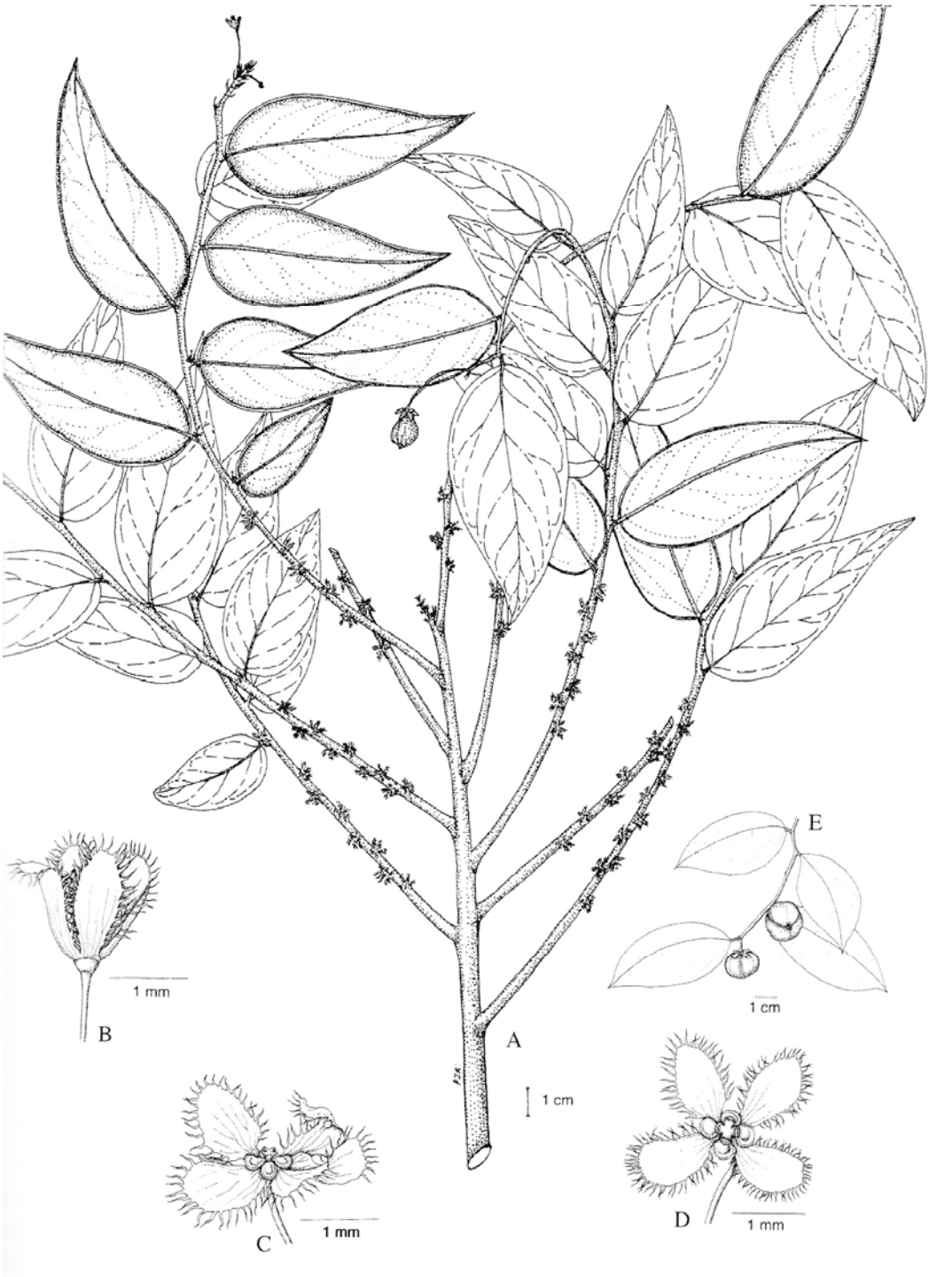

A3. *Phyllanthus acidus*: A. habit; B. inflorescences; C. staminate flower; D. fruit. (Chantaranothai, 2007).


A4. *Phyllanthus amarus*: A. habit; B. fruit (Chantaranothai, 2007).

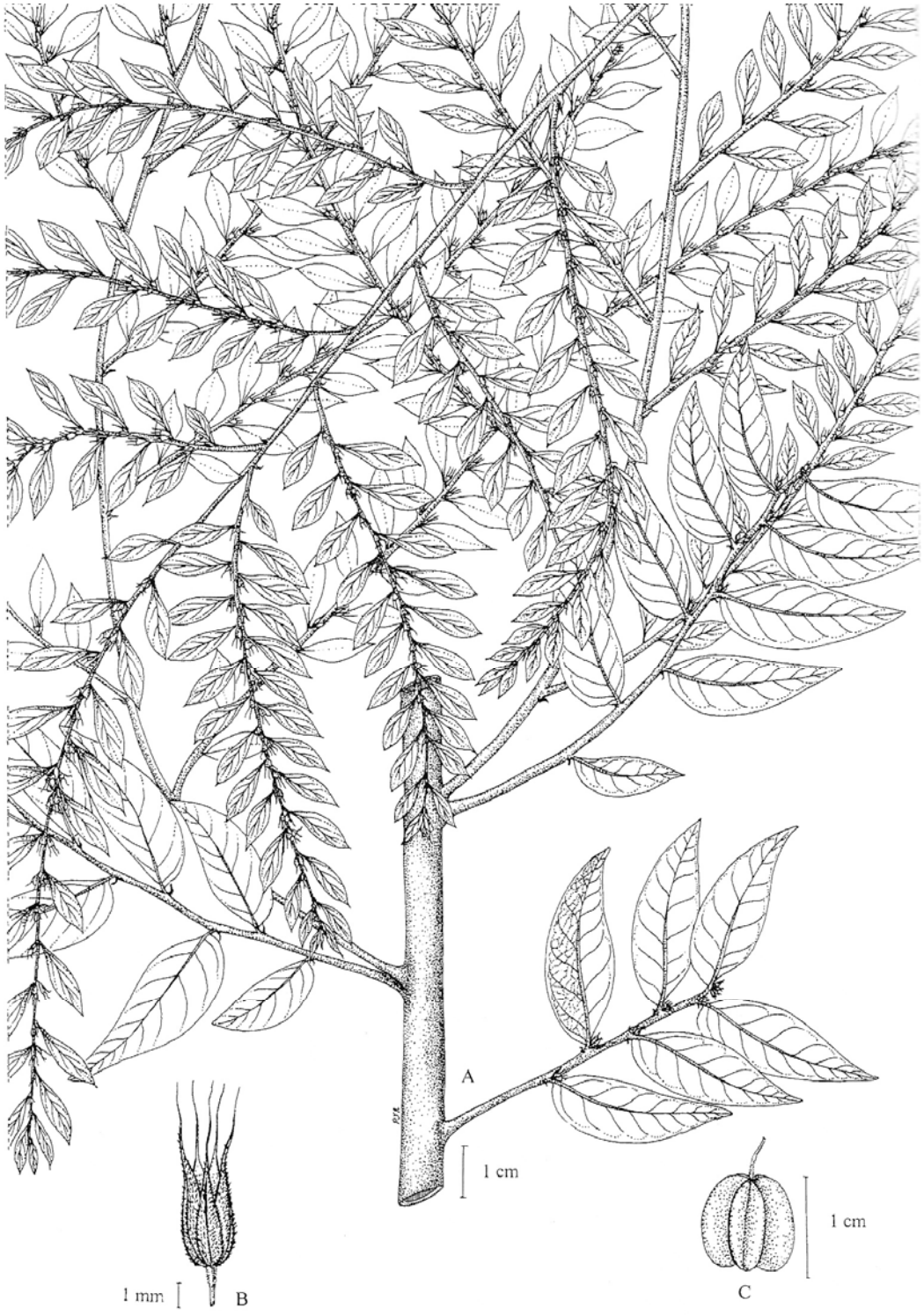

**A5**. *Phyllanthus collinsiae*: A. habit; B. leaves enlarged; C. staminate and pistillate flowers (Chantaranothai, 2007).




A6. *Phyllanthus columnaris*: A. habit; B. flowers; C. staminate flowers; D. fruits (Chantaranothai, 2007).

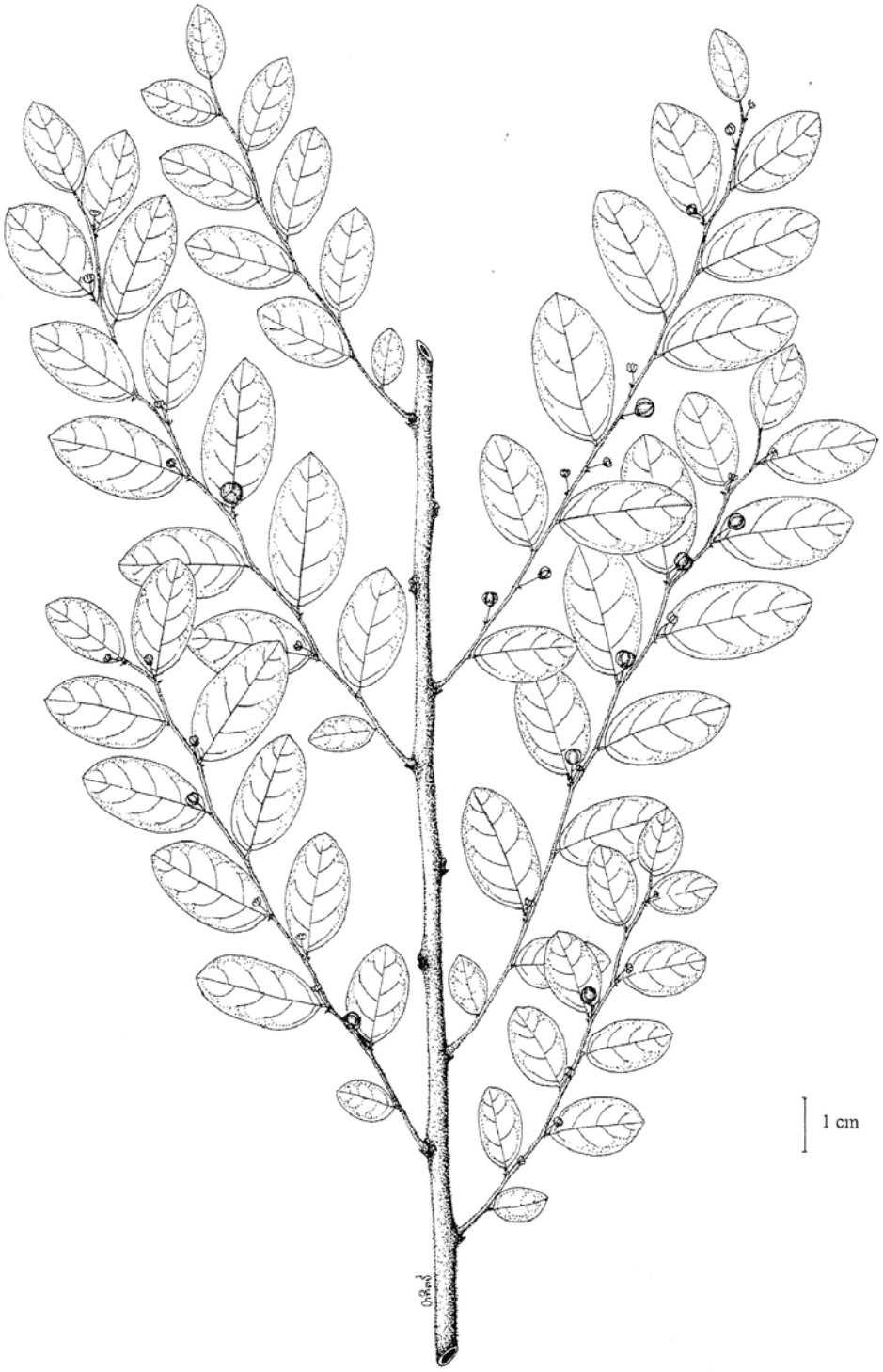



**A7**. *Phyllanthus elegans*: A. habit; B.-D. staminate flowers; E. fruits (Chantaranothai, 2007).




A8. *Phyllanthus mirabilis*: A. habit; B. staminate flowers; C. fruits (Chantaranothai, 2007).


A9. *Phyllanthus oxyphyllus*: habit (Chantaranothai, 2007).

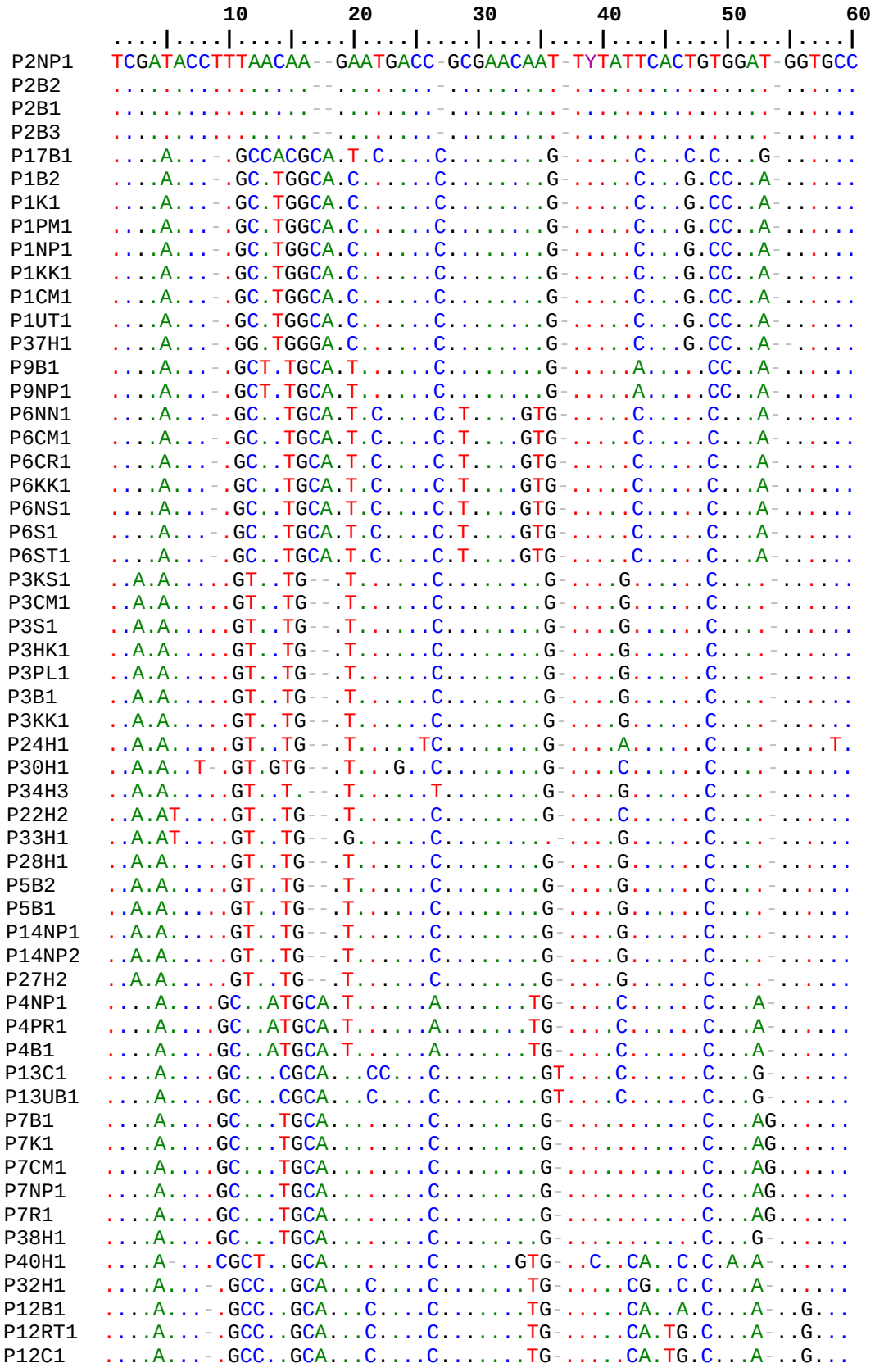



A10. *Phyllanthus reticulatus*: habit (Chantaranothai, 2007).


**A11.** *Phyllanthus urinaria*: A. fruiting branch; B. fruit (Chantaranothai, 2007).

# APPENDIX B

Complete sequence alignment for *Phyllanthus* species. Position 1 to 227 is the ITS1 region, the 5.8S nrDNA region corresponds to position 228 to 390, and the ITS2 region corresponds to position 391-605. The same sequences are indicated by dot (.). Hyphen (-) denotes alignment gap. The characters indicate nucleotide additives. Y=C/T, R=A/G, W=A/T, K=G/T, M=A/C, S=C/G.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P7B1
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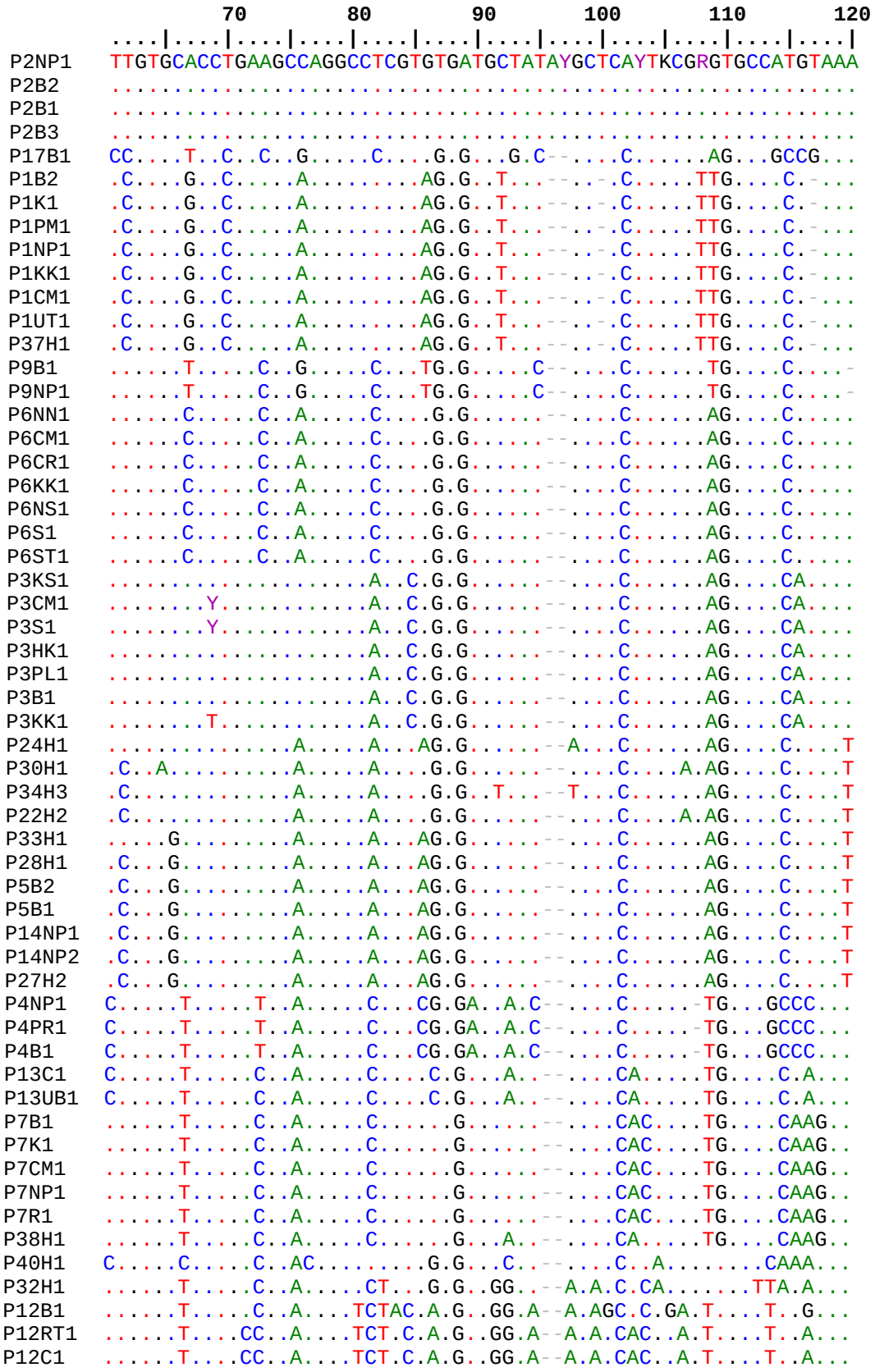
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P2B3
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P1K1 .C....G..C.....A.........AG.G..T.....--.C.....TTG....C.-...
P1PM1 .C....G..C.....A.........AG.G..T.....--.C.....TTG....C.-...
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P1KK1 .C....G..C.....A.........AG.G..T.....--.C.....TTG....C.-...
P1CM1 .C....G..C.....A.........AG.G..T.....--.C.....TTG....C.-...
P1UT1 .C....G..C.....A.........AG.G..T.....--.C.....TTG....C.-...
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P3KK1 ........T...........A..C.G.G.....--....C......AG....CA....
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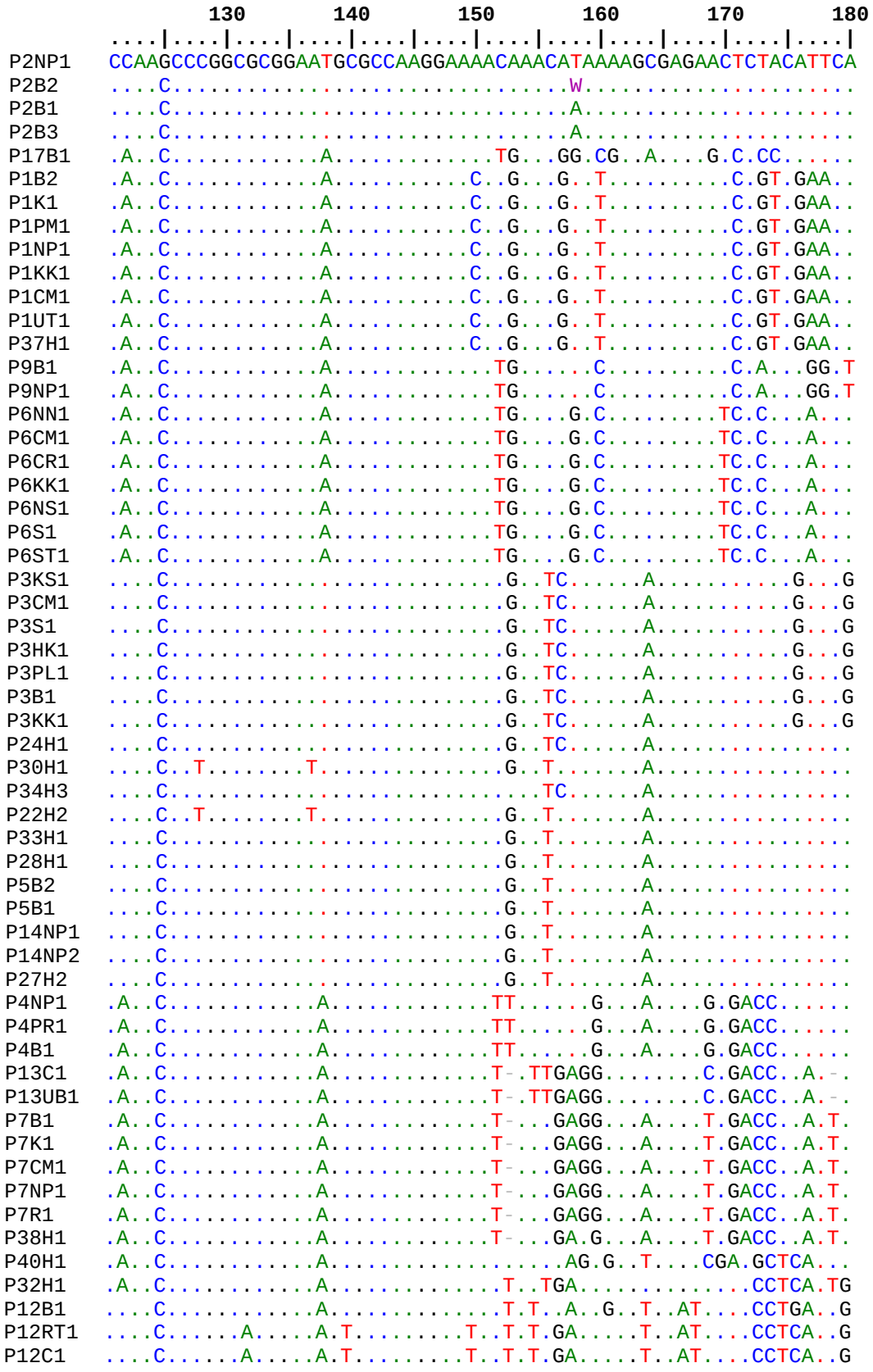
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P4NP1
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P4B1
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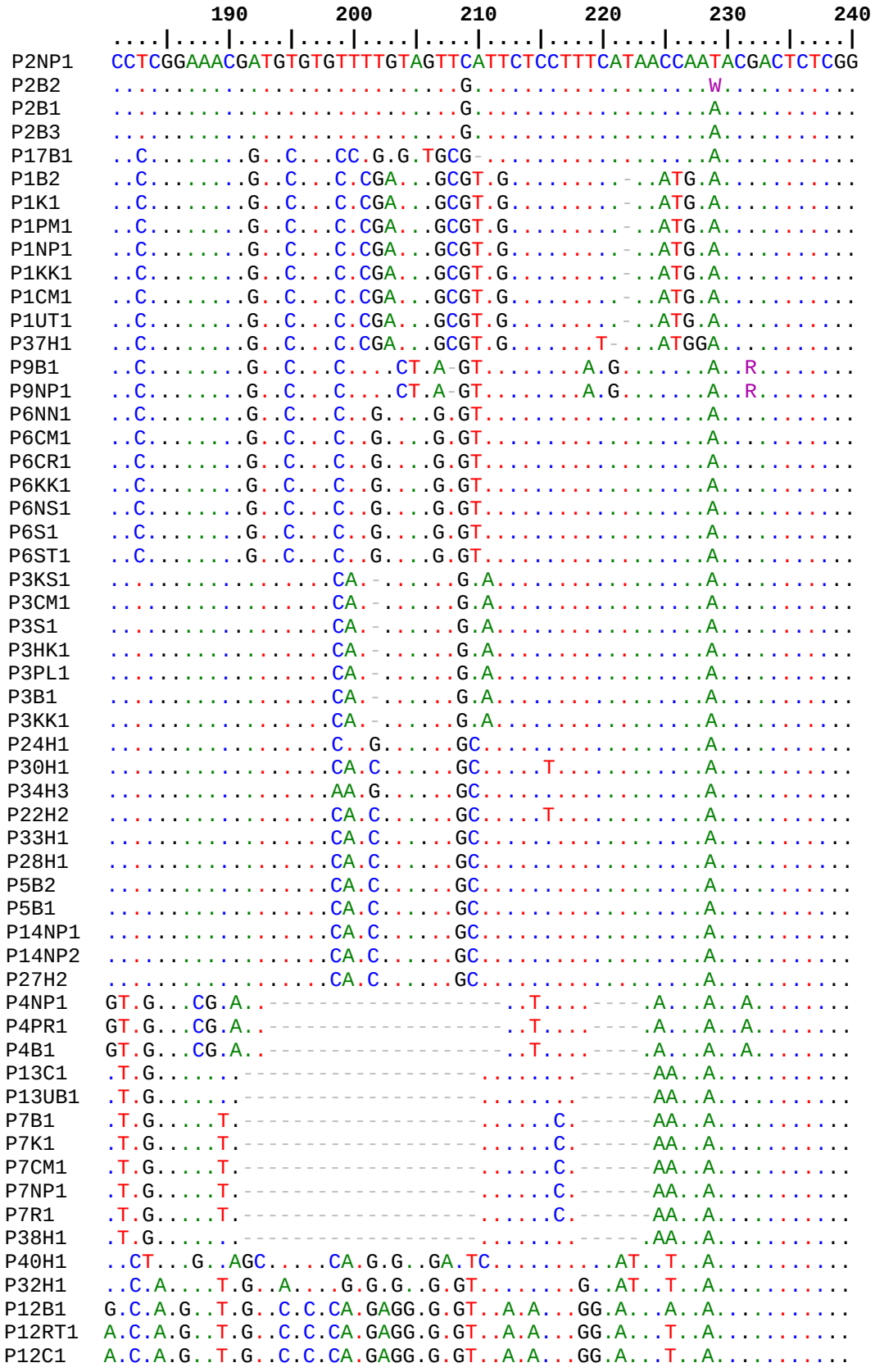
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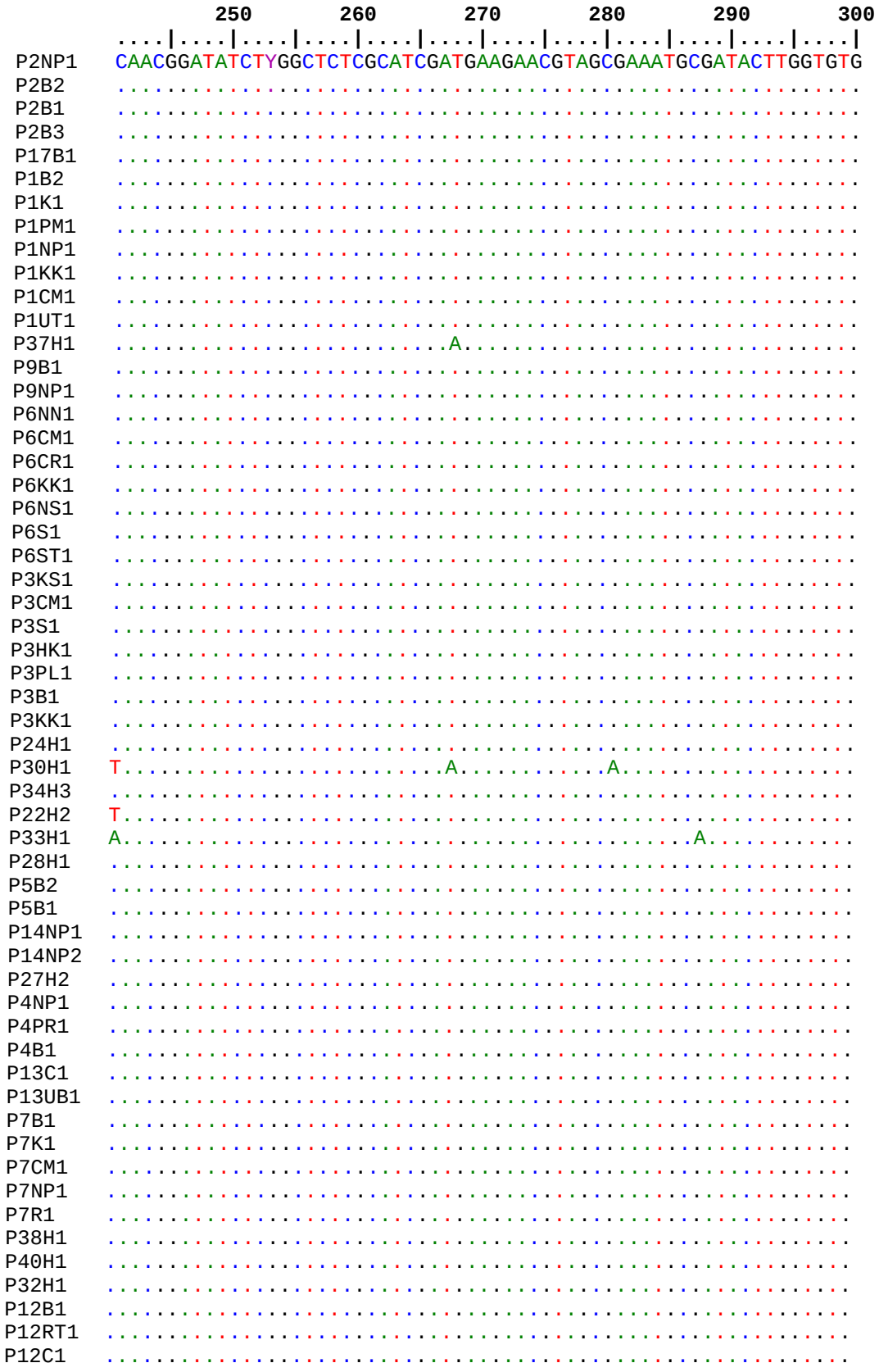
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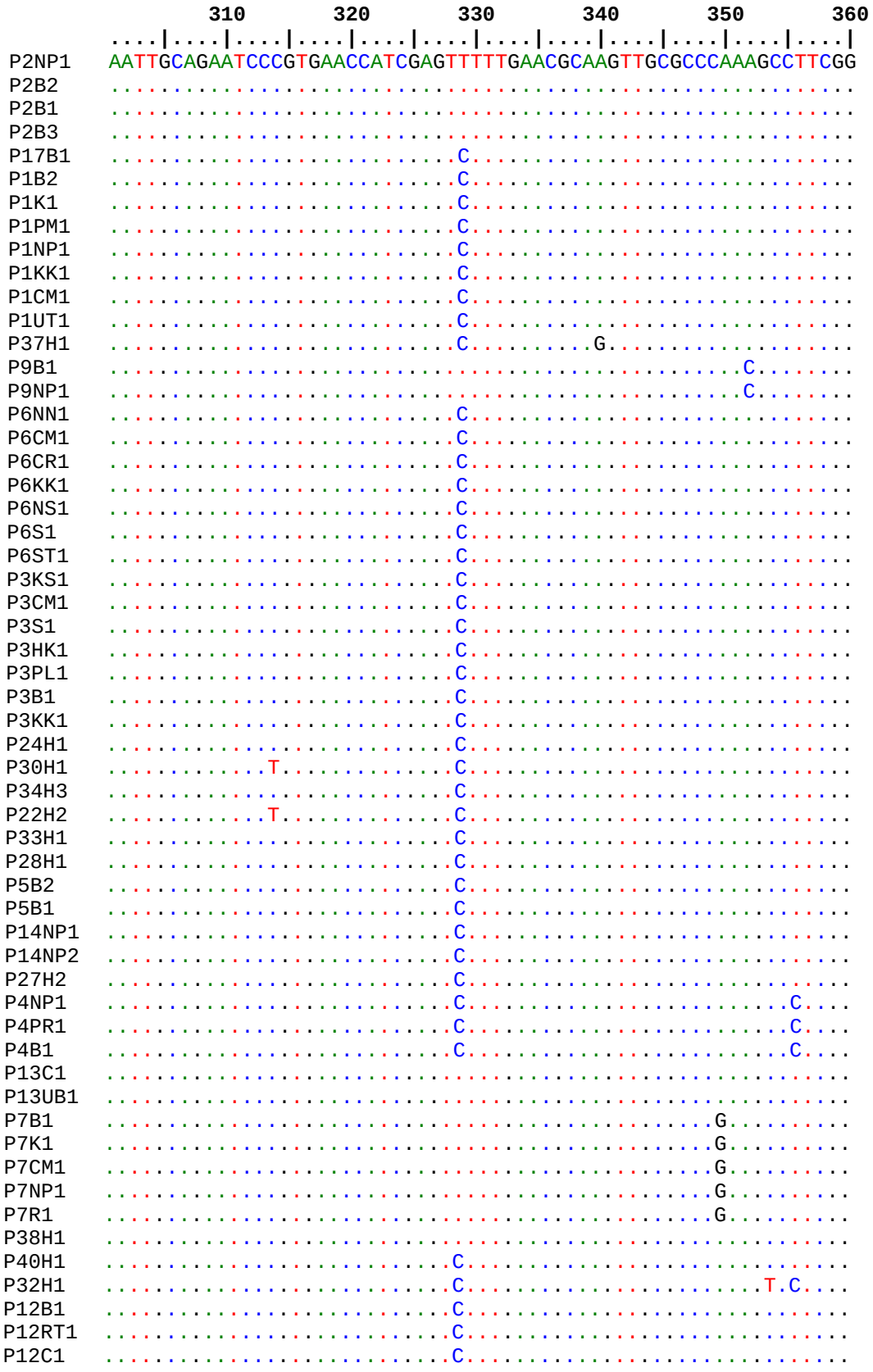
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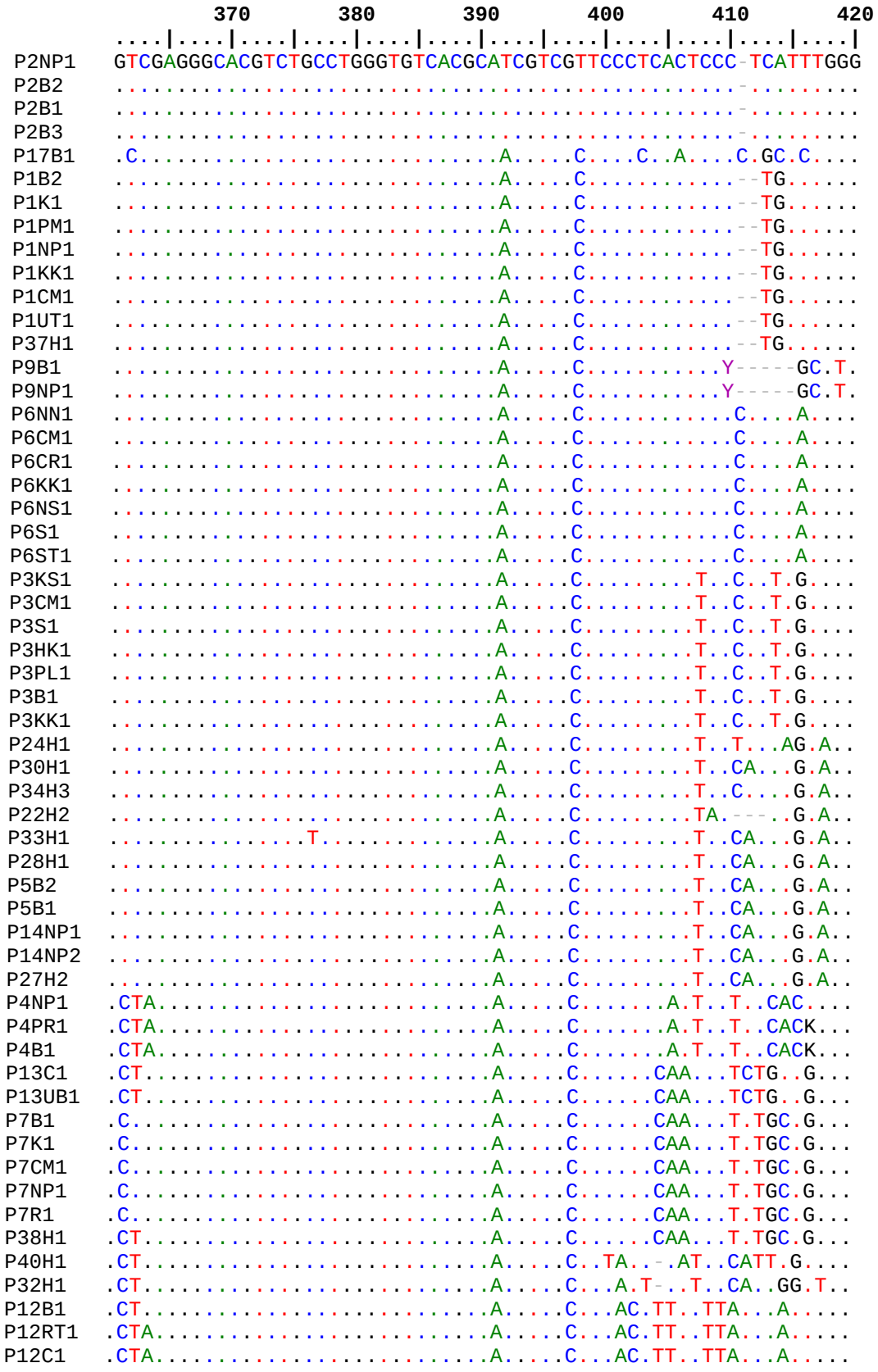
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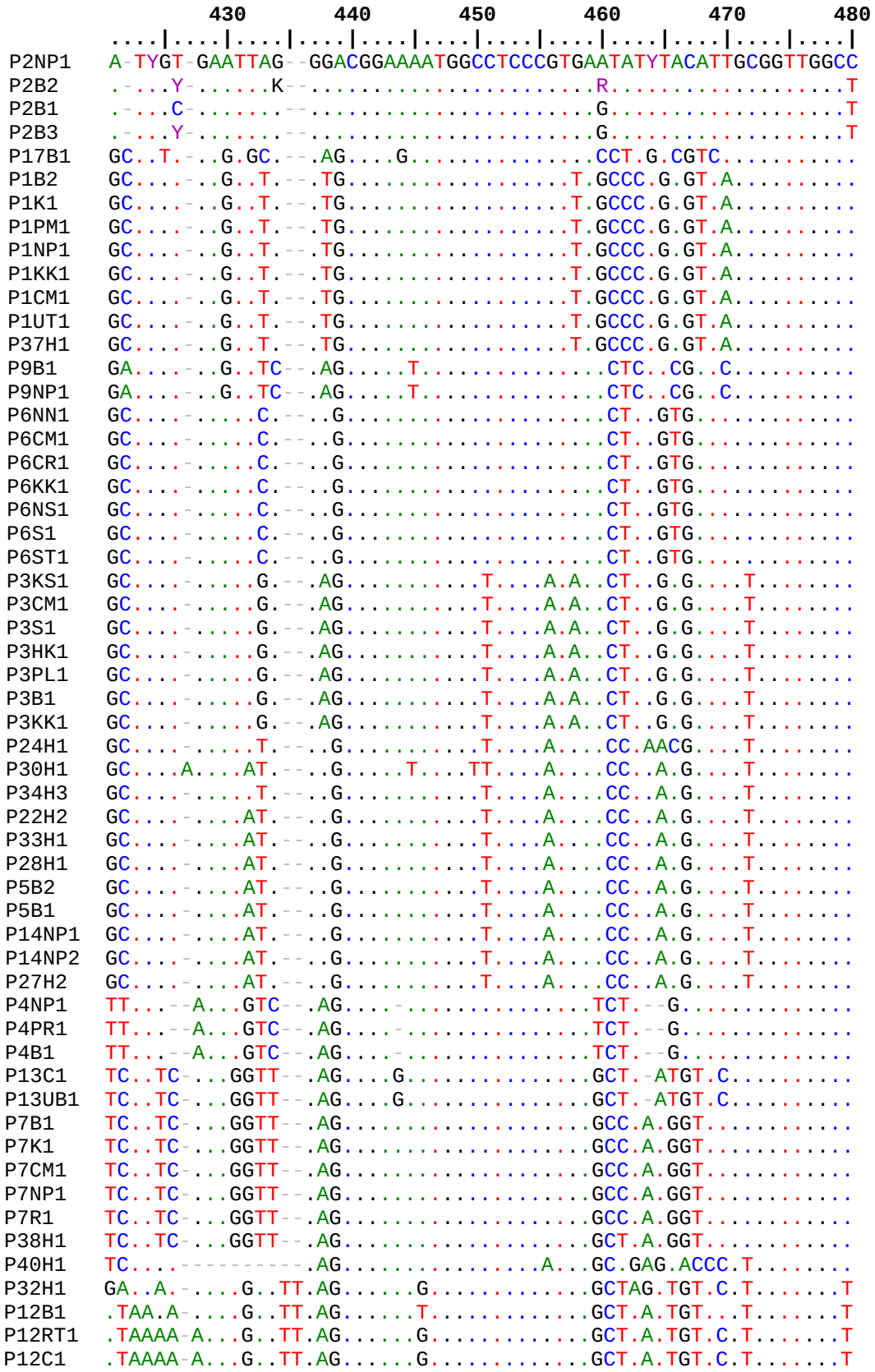
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P2B1 .-...C-.....--.....G.....T
P2B3 .-...Y-.....--.....G.....T
P17B1 GC..T.--.G.GC.--.AG....G.....CCT.G.CGTC.....
P1B2 GC....--..G..T.--.TG.....T.GCCC.G.GT.A.....
P1K1 GC....--..G..T.--.TG.....T.GCCC.G.GT.A.....
P1PM1 GC....--..G..T.--.TG.....T.GCCC.G.GT.A.....
P1NP1 GC....--..G..T.--.TG.....T.GCCC.G.GT.A.....
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P6CM1 GC....-----.C.---..G.....CT..GTG.....
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P6KK1 GC....-----.C.---..G.....CT..GTG.....
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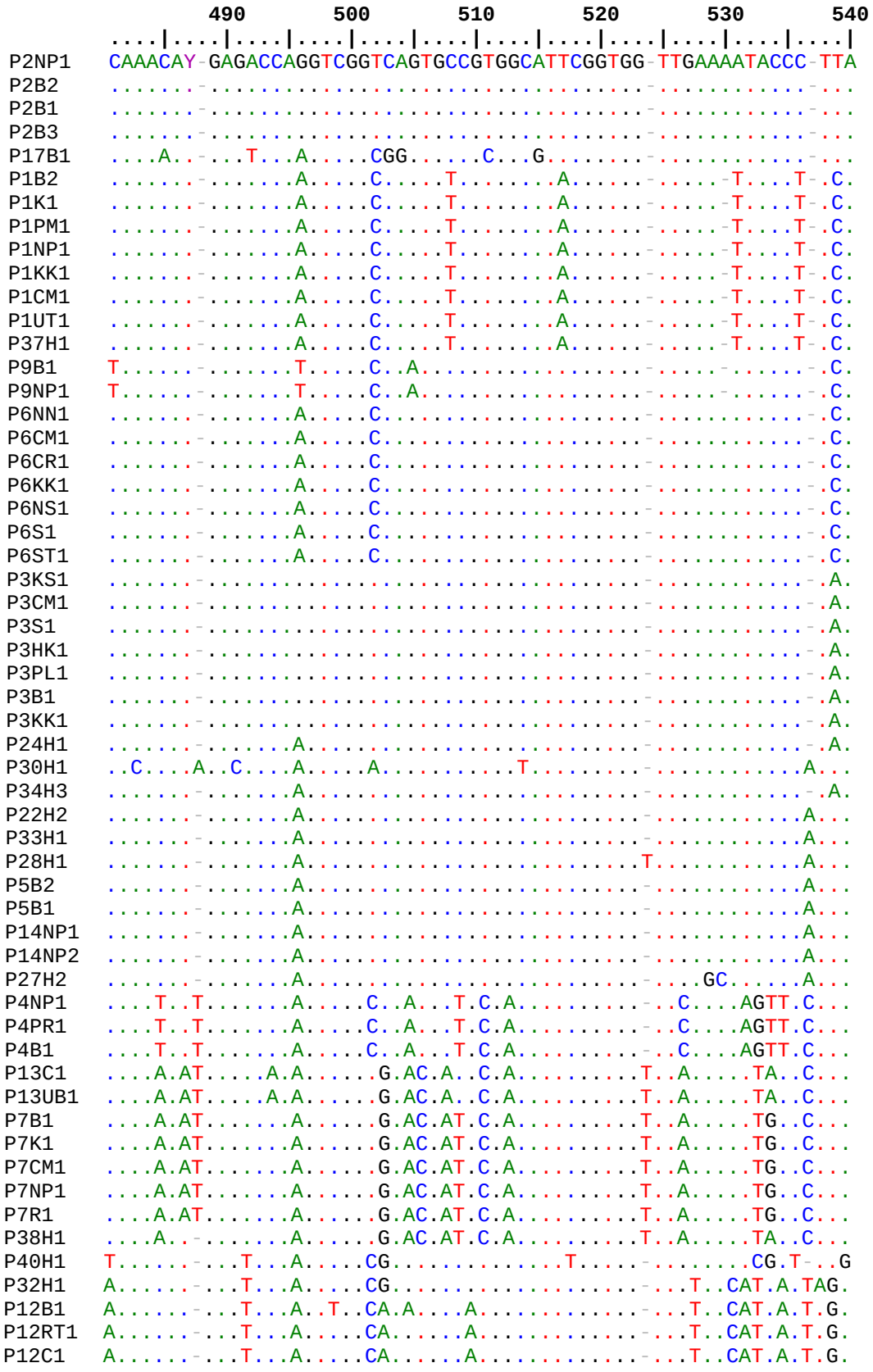
P3B1 GC....-----.G.--.AG.....T....A.A..CT..G.G....T.....
P3KK1 GC....-----.G.--.AG.....T....A.A..CT..G.G....T.....
P24H1 GC....-----.T.---..G.....T....A....CC.AACG....T.....
P30H1 GC....A....AT.---..G.....T....TT....A....CC..A.G....T.....
P34H3 GC....-----.T.---..G.....T....A....CC..A.G....T.....
P22H2 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P33H1 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P28H1 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P5B2 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P5B1 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P14NP1 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P14NP2 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P27H2 GC....-----.AT.---..G.....T....A....CC..A.G....T.....
P4NP1 TT.....A...GTC--.AG.....TCT.--G.....
P4PR1 TT.....A...GTC--.AG.....TCT.--G.....
P4B1 TT.....A...GTC--.AG.....TCT.--G.....
P13C1 TC..TC-...GGTT--.AG....G.....GCT.-ATGT.C.....
P13UB1 TC..TC-...GGTT--.AG....G.....GCT.-ATGT.C.....
P7B1 TC..TC-...GGTT--.AG.....GCC.A.GGT.....
P7K1 TC..TC-...GGTT--.AG.....GCC.A.GGT.....
P7CM1 TC..TC-...GGTT--.AG.....GCC.A.GGT.....
P7NP1 TC..TC-...GGTT--.AG.....GCC.A.GGT.....
P7R1 TC..TC-...GGTT--.AG.....GCC.A.GGT.....
P38H1 TC..TC-...GGTT--.AG.....GCT.A.GGT.....
P40H1 TC....----------.AG.....A...GC.GAG.ACCC.T.....
P32H1 GA..A.-....G..TT.AG.....G.....GCTAG.TGT.C.T.....T
P12B1 .TAA.A-....G..TT.AG.....T.....GCT.A.TGT...T.....T
P12RT1 .TAAAA-A...G..TT.AG.....G.....GCT.A.TGT.C.T.....T
P12C1 .TAAAA-A...G..TT.AG.....G.....GCT.A.TGT.C.T.....T


490
500
510
520
530
540
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P2B2
P2B1
P2B3
P17B1
P1B2
P1K1
P1PM1
P1NP1
P1KK1
P1CM1
P1UT1
P37H1
P9B1
P9NP1
P6NN1
P6CM1
P6CR1
P6KK1
P6NS1
P6S1
P6ST1
P3KS1
P3CM1
P3S1
P3HK1
P3PL1
P3B1
P3KK1
P24H1
P30H1
P34H3
P22H2
P33H1
P28H1
P5B2
P5B1
P14NP1
P14NP2
P27H2
P4NP1
P4PR1
P4B1
P13C1
P13UB1
P7B1
P7K1
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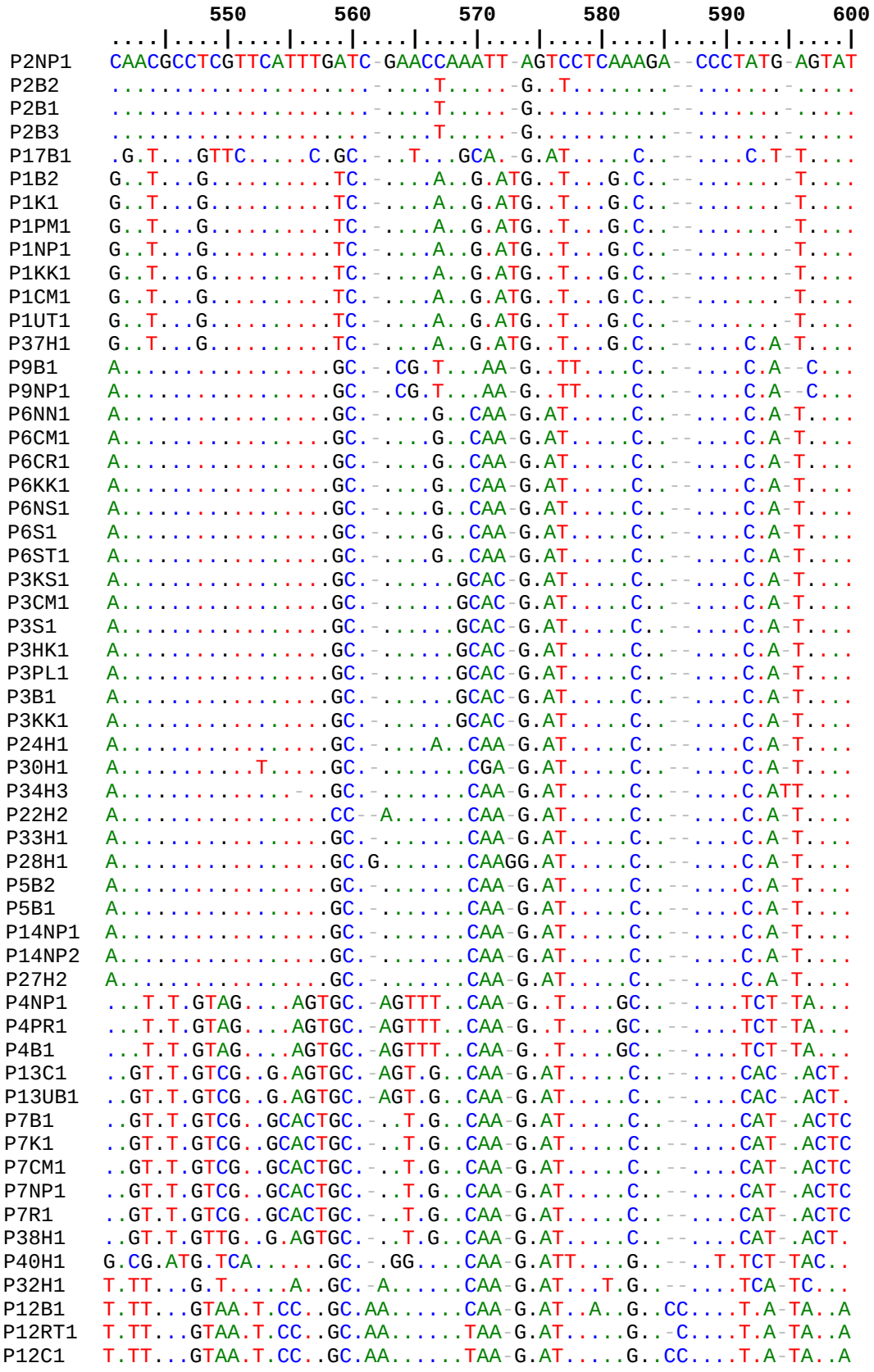
P7NP1
P7R1
P38H1
P40H1
P32H1
P12B1
P12RT1
P12C1


550
560
570
580
590
600
P2NP1 CAACGCCTCGTTCATTTGATC-GAACCAAATT-AGTCCTCAAAGA--CCCTATG-AGTAT
P2B2 .....-....T.....G..T.....--.....-.....
P2B1 .....-....T.....G.....--.....-.....
P2B3 .....-....T.....G.....--.....-.....
P17B1 .G.T...GTTC.....C.GC.-..T...GCA.-G.AT.....C..--....C.T-T....
P1B2 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P1K1 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P1PM1 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P1NP1 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P1KK1 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P1CM1 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P1UT1 G..T...G.....TC.-....A..G.ATG..T...G.C..--.....-T....
P37H1 G..T...G.....TC.-....A..G.ATG..T...G.C..--....C.A-T....
P9B1 A.....GC.-.CG.T...AA-G..TT....C..--....C.A--C...
P9NP1 A.....GC.-.CG.T...AA-G..TT....C..--....C.A--C...
P6NN1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P6CM1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P6CR1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P6KK1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P6NS1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P6S1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P6ST1 A.....GC.-....G..CAA-G.AT.....C..--....C.A-T....
P3KS1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P3CM1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P3S1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P3HK1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P3PL1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P3B1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P3KK1 A.....GC.-.....GCAC-G.AT.....C..--....C.A-T....
P24H1 A.....GC.-....A..CAA-G.AT.....C..--....C.A-T....
P30H1 A.....T.....GC.-.....CGA-G.AT.....C..--....C.A-T....
P34H3 A.....-..GC.-.....CAA-G.AT.....C..--....C.ATT....
P22H2 A.....CC--A.....CAA-G.AT.....C..--....C.A-T....
P33H1 A.....GC.-.....CAA-G.AT.....C..--....C.A-T....
P28H1 A.....GC.G.....CAAGG.AT.....C..--....C.A-T....
P5B2 A.....GC.-.....CAA-G.AT.....C..--....C.A-T....
P5B1 A.....GC.-.....CAA-G.AT.....C..--....C.A-T....
P14NP1 A.....GC.-.....CAA-G.AT.....C..--....C.A-T....
P14NP2 A.....GC.-.....CAA-G.AT.....C..--....C.A-T....
P27H2 A.....GC.-.....CAA-G.AT.....C..--....C.A-T....
P4NP1 ...T.T.GTAG....AGTGC.-AGTTT..CAA-G..T....GC..--....TCT-TA...
P4PR1 ...T.T.GTAG....AGTGC.-AGTTT..CAA-G..T....GC..--....TCT-TA...
P4B1 ...T.T.GTAG....AGTGC.-AGTTT..CAA-G..T....GC..--....TCT-TA...
P13C1 ..GT.T.GTCG..G.AGTGC.-AGT.G..CAA-G.AT.....C..--....CAC-.ACT.
P13UB1 ..GT.T.GTCG..G.AGTGC.-AGT.G..CAA-G.AT.....C..--....CAC-.ACT.
P7B1 ..GT.T.GTCG..GCACTGC.-..T.G..CAA-G.AT.....C..--....CAT-.ACTC
P7K1 ..GT.T.GTCG..GCACTGC.-..T.G..CAA-G.AT.....C..--....CAT-.ACTC
P7CM1 ..GT.T.GTCG..GCACTGC.-..T.G..CAA-G.AT.....C..--....CAT-.ACTC
P7NP1 ..GT.T.GTCG..GCACTGC.-..T.G..CAA-G.AT.....C..--....CAT-.ACTC
P7R1 ..GT.T.GTCG..GCACTGC.-..T.G..CAA-G.AT.....C..--....CAT-.ACTC
P38H1 ..GT.T.GTTG..G.AGTGC.-..T.G..CAA-G.AT.....C..--....CAT-.ACT.
P40H1 G.CG.ATG.TCA.....GC.-.GG....CAA-G.ATT....G..---..T.TCT-TAC..
P32H1 T.TT...G.T.....A..GC.-A.....CAA-G.AT...T.G..--....TCA-TC...
P12B1 T.TT...GTAA.T.CC..GC.AA.....CAA-G.AT..A..G..CC....T.A-TA..A
P12RT1 T.TT...GTAA.T.CC..GC.AA.....TAA-G.AT.....G..-C....T.A-TA..A
P12C1 T.TT...GTAA.T.CC..GC.AA.....TAA-G.AT.....G..CC....T.A-TA..A

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          ....|
P2NP1     TCGAC
P2B2      .....
P2B1      .....
P2B3      .....
P17B1     C....
P1B2      .....
P1K1      .....
P1PM1     .....
P1NP1     .....
P1KK1     .....
P1CM1     .....
P1UT1     .....
P37H1     .....
P9B1      C....
P9NP1     C....
P6NN1     C....
P6CM1     C....
P6CR1     C....
P6KK1     C....
P6NS1     C....
P6S1      C....
P6ST1     C....
P3KS1     C...Y
P3CM1     C...T
P3S1      C...T
P3HK1     C...T
P3PL1     C...T
P3B1      C...T
P3KK1     C....
P24H1     .....
P30H1     .....
P34H3     .....
P22H2     .....
P33H1     .....
P28H1     .....
P5B2      .....
P5B1      .....
P14NP1    .....
P14NP2    .....
P27H2     .....
P4NP1     .T...
P4PR1     .T...
P4B1      .T...
P13C1     .T...
P13UB1    .T...
P7B1      .T...
P7K1      .T...
P7CM1     .T...
P7NP1     .T...
P7R1      .T...
P38H1     .T...
P40H1     CT...
P32H1     .....
P12B1     C....
P12RT1    CT...
P12C1     CT...
```

# APPENDIX C

Genetic Distance of 52 *Phyllanthus* species and 6 outgroups calculated by Uncorrected ("p") distance matrix.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| 1 | *P. calycinus*[AY936674] | - | | | | | | |
| 2 | *P. taxodiifolius* | 0.14607 | - | | | | | |
| 3 | *P. acutissimus* | 0.14216 | 0.13468 | - | | | | |
| 4 | *P. cinereus*[AY936682] | 0.14088 | 0.13148 | 0.05828 | - | | | |
| 5 | *P. pulcher* | 0.14498 | 0.14631 | 0.05829 | 0.04807 | - | | |
| 6 | *P. pulcher*[AY936726] | 0.14507 | 0.14807 | 0.06017 | 0.05149 | 0.00332 | - | |
| 7 | *P. sikkimensis* | 0.13816 | 0.13004 | 0.04533 | 0.03995 | 0.02831 | 0.02485 | - |
| 8 | *P. roseus* | 0.18475 | 0.19768 | 0.18301 | 0.18163 | 0.18517 | 0.18984 | 0.18118 |
| 9 | *P. amarus* | 0.18107 | 0.19037 | 0.17546 | 0.17290 | 0.17621 | 0.18108 | 0.17243 |
| 10 | *P. amarus*[AY936668] | 0.18107 | 0.19037 | 0.17546 | 0.17290 | 0.17621 | 0.18108 | 0.17243 |
| 11 | *P. amarus*[AY936669] | 0.18285 | 0.19209 | 0.18058 | 0.17463 | 0.18129 | 0.18613 | 0.17751 |
| 12 | *Sauropus villosus* | 0.18287 | 0.18690 | 0.16572 | 0.16611 | 0.17269 | 0.17307 | 0.16227 |
| 13 | *Breynia cernua* | 0.17273 | 0.17934 | 0.15944 | 0.14982 | 0.16542 | 0.16748 | 0.15672 |
| 14 | *P. urinaria*[AY936736] | 0.18830 | 0.18906 | 0.19792 | 0.19619 | 0.19834 | 0.19840 | 0.18918 |
| 15 | *P. urinaria* | 0.18296 | 0.19405 | 0.19433 | 0.19600 | 0.19280 | 0.19284 | 0.18541 |
| 16 | *P. urinaria*[AY936735] | 0.18306 | 0.19408 | 0.19429 | 0.19595 | 0.19279 | 0.19284 | 0.18544 |
| 17 | *P. muellerianus*[AY936711] | 0.14584 | 0.15095 | 0.14629 | 0.14687 | 0.15219 | 0.15362 | 0.14143 |
| 18 | *P. reticulatus* | 0.14674 | 0.15093 | 0.14795 | 0.14877 | 0.15554 | 0.15702 | 0.14326 |
| 19 | *P. reticulatus*[AY936728] | 0.14666 | 0.15078 | 0.14793 | 0.14875 | 0.15721 | 0.15869 | 0.14493 |
| 20 | *P. casticum*[AY936676] | 0.14512 | 0.14633 | 0.14481 | 0.14890 | 0.15248 | 0.15396 | 0.14178 |
| 21 | *P. pervilleanus*[AY936723] | 0.14804 | 0.14915 | 0.14779 | 0.15182 | 0.15369 | 0.15515 | 0.14301 |
| 22 | *P. emblica* | 0.17494 | 0.17982 | 0.19088 | 0.17606 | 0.19147 | 0.19481 | 0.18400 |
| 23 | *P. emblica*[AY936689] | 0.17843 | 0.18000 | 0.19106 | 0.17622 | 0.19168 | 0.19503 | 0.18421 |
| 24 | *P. lingulatus* | 0.18929 | 0.20140 | 0.20517 | 0.19865 | 0.21068 | 0.21391 | 0.20539 |
| 25 | *P. columnaris* | 0.16131 | 0.17177 | 0.18082 | 0.17619 | 0.17977 | 0.18305 | 0.17568 |
| 26 | *P. polyphyllus*[AY936725] | 0.17156 | 0.17156 | 0.18072 | 0.17266 | 0.18131 | 0.18463 | 0.17721 |
| 27 | *P. oxyphyllus* | 0.16363 | 0.17200 | 0.17798 | 0.17158 | 0.18028 | 0.18361 | 0.17614 |
| 28 | *P. oxyphyllus*[AY936719] | 0.16522 | 0.17518 | 0.17947 | 0.17307 | 0.18176 | 0.18509 | 0.17764 |
| 29 | *P. microcarpus* | 0.16956 | 0.18295 | 0.18334 | 0.18194 | 0.19252 | 0.19583 | 0.18851 |
| 30 | *P. orientalis* | 0.17307 | 0.18158 | 0.18533 | 0.18076 | 0.18945 | 0.19271 | 0.18542 |
| 31 | *P. harmandii* | 0.16285 | 0.17469 | 0.17806 | 0.17355 | 0.18212 | 0.18535 | 0.17808 |
| 32 | *P. collinsiae* | 0.16288 | 0.17474 | 0.17867 | 0.17412 | 0.18278 | 0.18603 | 0.17874 |
| 33 | *P. angkorensis* | 0.16288 | 0.17474 | 0.17867 | 0.17412 | 0.18278 | 0.18603 | 0.17874 |
| 34 | *P. gracilipes* | 0.16623 | 0.17803 | 0.18200 | 0.17742 | 0.18608 | 0.18933 | 0.18204 |
| 35 | *P. mannianus*[AY936708] | 0.17055 | 0.18169 | 0.18284 | 0.18332 | 0.18663 | 0.18524 | 0.17948 |
| 36 | *P. debilis* | 0.18120 | 0.17023 | 0.17218 | 0.18078 | 0.17965 | 0.17823 | 0.17097 |
| 37 | *P. debilis*[AY936686] | 0.17752 | 0.16839 | 0.16713 | 0.17576 | 0.17463 | 0.17319 | 0.16593 |
| 38 | *P. kaessneri*[AY936700] | 0.17385 | 0.16453 | 0.17205 | 0.17701 | 0.18426 | 0.18788 | 0.17902 |
| 39 | *P. sepialis*[AY936732] | 0.16431 | 0.16468 | 0.16791 | 0.16537 | 0.17047 | 0.17384 | 0.16314 |
| 40 | *P. pinnatus*[AY936724] | 0.15682 | 0.15977 | 0.15525 | 0.15606 | 0.16759 | 0.17112 | 0.15878 |
| 41 | *P. acidus* | 0.15786 | 0.15739 | 0.15637 | 0.15369 | 0.16219 | 0.16550 | 0.15651 |
| 42 | *P. acidus*[AY936666] | 0.15786 | 0.15739 | 0.15637 | 0.15369 | 0.16219 | 0.16550 | 0.15651 |
| 43 | *P. chacoensis*[AY936677] | 0.16487 | 0.16577 | 0.16481 | 0.16209 | 0.17061 | 0.17200 | 0.16304 |
| 44 | *Margaritaria cyanosperma* | 0.21353 | 0.21149 | 0.19596 | 0.20395 | 0.19663 | 0.19660 | 0.19783 |
| 45 | Lingelsheimia sp. | 0.18252 | 0.19280 | 0.17947 | 0.17996 | 0.18680 | 0.18844 | 0.18452 |
| 46 | *Savia bahamensis* | 0.17971 | 0.19355 | 0.17865 | 0.17782 | 0.18219 | 0.18421 | 0.17352 |
| 47 | *Flueggea leucopyrus* | 0.18188 | 0.19775 | 0.17614 | 0.18025 | 0.17340 | 0.17202 | 0.17967 |
| 48 | *P. cochinchinensi*[AY936684] | 0.21746 | 0.19673 | 0.18862 | 0.19407 | 0.19617 | 0.19798 | 0.19106 |
| 49 | *P. welwitschianus* | 0.19888 | 0.19339 | 0.18718 | 0.19250 | 0.19133 | 0.19309 | 0.18269 |
| 50 | *P. welwitschianus*[AY936739] | 0.20241 | 0.19398 | 0.18718 | 0.19476 | 0.19190 | 0.19369 | 0.18320 |
| 51 | *P. gardnerianus*[AY936694] | 0.21230 | 0.22232 | 0.21217 | 0.21258 | 0.21267 | 0.21759 | 0.21017 |
| 52 | *P. virgatus* (1) | 0.21309 | 0.22452 | 0.21135 | 0.21350 | 0.21183 | 0.21668 | 0.21452 |
| 53 | *P. virgatus* (2) | 0.21252 | 0.22385 | 0.21240 | 0.21461 | 0.21291 | 0.21774 | 0.21564 |
| 54 | *P. chrysanthus*[AY936680] | 0.21529 | 0.21528 | 0.20896 | 0.21474 | 0.21609 | 0.22092 | 0.21048 |
| 55 | *P. virgatus*[AY936738] | 0.21684 | 0.21722 | 0.20725 | 0.21297 | 0.21444 | 0.21928 | 0.20881 |
| 56 | *P. wheeleri*[AY936740] | 0.21692 | 0.22682 | 0.21478 | 0.21506 | 0.21007 | 0.21403 | 0.20515 |
| 57 | *P. myrtifolius* | 0.19298 | 0.20337 | 0.17509 | 0.18024 | 0.18418 | 0.18886 | 0.17817 |
| 58 | *P. myrtifolius*[AY936712] | 0.19471 | 0.20140 | 0.17512 | 0.18035 | 0.18426 | 0.18773 | 0.17704 |

| | | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|
| 8 | *P. roseus* | - | | | | | | |
| 9 | *P. amarus* | 0.01450 | - | | | | | |
| 10 | *P. amarus*[AY936668] | 0.01450 | 0.00000 | - | | | | |
| 11 | *P. amarus*[AY936669] | 0.02256 | 0.00805 | 0.00805 | - | | | |
| 12 | *Sauropus villosus* | 0.16071 | 0.15235 | 0.15235 | 0.15718 | - | | |
| 13 | *Breynia cernua* | 0.15889 | 0.14896 | 0.14896 | 0.15051 | 0.06271 | - | |
| 14 | *P. urinaria*[AY936736] | 0.16237 | 0.15083 | 0.15083 | 0.14915 | 0.14388 | 0.14320 | - |
| 15 | *P. urinaria* | 0.16707 | 0.15556 | 0.15556 | 0.15388 | 0.14859 | 0.14626 | 0.02724 |
| 16 | *P. urinaria*[AY936735] | 0.16896 | 0.15746 | 0.15746 | 0.15418 | 0.15207 | 0.14975 | 0.03366 |

| | | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|
| 17 | *P. muellerianus*[AY936711] | 0.15475 | 0.14321 | 0.14321 | 0.14630 | 0.16004 | 0.14973 | 0.17859 |
| 18 | *P. reticulatus* | 0.15933 | 0.14935 | 0.14935 | 0.15245 | 0.16354 | 0.15149 | 0.18180 |
| 19 | *P. reticulatus*[AY936728] | 0.15771 | 0.14773 | 0.14773 | 0.15084 | 0.16028 | 0.14818 | 0.17994 |
| 20 | *P. casticum*[AY936676] | 0.15335 | 0.14340 | 0.14340 | 0.14653 | 0.16240 | 0.15035 | 0.18056 |
| 21 | *P. pervilleanus*[AY936723] | 0.16429 | 0.15433 | 0.15433 | 0.15746 | 0.17146 | 0.15628 | 0.18798 |
| 22 | *P. emblica* | 0.14873 | 0.14366 | 0.14366 | 0.14525 | 0.12437 | 0.12168 | 0.10496 |
| 23 | *P. emblica*[AY936689] | 0.15711 | 0.15045 | 0.15045 | 0.15205 | 0.13200 | 0.12629 | 0.10813 |
| 24 | *P. lingulatus* | 0.16278 | 0.16097 | 0.16097 | 0.16259 | 0.14486 | 0.13751 | 0.12954 |
| 25 | *P. columnaris* | 0.13909 | 0.13404 | 0.13404 | 0.13563 | 0.11589 | 0.11515 | 0.10779 |
| 26 | *P. polyphyllus*[AY936725] | 0.14699 | 0.14191 | 0.14191 | 0.14350 | 0.12557 | 0.11822 | 0.11106 |
| 27 | *P. oxyphyllus* | 0.13766 | 0.13256 | 0.13256 | 0.13416 | 0.11452 | 0.11368 | 0.10004 |
| 28 | *P. oxyphyllus*[AY936719] | 0.13913 | 0.13402 | 0.13402 | 0.13561 | 0.11618 | 0.11196 | 0.10334 |
| 29 | *P. microcarpus* | 0.15362 | 0.14858 | 0.14858 | 0.15168 | 0.12820 | 0.12351 | 0.11493 |
| 30 | *P. orientalis* | 0.14818 | 0.14311 | 0.14311 | 0.14470 | 0.12239 | 0.11968 | 0.10628 |
| 31 | *P. harmandii* | 0.14134 | 0.13629 | 0.13629 | 0.13785 | 0.11592 | 0.11323 | 0.10465 |
| 32 | *P. collinsiae* | 0.14006 | 0.13499 | 0.13499 | 0.13656 | 0.11602 | 0.11328 | 0.10473 |
| 33 | *P. angkorensis* | 0.14006 | 0.13499 | 0.13499 | 0.13656 | 0.11602 | 0.11328 | 0.10473 |
| 34 | *P. gracilipes* | 0.14146 | 0.13642 | 0.13642 | 0.13799 | 0.11931 | 0.11655 | 0.10797 |
| 35 | *P. mannianus*[AY936708] | 0.14661 | 0.13824 | 0.13824 | 0.13821 | 0.16047 | 0.14954 | 0.14153 |
| 36 | *P. debilis* | 0.13780 | 0.12946 | 0.12946 | 0.13242 | 0.13174 | 0.12925 | 0.14454 |
| 37 | *P. debilis*[AY936686] | 0.13743 | 0.12909 | 0.12909 | 0.13206 | 0.12853 | 0.12598 | 0.14142 |
| 38 | *P. kaessneri*[AY936700] | 0.14033 | 0.13363 | 0.13363 | 0.13661 | 0.15072 | 0.13582 | 0.15712 |
| 39 | *P. sepialis*[AY936732] | 0.12876 | 0.12210 | 0.12210 | 0.12368 | 0.12605 | 0.12074 | 0.12456 |
| 40 | *P. pinnatus*[AY936724] | 0.11761 | 0.10772 | 0.10772 | 0.10932 | 0.11960 | 0.11541 | 0.12994 |
| 41 | *P. acidus* | 0.11378 | 0.10555 | 0.10555 | 0.10711 | 0.10962 | 0.10362 | 0.11503 |
| 42 | *P. acidus*[AY936666] | 0.11378 | 0.10555 | 0.10555 | 0.10711 | 0.10962 | 0.10362 | 0.11503 |
| 43 | *P. chacoensis*[AY936677] | 0.12679 | 0.11373 | 0.11373 | 0.11528 | 0.11943 | 0.10872 | 0.12487 |
| 44 | *Margaritaria cyanosperma* | 0.20323 | 0.19315 | 0.19315 | 0.19787 | 0.21364 | 0.19412 | 0.22832 |
| 45 | *Lingelsheimia* sp. | 0.18921 | 0.17925 | 0.17925 | 0.18076 | 0.18449 | 0.17491 | 0.19759 |
| 46 | *Savia bahamensis* | 0.17649 | 0.16808 | 0.16808 | 0.17456 | 0.18685 | 0.18445 | 0.19727 |
| 47 | *Flueggea leucopyrus* | 0.17251 | 0.16420 | 0.16420 | 0.16729 | 0.19050 | 0.18482 | 0.19435 |
| 48 | *P. cochinchinensis*[AY936684] | 0.20423 | 0.19559 | 0.19559 | 0.19893 | 0.21770 | 0.20344 | 0.22266 |
| 49 | *P. welwitschianus*[AY936739] | 0.20079 | 0.19049 | 0.19049 | 0.19387 | 0.21682 | 0.19947 | 0.20968 |
| 50 | *P. welwitschianus* | 0.20120 | 0.19089 | 0.19089 | 0.19430 | 0.21559 | 0.19825 | 0.21001 |
| 51 | *P. gardnerianus*[AY936694] | 0.20265 | 0.19763 | 0.19763 | 0.19762 | 0.23359 | 0.22852 | 0.23609 |
| 52 | *P. virgatus* (1) | 0.22604 | 0.21775 | 0.21775 | 0.21776 | 0.23656 | 0.22708 | 0.24378 |
| 53 | *P. virgatus* (2) | 0.22704 | 0.21876 | 0.21876 | 0.21879 | 0.23760 | 0.22644 | 0.24369 |
| 54 | *P. chrysanthus*[AY936680] | 0.20830 | 0.20327 | 0.20327 | 0.20163 | 0.22429 | 0.22094 | 0.22668 |
| 55 | *P. virgatus*[AY936738] | 0.21179 | 0.20675 | 0.20675 | 0.20513 | 0.22430 | 0.22098 | 0.23190 |
| 56 | *P. wheeleri*[AY936740] | 0.20969 | 0.19976 | 0.19976 | 0.20298 | 0.21696 | 0.22029 | 0.22488 |
| 57 | *P. myrtifolius* | 0.18476 | 0.17484 | 0.17484 | 0.17645 | 0.19321 | 0.19265 | 0.20174 |
| 58 | *P. myrtifolius*[AY936712] | 0.18336 | 0.17339 | 0.17339 | 0.17502 | 0.19314 | 0.19258 | 0.20348 |

| | | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|
| 15 | *P. urinaria* | - | | | | | | |
| 16 | *P. urinaria*[AY936735] | 0.00641 | - | | | | | |
| 17 | *P. muellerianus*[AY936711] | 0.17814 | 0.17995 | - | | | | |
| 18 | *P. reticulatus* | 0.18462 | 0.18639 | 0.02889 | - | | | |
| 19 | *P. reticulatus*[AY936728] | 0.18113 | 0.18290 | 0.03209 | 0.00640 | - | | |
| 20 | *P. casticum*[AY936676] | 0.18018 | 0.18200 | 0.02890 | 0.02564 | 0.02723 | - | |
| 21 | *P. pervilleanus*[AY936723] | 0.18438 | 0.18622 | 0.04005 | 0.03680 | 0.03680 | 0.01122 | - |
| 22 | *P. emblica* | 0.11765 | 0.12242 | 0.14663 | 0.14664 | 0.14503 | 0.14698 | 0.15783 |
| 23 | *P. emblica*[AY936689] | 0.12249 | 0.12728 | 0.15015 | 0.15019 | 0.15177 | 0.15374 | 0.16457 |
| 24 | *P. lingulatus* | 0.13747 | 0.14254 | 0.16855 | 0.17036 | 0.16877 | 0.17084 | 0.18156 |
| 25 | *P. columnaris* | 0.11734 | 0.12081 | 0.15109 | 0.15429 | 0.15429 | 0.15151 | 0.16392 |
| 26 | *P. polyphyllus*[AY936725] | 0.12213 | 0.12556 | 0.15110 | 0.15428 | 0.15266 | 0.14826 | 0.15908 |
| 27 | *P. oxyphyllus* | 0.11126 | 0.11472 | 0.14838 | 0.14993 | 0.14830 | 0.14714 | 0.15799 |
| 28 | *P. oxyphyllus*[AY936719] | 0.11444 | 0.11786 | 0.14651 | 0.14808 | 0.14646 | 0.14528 | 0.15614 |
| 29 | *P. microcarpus* | 0.12289 | 0.12794 | 0.15557 | 0.15720 | 0.15556 | 0.15755 | 0.16843 |
| 30 | *P. orientalis* | 0.11738 | 0.12240 | 0.15634 | 0.15920 | 0.15759 | 0.15799 | 0.16879 |
| 31 | *P. harmandii* | 0.11249 | 0.11752 | 0.14467 | 0.14624 | 0.14461 | 0.14498 | 0.15583 |
| 32 | *P. collinsiae* | 0.11256 | 0.11757 | 0.14474 | 0.14635 | 0.14473 | 0.14509 | 0.15594 |
| 33 | *P. angkorensis* | 0.11256 | 0.11757 | 0.14474 | 0.14635 | 0.14473 | 0.14509 | 0.15594 |
| 34 | *P. gracilipes* | 0.11579 | 0.12080 | 0.14796 | 0.14954 | 0.14793 | 0.14829 | 0.15914 |
| 35 | *P. mannianus*[AY936708] | 0.13957 | 0.14634 | 0.14342 | 0.14648 | 0.14313 | 0.13567 | 0.14175 |
| 36 | *P. debilis* | 0.14421 | 0.14928 | 0.14006 | 0.13981 | 0.13655 | 0.13215 | 0.14148 |
| 37 | *P. debilis*[AY936686] | 0.14107 | 0.14611 | 0.13520 | 0.13827 | 0.13501 | 0.13055 | 0.13991 |
| 38 | *P. kaessneri*[AY936700] | 0.15843 | 0.15868 | 0.13796 | 0.14267 | 0.13617 | 0.13821 | 0.14104 |
| 39 | *P. sepialis*[AY936732] | 0.13080 | 0.13244 | 0.12003 | 0.11994 | 0.11497 | 0.12031 | 0.12642 |
| 40 | *P. pinnatus*[AY936724] | 0.13774 | 0.13957 | 0.11450 | 0.11932 | 0.11610 | 0.11158 | 0.12252 |
| 41 | *P. acidus* | 0.12599 | 0.12936 | 0.10102 | 0.10733 | 0.10411 | 0.10601 | 0.11694 |

| | | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|
| 42 | *P. acidus*[AY936666] | 0.12599 | 0.12936 | 0.10102 | 0.10733 | 0.10411 | 0.10601 | 0.11694 |
| 43 | *P. chacoensis*[AY936677] | 0.12932 | 0.13107 | 0.10103 | 0.11050 | 0.10728 | 0.10756 | 0.11690 |
| 44 | *Margaritaria cyanosperma* | 0.22276 | 0.22456 | 0.15001 | 0.15444 | 0.15460 | 0.15473 | 0.16301 |
| 45 | *Lingelsheimia* sp. | 0.19881 | 0.20068 | 0.15030 | 0.15813 | 0.15488 | 0.15340 | 0.16295 |
| 46 | *Savia bahamensis* | 0.18686 | 0.18877 | 0.14424 | 0.14886 | 0.14887 | 0.14267 | 0.15045 |
| 47 | *Flueggea leucopyrus* | 0.19082 | 0.19271 | 0.14808 | 0.15415 | 0.15250 | 0.15129 | 0.15572 |
| 48 | *P. cochinchinensis*[AY936684] | 0.22221 | 0.22097 | 0.16775 | 0.17401 | 0.17237 | 0.16956 | 0.17569 |
| 49 | *P. welwitschianus* | 0.21426 | 0.21297 | 0.17384 | 0.17854 | 0.18017 | 0.17876 | 0.18343 |
| 50 | *P. welwitschianus*[AY936739] | 0.21463 | 0.21334 | 0.17255 | 0.17719 | 0.17883 | 0.17746 | 0.18216 |
| 51 | *P. gardnerianus*[AY936694] | 0.23589 | 0.23629 | 0.17521 | 0.18308 | 0.18130 | 0.18530 | 0.19131 |
| 52 | *P. virgatus* (1) | 0.23546 | 0.23594 | 0.19652 | 0.20918 | 0.20739 | 0.20324 | 0.20920 |
| 53 | *P. virgatus* (2) | 0.23537 | 0.23586 | 0.19734 | 0.20999 | 0.20837 | 0.20411 | 0.20680 |
| 54 | *P. chrysanthus*[AY936680] | 0.22481 | 0.22521 | 0.19197 | 0.20462 | 0.20286 | 0.19872 | 0.20461 |
| 55 | *P. virgatus*[AY936738] | 0.23005 | 0.23049 | 0.19208 | 0.20163 | 0.19983 | 0.19893 | 0.20641 |
| 56 | *P. wheeleri*[AY936740] | 0.22311 | 0.22493 | 0.18876 | 0.19993 | 0.19811 | 0.18907 | 0.19834 |
| 57 | *P. myrtifolius* | 0.20316 | 0.20510 | 0.16425 | 0.17366 | 0.16863 | 0.16444 | 0.17202 |
| 58 | *P. myrtifolius*[AY936712] | 0.20487 | 0.20682 | 0.16443 | 0.17060 | 0.16557 | 0.16138 | 0.16898 |

| | | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
|---|---|---|---|---|---|---|---|---|
| 22 | *P. emblica* | - | | | | | | |
| 23 | *P. emblica*[AY936689] | 0.01125 | - | | | | | |
| 24 | *P. lingulatus* | 0.07417 | 0.08226 | - | | | | |
| 25 | *P. columnaris* | 0.04963 | 0.05764 | 0.05953 | - | | | |
| 26 | *P. polyphyllus*[AY936725] | 0.04644 | 0.05126 | 0.05632 | 0.03200 | - | | |
| 27 | *P. oxyphyllus* | 0.04330 | 0.05134 | 0.05161 | 0.02726 | 0.02247 | - | |
| 28 | *P. oxyphyllus*[AY936719] | 0.03848 | 0.04655 | 0.05001 | 0.02567 | 0.01765 | 0.00482 | - |
| 29 | *P. microcarpus* | 0.05808 | 0.06616 | 0.02744 | 0.04188 | 0.04027 | 0.03551 | 0.03394 |
| 30 | *P. orientalis* | 0.05130 | 0.05934 | 0.04655 | 0.03521 | 0.03841 | 0.03208 | 0.03048 |
| 31 | *P. harmandii* | 0.04484 | 0.05289 | 0.03526 | 0.02875 | 0.02880 | 0.02243 | 0.02081 |
| 32 | *P. collinsiae* | 0.04491 | 0.05297 | 0.03528 | 0.02880 | 0.02880 | 0.02245 | 0.02083 |
| 33 | *P. angkorensis* | 0.04491 | 0.05297 | 0.03528 | 0.02880 | 0.02880 | 0.02245 | 0.02083 |
| 34 | *P. gracilipes* | 0.04811 | 0.05619 | 0.03851 | 0.03200 | 0.03200 | 0.02564 | 0.02403 |
| 35 | *P. mannianus*[AY936708] | 0.14062 | 0.14576 | 0.16200 | 0.13556 | 0.13887 | 0.13771 | 0.13589 |
| 36 | *P. debilis* | 0.12924 | 0.13767 | 0.15054 | 0.12448 | 0.13079 | 0.13121 | 0.12938 |
| 37 | *P. debilis*[AY936686] | 0.12608 | 0.13453 | 0.14892 | 0.12289 | 0.12921 | 0.12965 | 0.12780 |
| 38 | *P. kaessneri*[AY936700] | 0.14171 | 0.15011 | 0.15617 | 0.13537 | 0.13837 | 0.13236 | 0.13053 |
| 39 | *P. sepialis*[AY936732] | 0.11055 | 0.11893 | 0.13202 | 0.10741 | 0.11388 | 0.10773 | 0.10922 |
| 40 | *P. pinnatus*[AY936724] | 0.10811 | 0.11632 | 0.13220 | 0.09995 | 0.10155 | 0.10189 | 0.10016 |
| 41 | *P. acidus* | 0.08671 | 0.09494 | 0.11076 | 0.08351 | 0.08510 | 0.08217 | 0.08040 |
| 42 | *P. acidus*[AY936666] | 0.08671 | 0.09494 | 0.11076 | 0.08351 | 0.08510 | 0.08217 | 0.08040 |
| 43 | *P. chacoensis*[AY936677] | 0.10921 | 0.11588 | 0.13001 | 0.10280 | 0.10759 | 0.10475 | 0.10293 |
| 44 | *Margaritaria cyanosperma* | 0.20237 | 0.20941 | 0.23857 | 0.21339 | 0.21176 | 0.20940 | 0.20732 |
| 45 | *Lingelsheimia* sp. | 0.17366 | 0.18053 | 0.20887 | 0.18342 | 0.18489 | 0.17908 | 0.17717 |
| 46 | *Savia bahamensis* | 0.17676 | 0.18468 | 0.20675 | 0.18620 | 0.17977 | 0.17555 | 0.17368 |
| 47 | *Flueggea leucopyrus* | 0.17702 | 0.18490 | 0.19609 | 0.18161 | 0.17519 | 0.17094 | 0.16906 |
| 48 | *P. cochinchinensis*[AY936684] | 0.18892 | 0.18922 | 0.21992 | 0.20204 | 0.19712 | 0.19931 | 0.19745 |
| 49 | *P. welwitschianus* | 0.19219 | 0.19247 | 0.21830 | 0.20048 | 0.19553 | 0.19443 | 0.19260 |
| 50 | *P. welwitschianus*[AY936739] | 0.19088 | 0.19115 | 0.21698 | 0.19754 | 0.19260 | 0.19153 | 0.18968 |
| 51 | *P. gardnerianus*[AY936694] | 0.21907 | 0.21920 | 0.24575 | 0.21727 | 0.22050 | 0.21316 | 0.21460 |
| 52 | *P. virgatus* (1) | 0.22687 | 0.22377 | 0.25139 | 0.22669 | 0.22989 | 0.22560 | 0.22377 |
| 53 | *P. virgatus* (2) | 0.22787 | 0.22482 | 0.25286 | 0.22774 | 0.23092 | 0.22661 | 0.22477 |
| 54 | *P. chrysanthus*[AY936680] | 0.21296 | 0.21313 | 0.23651 | 0.21123 | 0.21769 | 0.21034 | 0.21177 |
| 55 | *P. virgatus*[AY936738] | 0.21312 | 0.21327 | 0.23347 | 0.20661 | 0.21469 | 0.20407 | 0.20877 |
| 56 | *P. wheeleri*[AY936740] | 0.21624 | 0.21855 | 0.23984 | 0.20968 | 0.21603 | 0.20879 | 0.21026 |
| 57 | *P. myrtifolius* | 0.19141 | 0.19479 | 0.21806 | 0.18799 | 0.19445 | 0.18367 | 0.18512 |
| 58 | *P. myrtifolius*[AY936712] | 0.19327 | 0.19992 | 0.21684 | 0.18991 | 0.19312 | 0.18235 | 0.18379 |

| | | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
|---|---|---|---|---|---|---|---|---|
| 29 | *P. microcarpus* | - | | | | | | |
| 30 | *P. orientalis* | 0.02891 | - | | | | | |
| 31 | *P. harmandii* | 0.02094 | 0.01277 | - | | | | |
| 32 | *P. collinsiae* | 0.02094 | 0.01278 | 0.00412 | - | | | |
| 33 | *P. angkorensis* | 0.02094 | 0.01278 | 0.00412 | 0.00000 | - | | |
| 34 | *P. gracilipes* | 0.02417 | 0.01597 | 0.00318 | 0.00319 | 0.00319 | - | |
| 35 | *P. mannianus*[AY936708] | 0.14661 | 0.14706 | 0.13872 | 0.13885 | 0.13885 | 0.14206 | - |
| 36 | *P. debilis* | 0.13937 | 0.13408 | 0.12416 | 0.12425 | 0.12425 | 0.12747 | 0.07692 |

| | | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
|---|---|---|---|---|---|---|---|---|
| 37 | *P. debilis*[AY936686] | 0.13776 | 0.13247 | 0.12256 | 0.12264 | 0.12264 | 0.12586 | 0.07524 |
| 38 | *P. kaessneri*[AY936700] | 0.14192 | 0.14153 | 0.13313 | 0.13179 | 0.13179 | 0.13501 | 0.09501 |
| 39 | *P. sepialis*[AY936732] | 0.12259 | 0.11556 | 0.10888 | 0.10902 | 0.10902 | 0.11223 | 0.08029 |
| 40 | *P. pinnatus*[AY936724] | 0.11698 | 0.10966 | 0.10308 | 0.10319 | 0.10319 | 0.10640 | 0.09920 |
| 41 | *P. acidus* | 0.09561 | 0.08996 | 0.08344 | 0.08351 | 0.08351 | 0.08670 | 0.09906 |
| 42 | *P. acidus*[AY936666] | 0.09561 | 0.08996 | 0.08344 | 0.08351 | 0.08351 | 0.08670 | 0.09906 |
| 43 | *P. chacoensis*[AY936677] | 0.11509 | 0.11245 | 0.10273 | 0.10279 | 0.10279 | 0.10598 | 0.10552 |
| 44 | *Margaritaria cyanosperma* | 0.22087 | 0.22102 | 0.21278 | 0.21289 | 0.21289 | 0.21609 | 0.20821 |
| 45 | *Lingelsheimia* sp. | 0.18929 | 0.19105 | 0.18274 | 0.18292 | 0.18292 | 0.18611 | 0.18997 |
| 46 | *Savia bahamensis* | 0.19381 | 0.19391 | 0.18415 | 0.18424 | 0.18424 | 0.18744 | 0.18446 |
| 47 | *Flueggea leucopyrus* | 0.18464 | 0.18495 | 0.17530 | 0.17532 | 0.17532 | 0.17850 | 0.17568 |
| 48 | *P. cochinchinensis*[AY936684] | 0.20617 | 0.20316 | 0.19806 | 0.19819 | 0.19819 | 0.20140 | 0.21090 |
| 49 | *P. welwitschianus* | 0.20337 | 0.19873 | 0.19520 | 0.19539 | 0.19539 | 0.19861 | 0.20877 |
| 50 | *P. welwitschianus*[AY936712] | 0.20209 | 0.19743 | 0.19393 | 0.19410 | 0.19410 | 0.19732 | 0.20582 |
| 51 | *P. gardnerianus*[AY936694] | 0.22636 | 0.22799 | 0.22123 | 0.21995 | 0.21995 | 0.22316 | 0.21430 |
| 52 | *P. virgatus* (1) | 0.23585 | 0.23897 | 0.23376 | 0.23258 | 0.23258 | 0.23578 | 0.22960 |
| 53 | *P. virgatus* (2) | 0.23685 | 0.23999 | 0.23473 | 0.23356 | 0.23356 | 0.23675 | 0.22897 |
| 54 | *P. chrysanthus*[AY936680] | 0.22031 | 0.22189 | 0.21829 | 0.21706 | 0.21706 | 0.22025 | 0.21667 |
| 55 | *P. virgatus*[AY936738] | 0.21728 | 0.21888 | 0.21528 | 0.21405 | 0.21405 | 0.21724 | 0.22030 |
| 56 | *P. wheeleri*[AY936740] | 0.21719 | 0.21876 | 0.21386 | 0.21396 | 0.21396 | 0.21717 | 0.22341 |
| 57 | *P. myrtifolius* | 0.19843 | 0.19717 | 0.19220 | 0.19237 | 0.19237 | 0.19555 | 0.19802 |
| 58 | *P. myrtifolius*[AY936712] | 0.19713 | 0.19909 | 0.19089 | 0.19105 | 0.19105 | 0.19423 | 0.19953 |

| | | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
|---|---|---|---|---|---|---|---|---|
| 36 | *P. debilis* | - | | | | | | |
| 37 | *P. debilis*[AY936686] | 0.00325 | - | | | | | |
| 38 | *P. kaessneri*[AY936700] | 0.09439 | 0.09090 | - | | | | |
| 39 | *P. sepialis*[AY936732] | 0.08157 | 0.07818 | 0.07465 | - | | | |
| 40 | *P. pinnatus*[AY936724] | 0.09458 | 0.09307 | 0.09443 | 0.07147 | - | | |
| 41 | *P. acidus* | 0.08766 | 0.08445 | 0.08743 | 0.06784 | 0.03537 | - | |
| 42 | *P. acidus*[AY936666] | 0.08766 | 0.08445 | 0.08743 | 0.06784 | 0.03537 | 0.00000 | - |
| 43 | *P. chacoensis*[AY936677] | 0.09571 | 0.09247 | 0.09230 | 0.07274 | 0.04659 | 0.03035 | 0.03035 |
| 44 | *Margaritaria cyanosperma* | 0.19391 | 0.18911 | 0.19381 | 0.19095 | 0.18284 | 0.17183 | 0.17183 |
| 45 | *Lingelsheimia* sp. | 0.17679 | 0.17367 | 0.17288 | 0.17673 | 0.16229 | 0.15318 | 0.15318 |
| 46 | *Savia bahamensis* | 0.17827 | 0.17692 | 0.16485 | 0.16493 | 0.14732 | 0.14359 | 0.14359 |
| 47 | *Flueggea leucopyrus* | 0.18219 | 0.17905 | 0.16536 | 0.16684 | 0.15137 | 0.14245 | 0.14245 |
| 48 | *P. cochinchinensis*[AY936684] | 0.19434 | 0.19122 | 0.19728 | 0.19220 | 0.17878 | 0.16399 | 0.16399 |
| 49 | *P. welwitschianus* | 0.19727 | 0.19415 | 0.19025 | 0.19358 | 0.17190 | 0.16363 | 0.16363 |
| 50 | *P. welwitschianus*[AY936739] | 0.19431 | 0.19119 | 0.18904 | 0.19220 | 0.17056 | 0.16070 | 0.16070 |
| 51 | *P. gardnerianus*[AY936694] | 0.21669 | 0.21168 | 0.20398 | 0.19610 | 0.20225 | 0.18761 | 0.18761 |
| 52 | *P. virgatus* (1) | 0.22380 | 0.21891 | 0.22080 | 0.20930 | 0.20427 | 0.19410 | 0.19410 |
| 53 | *P. virgatus* (2) | 0.22486 | 0.21998 | 0.21859 | 0.20756 | 0.20529 | 0.19508 | 0.19508 |
| 54 | *P. chrysanthus*[AY936680] | 0.21254 | 0.20752 | 0.20154 | 0.19159 | 0.18995 | 0.18318 | 0.18318 |
| 55 | *P. virgatus*[AY936738] | 0.21447 | 0.20942 | 0.20649 | 0.19827 | 0.19343 | 0.18659 | 0.18659 |
| 56 | *P. wheeleri*[AY936740] | 0.20440 | 0.20128 | 0.20874 | 0.19487 | 0.18952 | 0.17823 | 0.17823 |
| 57 | *P. myrtifolius* | 0.18723 | 0.18234 | 0.18337 | 0.16837 | 0.16042 | 0.15549 | 0.15549 |
| 58 | *P. myrtifolius*[AY936712] | 0.18868 | 0.18381 | 0.18191 | 0.16859 | 0.16048 | 0.15722 | 0.15722 |

| | | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
|---|---|---|---|---|---|---|---|---|
| 43 | *P. chacoensis*[AY936684] | - | | | | | | |
| 44 | *Margaritaria cyanosperma* | 0.17174 | - | | | | | |
| 45 | *Lingelsheimia* sp. | 0.15799 | 0.13421 | - | | | | |
| 46 | *Savia bahamensis* | 0.14358 | 0.14179 | 0.11419 | - | | | |
| 47 | *Flueggea leucopyrus* | 0.15059 | 0.14654 | 0.11720 | 0.09273 | - | | |
| 48 | *P. cochinchinensis*[AY93 | 0.17708 | 0.19994 | 0.18457 | 0.18224 | 0.17605 | - | |
| 49 | *P. welwitschianus* | 0.17659 | 0.21461 | 0.17975 | 0.17576 | 0.18056 | 0.06799 | - |
| 50 | *P. welwitschianus*[AY936739] | 0.17537 | 0.20852 | 0.17681 | 0.17767 | 0.18092 | 0.06641 | 0.00971 |
| 51 | *P. gardnerianus*[AY936694] | 0.19902 | 0.23058 | 0.20848 | 0.20453 | 0.19739 | 0.20026 | 0.20498 |
| 52 | *P. virgatus* (1) | 0.20380 | 0.23909 | 0.21882 | 0.20954 | 0.20725 | 0.20725 | 0.21169 |
| 53 | *P. virgatus* (2) | 0.20470 | 0.23801 | 0.22061 | 0.20936 | 0.20395 | 0.20828 | 0.21278 |
| 54 | *P. chrysanthus*[AY936680] | 0.19768 | 0.23313 | 0.21174 | 0.20174 | 0.20305 | 0.20378 | 0.20197 |
| 55 | *P. virgatus*[AY936738] | 0.20107 | 0.23230 | 0.21217 | 0.20050 | 0.20352 | 0.20266 | 0.20247 |
| 56 | *P. wheeleri*[AY936740] | 0.18823 | 0.23823 | 0.20328 | 0.19072 | 0.20147 | 0.20063 | 0.20523 |
| 57 | *P. myrtifolius* | 0.16519 | 0.20366 | 0.17285 | 0.18086 | 0.17231 | 0.16448 | 0.16748 |
| 58 | *P. myrtifolius*[AY936712] | 0.16703 | 0.20208 | 0.16830 | 0.17793 | 0.16932 | 0.16259 | 0.16559 |

| | | 50 | 51 | 52 | 53 | 54 | 55 | 56 |
|---|---|---|---|---|---|---|---|---|
| 50 | *P. welwitschianus*[AY936739] | - | | | | | | |
| 51 | *P. gardnerianus*[AY936694] | 0.20390 | - | | | | | |
| 52 | *P. virgatus* (1) | 0.21069 | 0.09445 | - | | | | |
| 53 | *P. virgatus* (2) | 0.21179 | 0.09467 | 0.00318 | - | | | |
| 54 | *P. chrysanthus*[AY936680] | 0.20091 | 0.08207 | 0.06537 | 0.06532 | - | | |
| 55 | *P. virgatus*[AY936738] | 0.20143 | 0.08687 | 0.07009 | 0.07006 | 0.02871 | - | |
| 56 | *P. wheeleri*[AY936740] | 0.20745 | 0.14112 | 0.14156 | 0.14156 | 0.13662 | 0.14017 | - |
| 57 | *P. myrtifolius* | 0.16632 | 0.10933 | 0.11485 | 0.11478 | 0.10540 | 0.10704 | 0.10920 |
| 58 | *P. myrtifolius*[AY936712] | 0.16441 | 0.11109 | 0.11970 | 0.11965 | 0.11025 | 0.11192 | 0.11573 |

| | | 57 | 58 |
|---|---|---|---|
| 57 | *P. myrtifolius* | - | |
| 58 | *P. myrtifolius*[AY936712] | 0.00638 | - |

# VITA

Miss Juthatip Manissorn was born on January 25, 1981 in Sakon Nakhon Province, Thailand. She received her Bachelor's Degree of Botany, Second Class Honors in 2003 from the Faculty of Sciences, Chulalongkorn University, Thailand. Throughout her Ph.D. program she was full grant supported from the 2003 Royal Golden Jubilee (RGJ) Ph.D. Scholarship from Thailand Research Fund Thailand (TRF).