Macroscopic-microscopic characteristics and AFLP fingerprint for identification of Erythroxylum novogranatense, E. cambodianum and E. cuneatum endemic to Thailand

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ABSTRACT
Erythroxylum novogranatense (Morris) Hieron, E. cambodianum Pierre and E. cuneatum (Miq.) Kurz in family Erythroxylaceae was traditionally used as an antipyretic, general stimulant and gastrointestinal diseases. Due to their morphological similarity, the correct identification was necessary for the quality control in herbal medicine. E. novogranatense (Morris) Hieron, E. cambodianum Pierre and E. cuneatum (Miq.) Kurz endemic to Thailand according to WHO standard guideline and amplified fragment length polymorphism (AFLP) fingerprint. Morphological characters of E. novogranatense, E. cambodianum and E. cuneatum were similar in their flower, fruit and seed but different in stem and leaf. Microscopic characteristics from these three species, including constant leaf numbers, showed individual values. The stomata were classified as paracytic type. The midrib transverse section showed distinct characters of the epidermis, palisade cell, stomata, spongy cell, parenchyma, xylem vessel, phloem tissue and collenchyma. AFLP fingerprint showed highly polymorphisms 97.42% with the number of bands (349 bands) ranging between 50-750 bands. Primer E+ACG/M+CTT had the highest number of AFLP bands (91 bands). The dendrogram generated from UPGMA could separate these three species. In summary, the combination of morphological characteristics, microscopic investigation and AFLP fingerprinting can be used to identify plant species and determine the genetic relationship among three Erythroxylum species.

INTRODUCTION
The Erythroxylum (Erythroxylon) is the most well-known genus in the family Erythroxylaceae. Approximately 230 species are widely distributed in South America, Africa and Asia, including some parts of India, China and Thailand (Aynilian et al., 1974; Bieri et al., 2006). The presence of tropane alkaloids characterised the herbal plants in Erythroxylum species, tannins, flavonoids, diterpenes and phenylpropanoids (Bohm et al., 1988; Ansell et al., 1993; Zuanazzi et al., 2001). These plants have been used in folk medicine in South America as a general stimulant and for various gastrointestinal ailments (Bonefeld et al., 1986). In Thailand, E. novogranatense (Morris) Hieron known as Coca, E. cambodianum Pierre known as Hun-Hai and E. cuneatum (Miq.) Kurz known as Krai-Thong were used as folk medicine. Three species are closely related with their morphological characteristics...
such as stem, flower, fruit and seed (Figure 1). As a medicinal used, these species were also well known in Thai traditional medicine for anti-fever as well as an anti-inflammatory agent. However, scientific standards and pharmacognostic parameters have not been reported to ascertain the identity of these herbs.

The quality control of herbal plants is essential for assurance on quality, safety and efficacy of herbal medicines as recommended by the World Health Organization (WHO). Nowadays, various methods have been used for medicinal plants identification. The pharmacognostic specification is a useful technique for identification of herbal plant identity. The DNA-based techniques have been widely used for genetic diversity of herbal drug. This technique is being developed to more advantages over typical phenotype markers and reliable for informative polymorphisms (Joshi et al., 2004). Amplified fragment length polymorphism (AFLP) is one of molecular technique has been used in DNA markers as its strengths on reproducibility, high genomic abundance, highly polymorphic bands and replicable tags. This method is widely used for genetic diversity studies and phylogenetic relationship for identification of species level (K et al., 2005).

The similarities morphology of these 3 Erythroxylum led to confusion in their identification. Several characteristics, as well as molecular techniques, have been employed in this study for taxonomic identification and quality control of these plants.

MATERIALS AND METHODS

Plant materials

Both fresh mature and fresh young leaves of 3 species of genus Erythroxylum were collected from various geographical areas in Thailand. Plant specimens were authenticated by botanist, compared to the herbarium specimens and voucher specimens were kept at College of Public Health Sciences (CPHS), Chulalongkorn University, Thailand. Table 1 is a list of three Erythroxylum species used in this study.

Macroscopic-microscopic determination

Macroscopic-microscopic examinations of three Erythroxylum species were examined. Macroscopic characters were considered on the shape, size, colour and other visual inspections. Morphological characteristics were observed and described as botanical characters. Microscopic characters were physiologically seen under of microscope according to WHO standard guideline (WHO, 1998). For leaf preparation, the representative pieces of the sample were selected and cut into suitable length (1 square centimetre) for anatomical character study under a microscope. For leaf measurement, matured leaves were immersed in 3% sodium hypochlorite solution for chlorophyll removal and gently warming with chloral hydrate solution until it transparent (Mukherjee, 2008) and then mounted in 50% glycerin on a glass slide and observed under the microscope. Leaf constant numbers were examined with some modification according to the method previously described by Evans (Evans and Trease, 2009). Thirty fields for an individual specimen which were representative plant areas of photographs were determined in constant values. The city of each picture was calculated in square millimetre using Axio Vision program and then recorded. Leaf constant numbers in each parameter were analysed and presented as mean ± standard deviation (SD).

DNA isolation and AFLP fingerprint

Each sample (50-100 mg) of fresh young leaves were ground in liquid nitrogen to obtain a fine powder for isolation of DNA. Genomic DNA was individually extracted using a modified CTAB technique (Doyle and Doyle, 1990) and employed a DNA purification step to remove a large amount of polysaccharide. The genomic DNA was measured using a spectrophotometer (Nanodrop Technologies, USA) and stored for DNA template to qualify the DNA.

AFLP fingerprint was performed as described by Vos et al., (Vos et al., 1995) with some modifications. EcoRI digested genomic DNA (100 ng) (10 U/μl) and Tru9I (10 U/μl) (Boehringer Mannheim, Germany) in buffer A (Promega, USA) at 37 ºC for one h. The ligation process was carried out at 37 ºC for three h to generate DNA template-adapter for amplification. Pre-amplification using EcoRI+A and Msel+C primers (Eurofins MWG Operon, Germany) and followed by selective amplification using three selective nucleotides of EcoRI and Msel primers were performed using PCR Thermal Cycler (Thermo Electron Corporation, USA). The particular amplification products of primers combination were size-fractionated on 6% denaturing polyacrylamide gel electrophoresis, stained with silver nitrate and AFLP fingerprints were analysed (Bassam et al., 1991).

For data analysis, the presence or absence of AFLP polymorphic bands was visually scored to generate a binary data set. The Jaccard’s coefficient similarity matrix was computed (Jaccard, 1908) and the Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogram was constructed using FreeTree software (Pavlicek et al., 1999). Bootstrap replication of 1,000 re-sampling data subset was
Table 1: List of three Erythroxylum species

| No | Scientific Name                          | Vernacular Name       | Location          | Collecting date  |
|----|-----------------------------------------|-----------------------|-------------------|-----------------|
| 1  | Erythroxylum novogranatense (Morris) Hieron | Coca                  | 1. Bangkok (ENO-1) | December, 2014  |
|    |                                          |                       | 2. Nonthaburi (ENO-2) | October, 2014   |
|    |                                          |                       | 3. Chiang Mai (ENO-3) | July, 2014      |
| 2  | Erythroxylum cambodianum Pierre          | Hun Hai               | 1. Don Mot Daeng, Ubon Ratchathani (ECA-1) | November, 2014 |
| 3  | Erythroxylum cuneatum (Miq.) Kurz        | Krai Thong            | 2. Det Udom, Ubon Ratchathani (ECA-2) | November, 2014 |
|    |                                          |                       | 3. Bungkan (ECA-3) | August, 2014    |
|    |                                          |                       | 1. Songkhla (ECU-1) | January, 2014   |
|    |                                          |                       | 2. Hat Yai, Songkhla (ECU-2) | January, 2014   |

RESULTS AND DISCUSSION

Macroscopic Evaluation

Herbal medicine has been practised worldwide and now recognised by WHO as essential for primary healthcare. Herbal based drugs play a crucial role in the healthcare management system. The correct identification of medicinal plants is the first step in quality control. Pharmacognostic specification and authentication are needed for reliable identification to ensure the safety and efficacy of their medicinal properties. Macroscopic and microscopic is one of the methods for the examination of plant identification.

The macroscopic examinations are based on their morphological features are always used to distinguish various species or evaluate their quality. Morphological characters of E. novogranatense, E. cambodianum and E. cuneatum were similar in flower, fruit and seed, but they were different in stem and leaf shape. The stem of E. novogranatense and E. cambodianum were shrub, but E. cuneatum was shrub or small tree. While the shape of E. novogranatense is obovate, E. cambodianum is elliptic or obovate, and E. cuneatum is obovate or elliptic to elliptic-lanceolate. The important character of these three species is more conspicuous of two longitudinal curved lines (false midrib) on each side of the midrib on the lower face of the leaf. The morphological characteristics of three Erythroxylum species are summarised in Table 2.

Microscopic Evaluation

Microscopic observation is based on the optical system by the microscope. This method refers to the analysis of size, shape and relative structure of different cells and internal features in medicinal plants. It can provide supporting evidence for identification and standardisation of herbal drugs.

Anatomical and histological studies of the leaf can be used for discrimination of differential internal structures in each species. Midrib transverse section of E. novogranatense (Figure 2), E. cambodianum (Figure 3) and E. cuneatum (Figure 4) showed distinct characters of the epidermis, palisade cell, stomata, spongy cell, parenchyma, xylem vessel, phloem tissue and collenchyma. Three Erythroxylum species demonstrated distinguished arrangement of vessel members. The arrangement of xylem, phloem and fibre of E. cuneatum and E. cambo-
Table 2: Morphological characters of E. novogranatense, E. cambodianum and E. cuneatum

| Plant part | Morphological characteristics | E. novogranatense | E. cambodianum | E. cuneatum |
|------------|-------------------------------|-----------------|----------------|-------------|
| Stem       | Shrub to 1-2 m tall, brown to black colour | Shrub to 50-90 cm tall, gray to brown colour | Shrub or small tree to 10 m tall, brown to black colour |
| Leaf       | Leaves are thin, obovate shape, alternate, 2-3.5 cm wide and 3-5 cm long, entire margin, more conspicuous of two longitudinal curved lines (false midrib) on each side of the midrib, bright green above | Elliptic or obovate shape, alternate, 2.5-3 cm wide and 10-15 cm long, entire margin, dark green colour; false midrib is even more visible on the under face of the leaf | Obovate or elliptic to elliptic-lanceolate, alternate, 2-3 cm wide and 5-10 cm long, entire margin, dark green to greenish brown above and light green on the underside, false midrib is even more visible on the under face of the leaf |
| Flower     | Flowers are small, and disposed in clusters on short stalks, corolla is composed of five white or yellowish-white petals, 1.5-2 mm wide and 3-5 mm long | Flower in clusters on short stalks, petals white or greenish white to light green, 1.5-2 mm wide and 3-5 mm long, flowers on leafy branches | Flower in clusters of 2-6, calyx lobes triangular to 0.5-1.2 mm long, petals white or greenish white to light green, 1.5-2 mm wide and 3-4 mm long |
| Fruit      | Fruits on leafy branches, elliptical drupe, bright red colour when ripe, curved shape, 3-5 mm wide and 8-12 mm long | Ripening shiny bright red, ellipsoid, curved, triangular, 3-5 mm wide and 8-12 mm long | Ripening shiny bright red, ellipsoid, curved, triangular, 2.5-4.5 mm wide and 8-10 mm long |
| Seed       | Seed in fertile locule, flattened to planoconvex, 1-3 mm wide and 5-9 mm long | Seed in fertile locule, flattened, 1.5-3 mm wide and 6-10 mm long | Seed in fertile locule, flattened, 1-2.5 mm wide and 5-10 mm long |

dianum were shown circular arrangement but not shown this character in E. novogranatense. These internal characteristics can be used for classification of these three Erythroxylum species. According to the results, microscopic evaluation of these three species revealed the different morphological characteristics but contained almost similar cell components.

The form and surrounding cells arrangements of the stomata is one of microscopic character that can be distinguished each plant species. Four types of stomata; anomocytic, anisocytic, diacytic, and paracytic are often available for matured leaves that classify the differentially of the species (WHO, 1998). The type of stomata in each Erythroxylum species was classified as paracytic type or rubiaceous type (two subsidiary cells are long parallel to the axis of the stomata) and presented only on the lower (abaxial) epidermis of these three Erythroxylum species. The structure and shape of the epidermis and stomatal type are the first investigations in the microscopic analysis of leaf identification on surface area (Jones, 1986; Baranova, 1992). Based on characteristics of epidermal cells on lower epidermis, E. cambodianum showed polygonal cells shape, whereas E. novogranatense and E. cuneatum were irregular shape (Figure 5). Constant values of leaves are used to evaluation of character for different species or some closely related species by microscopic examination. Leaf measurement is one method that is a constant number used to examine the identification of each plant and useful parameters to distinguish in species level by qualitative microscopic evaluation (WHO, 1998). Leaf constant numbers were examined under the microscope. Leaf constant numbers of these three species
Table 3: Leaf constant numbers of *E. novogranatense*, *E. cambodianum* and *E. cuneatum*

| Erythroxylum species | Stomatal number on abaxial epidermis (mm$^2$) (Min-Max) | Stomatal index on abaxial epidermis Mean ± SD (Min-Max) | Epidermal cell number Mean ± SD (Min-Max) | Epidermal area mean ± SD (μm$^2$) (Min-Max) | Palisade ratio Mean ± SD (Min-Max) |
|----------------------|-----------------------------------------------------------|--------------------------------------------------------|------------------------------------------|---------------------------------------------|-----------------------------------|
| E. novogranatense    | 137.60 ± 12.46 (112-164)                                  | 9.69 ± 0.93 (7.51-12.05)                                | 1,285.33 ± 77.79 (1,168-1,240)          | 882.77 ± 43.59 (803.80-965.25)             | 8.91 (7.75-9.50)                  |
| E. cambodianum       | 111.87 ± 13.31 (84-144)                                   | 15.70 ± 1.43 (12.96-18.45)                              | 600.00 ± 36.59 (548-680)                | 1,325.10 ± 47.81 (1,243.78-1,436.78)      | 9.88 (7.50-12.50)                |
| E. cuneatum          | 131.20 ± 27.01 (80-172)                                   | 17.55 ± 1.73 (13.81-20.77)                              | 613.07 ± 58.83 (484-716)                | 975.63 ± 42.31 (915.75-1,082.25)          | 7.95 (7.25-9.00)                 |

Table 4: AFLP information from three Erythroxylum species resulted from AFLP analysis

| Primer combination     | No. of AFLP band | Size range (bps) | No. of polymorphic bands | Polymorphism (%) |
|------------------------|------------------|------------------|--------------------------|------------------|
| E+ACG/M+CTT            | 91               | 50 - 750         | 90                       | 98.90            |
| E+ACG/M+CTG            | 64               | 50 - 750         | 62                       | 96.88            |
| E+ACG/M+CAA            | 63               | 50 - 750         | 59                       | 93.65            |
| E+ACG/M+GCC            | 58               | 50 - 750         | 58                       | 100.00           |
| E+ACC/M+CTA            | 73               | 50 - 750         | 71                       | 97.26            |
| Total                  | 349              | 50 - 750         | 340                      | 97.42            |

Table 5: Similarity index of *E. cambodianum*, *E. cuneatum* and *E. novogranatense*

| Species | ECA-1 | ECA-2 | ECA-3 | ECU-1 | ECU-2 | ENO-1 | ENO-2 | ENO-3 | SNV |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-----|
| ECA-1   | 1.000 |       |       |       |       |       |       |       |     |
| ECA-2   | 0.715 | 1.000 |       |       |       |       |       |       |     |
| ECA-3   | 0.692 | 0.859 | 1.000 |       |       |       |       |       |     |
| ECU-1   | 0.419 | 0.439 | 0.426 | 1.000 |       |       |       |       |     |
| ECU-2   | 0.416 | 0.443 | 0.424 | 0.850 | 1.000 |       |       |       |     |
| ENO-1   | 0.316 | 0.301 | 0.295 | 0.270 | 0.272 | 1.000 |       |       |     |
| ENO-2   | 0.311 | 0.291 | 0.284 | 0.275 | 0.287 | 0.923 | 1.000 |       |     |
| ENO-3   | 0.311 | 0.291 | 0.279 | 0.270 | 0.282 | 0.923 | 0.988 | 1.000 |     |
| SNV     | 0.168 | 0.173 | 0.182 | 0.178 | 0.190 | 0.176 | 0.185 | 0.179 | 1.00|

ECA = *E. cambodianum*, ECU = *E. cuneatum*, ENO = *E. novogranatense* and SNV = *Strychnos nux-vomica*
The value of the stomatal number is one of the parameters which can be distinguished at the plant species level. In this study, the stomatal number of *E. novogranatense* (137.60 ± 12.46) was higher than *E. cuneatum* (131.20 ± 27.01) and *E. cambodianum* (111.87 ± 13.31). The stomatal index of *E. cuneatum* (17.55 ± 1.73) was higher than *E. cambodianum* (10.41 ± 0.80) and *E. novogranatense* (9.69 ± 0.93). However, stomata number can be varied with other conditions while the stomatal index is highly constant, which is not affected by age, size and environmental factors (Evans and Trease, 2009). Palisade ratio is one of the criteria used for identification for differentiation in plant species. It is an average number of palisade cells presented beneath each upper epidermal cell. The palisade ratio of *E. cambodianum* (9.88 ± 1.14) was higher than *E. novogranatense* (8.91 ± 0.52) and *E. cuneatum* (7.95 ± 0.47). The epidermal cell number of *E. novogranatense* (747.20 ± 34.46) was higher than *E. cuneatum* (613.07 ± 58.83) and *E. cambodianum* (600.00 ± 36.59). The epidermal cell area of *E. novogranatense* (882.77 ± 43.59 μm²) was less than *E. cuneatum* (975.63 ± 42.31 μm²) and *E. cambodianum* (1,325.10 ± 47.81 μm²).

As the results in a recent study, determination of leaf constants was considered as one useful parameter for species identification. The reviews of *E. novogranatense*, *E. cambodianum* and *E. cuneatum* have not been previously reported in constant numbers. This recent study is the first report of these three species. However, these constant values have been widely used for Thai medicinal plants identification and previous research used for identification.
of Datura species (Issaravanich et al., 2013), Cassia species (Sihanat et al., 2015) as well as Mangifera indica (Palanuvej et al., 2016).

In the current study, the different of macroscopic-microscopic evaluation can be served as an important tool for species identification.

**Genetic Relationship**

For AFLP fingerprint in a recent study, a total of 48 AFLP primers combination were screened. The totals of 349 bands (50-750 base pairs in size) were obtained from 5 primer combinations of which 340 bands were polymorphic (97.42%). An average AFLP banding was 69.80 bands per each primer combination. The highest number of AFLP bands (91 groups) was obtained from E+ACG/M+CTT and the lowest (58 bands) from E+ACG/M+CSC primer combination (Table 4). The AFLP profile of E. novogranatense, E. cambodianum and E. cuneatum obtained from E+ACG/M+CTT was shown in Figure 6.

For the genetic relationship, the genetic diversity
Figure 4: Transverse section of leaf midrib of E. cuneatum

Figure 5: Photographs of stomatal cells of three Erythroxylum species on lower surface of E. novogranatense (a), E. cambodianum (b) and E. cuneatum (c).
estimates (GDEs) were used for UPGMA clustering by calculated by Jaccard’s similarity matrix (Table 5). The similarity index varied from 0.27 to 0.99. The highest similarity index (0.988) was found between E. novogranatense in location 2 (ENO-2) and E. novogranatense in location 3 (ENO-3). In contrast, the lowest similarity index (0.270) was found between E. cuneatum in location 1 (ECU-1) and E. novogranatense in location 3 (ENO-3).

According to the dendrogram, plant samples in genus Erythroxylum; E. cambodianum, E. cuneatum and E. novogranatense collected from 3 different locations were classified as three distinct groups. Cluster A was composed of E. cambodianum from three locations (ECA-1, ECA-2 and ECA-3). Cluster B was composed of E. cuneatum from two locations (ECU-1 and ECU-2). Cluster C was composed of E. novogranatense from three locations (ECA-1, ECA-2 and ECA-3) and Strychnos nux-vomica (SNV) was used as an out-group plant which separated from three Erythroxylum species (Figure 7). The result of genetic similarity index showed that these three species could be clustering into three groups from their differentiated morphological characteristics and leaf constant numbers.

Therefore, AFLP marker is a powerful approach to detect DNA polymorphism for identification of species level, applicable to all organisms without previous sequence information and generally results in highly informative fingerprints. However, another DNA-based method such as sequence characterised amplified region (SCAR) marker, or
another technique should be further developed for simpler to identification in these three species. DNA fingerprinting method such as AFLP marker can be used for improvements over botanical characteristics and chemotaxonomic analysis in terms of time, cost, and analyse genetic diversity within among relatively plant species level (Welsh and McClelland, 1990). AFLP analysis was a powerful tool and very effective to analyse genetic variation in the medicinal plant as well as Erythroxylum samples in the previously reported to characterise and can identify the varieties of coca between the cultivated taxa of Erythroxylum (Johnson et al., 2003). AFLP method has more advantages in the ability to work without sequence information, multi-locus detection, small amount of DNA needed, highly informative fingerprints and high reproducibility (Karihaloo, 2015). This technique is a valuable essential marker for evaluation of identification and genetic relationships. AFLP fingerprint has been successful when used for plants diversity (Mba and Tohme, 2005). In other words, discrimination between closely related species and authentication of some other medicinal plants such as Zingiber species (Ghosh et al., 2011), Capparis species Aichi-Yousfi et al. (2016) and Boesenbergia species (Techaprasan et al., 2008).

CONCLUSIONS

In this study can be concluded that morphological characteristics, leaf constant numbers and AFLP fingerprinting have successfully used for identification and distinguish of these three Erythroxylum species. The correlation was found between the morphological and molecular analysis. The results of the recent study can use in genetic variation analysis among closely related species. The combination of morphological characteristics, microscopic investigation, and DNA fingerprinting can be useful for identification of plant species to provide supporting evidence for the quality control of medicinal plants.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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