Morphological and anatomical characters variation of *Indigofera* accessions from Java, Indonesia

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Abstract. Muzzazinah, Suratman, Nurmiyati, Ariani SRD. 2021. Morphological and anatomical characters variation of Indigofera accessions from Java, Indonesia. Biodiversitas 22: 2104-2116. The objective of this research was to evaluate morphological and anatomical character variation of *Indigofera* accessions from Java, Indonesia. A total of 15 accessions from three species of *Indigofera* were collected from centers of Batik manufacturing in Java located in the following locations: West Java (Pangandaran), Yogyakarta (Bantul, Kulonprogo), Central Java (Sragen, Sukoharjo, Karanganyar) and East Java (Tuban). There were 14 tested morphological characters based on measurement of vegetative and generative organs. Anatomical characters were observed from paradermal sections and there were 12 tested characters. Analysis of variance was performed for observed morphological and anatomical characters data in order to test the significance of variation among accessions using SPSS software. The characters' mean values then were used to perform principal component analysis and cluster analysis using PAST software. A cluster analysis was conducted by applying an Unweighted Pair Group Method with Arithmetic Averages (UPGMA) in order to group the accessions based on morphological and anatomical character similarity. The analysis of variance for the evaluated morphological and anatomical characters revealed highly significant differences among accessions for all of the tested characters suggesting that there was a high degree of phenotypic diversity among *Indigofera* accessions. Principal component analysis indicated that the first 2 components accounted for 90.72 % and 88.96 % of the total variation for morphological and anatomical data sets, respectively. Cluster analysis using morphological characters data revealed three main clusters. Four main clusters were then highlighted in cluster analysis of the anatomical character data. The grouping of the accession into clusters showed that there was no correlation between ecological habitat origin and the character diversity expressed among Indigofera accession. However, accessions that came from the same species had the tendency to cluster together.

Keywords: Anatomy, *Indigofera* accessions, Java, morphology, variation

INTRODUCTION

*Indigofera* is one of the largest genera in Fabaceae, including about 700 species (Airy Shaw 1985; Lewis et al. 2005; Maberley 2008). The genus is widespread in the tropical and subtropical regions of the Old and New World (Maberley 2008). The center of genetic diversity of this genus is in Africa and Madagascar, with up to 520 species (Gao et al. 2010). Other centers of diversity are found in India, South East Asia, and the New World (Schrire 1998; Schrire 2005; Schrire et al. 2009).

Several species in this genus have economic uses ranging from fodder, human food, ornamental plants, cover crops and green manures (Schrire 2005; Tamliselvi et al. 2011). *Indigofera* species is also known as a medicinal plant. For example, *Indigofera tinctoria* is used in constipation, liver disease, heart palpitation and gout treatment (Motamarri et al. 2012), anti-hyperglycaemic (Bangar and Saralaya 2011), antimicrobial activity on *Staphylococcus aureus*, *Bacillus pumilus* and *Streptococcus pyogenes* (Renukadevi and Sultana 2011), anti-inflammatory (Tyagi et al. 2010), antihepatotoxic (Felicia and Muthulingam 2012), and anthelminthic (Balamurugan and Selvarajan 2009). *Indigofera* is one of the oldest coloring agents known to man and is among the most widely used natural dye in the world (Howard 1988; Burkili 1995) such as *I. suffruticosa* and *I. tinctoria* which have been widely used as natural dye in the traditional textile manufacturing (for example Batik in Indonesia, especially in Java) (Muzzazinah et al. 2016).

In order to ascertain the level of variation among and within species, a variety of markers such as morphology, anatomy, biochemistry, and molecular are used (Suratman et al. 2016; Pitoyo et al. 2018; Restykania et al. 2019). Although morphological characters have been widely used in germplasm characterization, but they have a number of limitations, including low polymorphism, low heritability, late expression, and vulnerability to environmental influences (Smith and Smith 1992). However, besides their limitation, morphological character is still routinely used for preliminary evaluation of plant variations because it is fast, simple, inexpensive (Beyene et al. 2005; Jingura and Kamusoko 2015) and can be used as a general approach for assessing genetic diversity among morphologically distinguishable accessions (Said and Ehsan 2010; Suratman et al. 2016). In the characterization and identification of
plant groups, the use of anatomic features is also valuable (Nwachukwu and Mbagwu 2006; Munir et al. 2011; Rahayu et al. 2012; Chikmawati 2013), especially in distinguishing two or more taxa that have high morphological character similarities by showing the pattern of relationships of superficial convergence in morphological studies (Nwachukwu et al. 2016).

The morphological and anatomical character variation among *Indigofera* accessions from Java has not been investigated in detail. Information on genetic diversity and relationship among accessions of this plant will provide important input into determining resourceful management strategies and guiding the improvement of plants through plant breeding programs, especially in facilitating breeding material selection (Restykania et al. 2019). Therefore, this study aimed to evaluate morphological and anatomical character variation of *Indigofera* accessions from Java (Indonesia).

### MATERIALS AND METHODS

#### Plant material

A total of 15 accessions from three species of *Indigofera* used in this study were collected from centers of Batik manufacturing in Java located in the following locations: West Java (Pangandaran), Yogyakarta (Bantul, Kulonprogo), Central Java (Sragen, Sukoharjo, Karanganyar) and East Java (Tuban) (Table 1, Figure 1).

#### Field experiment

The seeds of 15 *Indigofera* accessions were carefully removed from the pods without scarification and selected after floatation test (Osawaru et al. 2013). Seeds were then sown in trays in a nursery (Hassen et al. 2006). After establishment, seedlings were then transplanted to experimental garden in Magelang District, Central Java (80 m asl). The study was laid out as a Randomised Complete Block Design (RCBD) with five replications. The plot size was 4.5 m² (1.5 m × 3 m) and the spacing was 40 cm between plants. Five plants for each accession then used in this study.

### Table 1. The location of Indigofera accessions collected from centers of Batik manufacturing in Java (Indonesia) with climatic data or each locality

| No. | Species | Acc. code | Location | Latitude Longitude | Alt. (m asl) | Temp. (°C) | Hum. (%) | Light intensity (lux) |
|-----|---------|-----------|----------|--------------------|-------------|-----------|----------|---------------------|
| 1   | *I. tinctoria* | A1PNG | Pangandaran, Pangandaran, West Java | S 07°38'10"; E 108°39'11" | 24 | 30 | 81 | 22,500 |
| 2   | *I. tinctoria* | A1KPG | Trisik, Galur, Kulonprogo, Yogyakarta | S 07°57'18"; E 110°11'06" | 28 | 31 | 74 | 39,400 |
| 3   | *I. suffruticosa* | A2KPG | Trisik, Galur, Kulonprogo, Yogyakarta | S 07°57'18"; E 110°11'06" | 28 | 31 | 74 | 39,400 |
| 4   | *I. tinctoria* | A1BTL1 | Pacar, Sewon, Bantul, Yogyakarta | S 07°52'36"; E 110°22'49" | 81 | 28.5 | 84 | 8,090 |
| 5   | *I. suffruticosa* | A2BTL1 | Pacar, Sewon, Bantul, Yogyakarta | S 07°52'36"; E 110°22'49" | 81 | 28.5 | 84 | 8,090 |
| 6   | *I. galegoidea* | A3BTL1 | Pacar, Sewon, Bantul, Yogyakarta | S 07°52'36"; E 110°22'49" | 81 | 28.5 | 84 | 8,090 |
| 7   | *I. tinctoria* | A1BTL2 | Giriloyo, Imogiri, Bantul, Yogyakarta | S 07°54'36"; E 110°23'54" | 71 | 30 | 74 | 9,580 |
| 8   | *I. suffruticosa* | A2BTL2 | Giriloyo, Imogiri, Bantul, Yogyakarta | S 07°54'36"; E 110°23'54" | 71 | 30 | 74 | 9,580 |
| 9   | *I. tinctoria* | A1SKH | Telukan, Grogo, Sukoharjo, Central Java | S 07°37'23"; E 110°49'18" | 100 | 35 | 75 | 7,350 |
| 10  | *I. tinctoria* | A1KRA | Ngringo, Jaten, Karanganyar, Central Java | S 07°33'30"; E 110°52'36" | 179 | 27.5 | 80.5 | 3,500 |
| 11  | *I. suffruticosa* | A2KRA | Ngringo, Jaten, Karanganyar, Central Java | S 07°33'30"; E 110°52'36" | 179 | 27.5 | 80.5 | 3,500 |
| 12  | *I. tinctoria* | A1SRA | Pilang, Masaran, Sragen, Central Java | S 07°27'06"; E 110°54'22" | 125 | 39 | 65 | 16,350 |
| 13  | *I. tinctoria* | A1TNBN1 | Margorejo, Kerek, Tuban, East Java | S 06°53'06"; E 111°52'38" | 27 | 28 | 99 | 2,000 |
| 14  | *I. tinctoria* | A1TNBN2 | Jarorejo, Kerek, Tuban, East Java | S 06°53'12"; E 111°52'40" | 27 | 27 | 99.5 | 12,900 |
| 15  | *I. tinctoria* | A1TNBN3 | Kedungrejo, Kerek, Tuban, East Java | S 06°53'43"; E 111°52'35" | 27 | 27.5 | 99.5 | 13,900 |

**Figure 1.** Map of the collection areas for *Indigofera* accessions from centers of Batik manufacturing in Java (Indonesia). Note: The number (1 to 15) indicated location of each collected accession as presented in Table 1.
Data collection

Data was gathered from the evaluated morphological and anatomical characters of Indigofera accession plants. Morphological characters were characterized based on measurements of vegetative and generative structures of the Indigofera plant such as stems, leaves, flowers, fruits and seeds. There were 14 measured morphological characters included plant height, canopy diameter, stem diameter, number of branches, length of leaf rachis, leaflets number per leaf rachis, leaflet length, leaflet width, inflorescence length, number of flowers in an inflorescence, number of fruits in a raceme, fruits length, fruits width and number of seeds in a pod.

Leaf anatomy characters were observed from paradermal sections. The preparation of microscope slides of the leaf paradermal section was carried out according to Munir et al. (2011), Arzani et al. (2013), Chikmawati (2013), and Kumar et al. (2014). Light microscopic (Model: Olympus, magnification of 10 x for ocular and a 40 x for objective) observations were used to observe the specimens (Pitoyo et al. 2018). There were 12 measured anatomical characters included stomatal density, stomatal length, stomatal width, trichome density, trichome length, and trichome width on both surfaces (abaxial and adaxial).

Data analysis

Analysis of variance was performed for observed quantitative morphological and anatomical characters data in order to test the significance of variation among accessions. Analysis of variance was performed by Duncan’s multiple range test (p < 0.05) using SPSS 20.0 version software for all measured characters. The characters’ mean values then were used to perform principal component analysis and cluster analysis using PAST 4.03 version software. A cluster analysis was conducted by applying an Unweighted Pair Group Method with Arithmetic Averages (UPGMA) in order to group the accessions based on morphological and anatomical character similarity.

RESULTS AND DISCUSSION

Analysis of morphological characters

The analysis of variance for the evaluated morphological characters is presented in Table 2 and Table 3. The results show that the accessions are significantly different at 5% probability with respect to the 14 morphological characters studied. The analysis of variance revealed highly significant differences among accessions for all of the tested characters. Canopy diameter (51.4-120.4 cm) and plant height (46.4-114.8 cm) showed wide variation while inflorescence length (20.2-76.4 mm), length of leaf rachis (23.6-48.6 cm), fruits length (11.8-65.8 mm), number of flowers in an inflorescence (19.4-30.4), stem diameter (10.8-18.6 mm), number of fruits in a raceme (9.8-20.2), leaflet length (11.4-20.8 mm), number of branch (8.4-17.8), leaflet width (6.2-11.6 mm), leaflets number per leaf rachis (7.4-12.6), number of seeds in a pod (4-12.4) and fruits width (0.88-2.36 mm) showed a narrower range of phenotypic variation. The fruit length had the highest coefficient of variance (45.2%) then followed by plant height (38.29%), inflorescence length (32.5 %), number of seeds in a pod (31.16%), length of leaf rachis (25.88 %), canopy diameter (25.88 %), leaflet length (24.45 %), stem diameter (22.46%), number of fruits in a raceme (21.59%), number of branch (21.38%), leaflet width (21.15%), fruits width (19.99%), leaflets number per leaf rachis (16.87%) and number of flowers in an inflorescence (11.83%).

Table 2. Estimates of variability of morphological characters among Indigofera accessions in Java

| Morphological characters          | Mean  | Min  | Max  | Standard deviation | Coefficient of variance (%) | P* (F-test) |
|-----------------------------------|-------|------|------|--------------------|-----------------------------|-------------|
| Plant height (cm)                 | 71.79 | 46.4 | 114.8| 27.48              | 38.29                       | < 0.01      |
| Canopy diameter (cm)              | 75.17 | 51.4 | 120.4| 19.45              | 25.88                       | < 0.01      |
| Stem diameter (mm)                | 13.67 | 10.8 | 18.6 | 3.07               | 22.46                       | < 0.01      |
| Number of branches                | 11.97 | 8.4  | 17.8 | 2.56               | 21.38                       | < 0.01      |
| Length of leaf rachis (cm)        | 30.08 | 23.6 | 48.6 | 7.79               | 25.88                       | < 0.01      |
| Leaflets number per leaf rachis   | 9.4   | 7.4  | 12.6 | 1.58               | 16.87                       | < 0.01      |
| Leaflet length (mm)               | 14.94 | 11.4 | 20.8 | 3.65               | 24.45                       | < 0.01      |
| Leaflet width (mm)                | 9.4   | 6.2  | 11.6 | 1.99               | 21.15                       | < 0.01      |
| Inflorescence length (mm)         | 42.87 | 20.2 | 76.4 | 13.93              | 32.5                        | < 0.01      |
| Number of flowers in an inflorescence| 27.36 | 19.4 | 30.4 | 3.23               | 11.83                       | < 0.01      |
| Number of fruits in a raceme      | 13.10 | 9.8  | 20.2 | 2.83               | 21.39                       | < 0.01      |
| Fruits length (mm)                | 27.59 | 11.8 | 65.8 | 12.47              | 45.2                        | < 0.01      |
| Fruits width (mm)                 | 1.68  | 0.88 | 2.36 | 0.33               | 19.99                       | < 0.01      |
| Number of seed in a pod           | 7.89  | 4    | 12.4 | 2.45               | 31.14                       | < 0.01      |

Note: *p < 0.01= highly significant
| Accessions | PIH | CaD | StD | NBr | LrL | NLf | LfL | LFW | IFl | NFf | NFr | FrL | FrW | NuS |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A1PNG      | 55.2±0.4 | 71.4±1.5 | 11.6±0.9 | 8.4±1.1 | 25.6±1.6 | 8.9±1.7 | 13.6±1.1 | 10.4±0.8 | 44.6±3.2 | 30.4±1.5 | 11.6±1.5 | 28.2±1.3 | 1.7±0±2.3 | 8.6±2.6 |
| A1KPG      | 46.8±2.6 | 56.2±0.8 | 10.8±1.3 | 9.6±1.4 | 23.6±0.8 | 9.4±1.7 | 13.2±0.8 | 10.8±0.8 | 41.2±1.3 | 28.4±1.5 | 10.2±1.3 | 26.4±0.8 | 1.7±0±1.5 | 9.0±3.1 |
| A2KPG      | 108.6±2.0 | 91.2±4.8 | 18.4±0.5 | 14.2±0.8 | 37.4±0.7 | 10.6±0.8 | 20.2±1.0 | 6.2±0.8 | 21.8±1.9 | 24.6±1.4 | 14.2±1.3 | 21.6±1.5 | 1.4±0±0.9 | 4.0±1.0 |
| A1BTL1     | 53.2±0.4 | 64.2±1.9 | 11.6±1.4 | 10.8±1.7 | 24.4±1.5 | 8.2±2.3 | 13.4±0.5 | 11.0±0.7 | 45.8±3.5 | 30.2±1.9 | 11.6±1.4 | 28.8±0.8 | 1.8±0±1.3 | 8.0±3.6 |
| A2BTL1     | 114.8±1.0 | 103.2±0.8 | 18.2±0.4 | 14.8±1.8 | 37.6±1.6 | 10.2±1.0 | 20.4±1.4 | 6.8±0.8 | 20.6±1.8 | 24.8±0.8 | 17.8±1.0 | 14.4±1.5 | 1.2±0±1.1 | 4.4±0.8 |
| A3BTL1     | 99.4±2.0 | 120.4±1.4 | 15.2±0.4 | 17.8±1.3 | 48.6±1.1 | 12.2±1.0 | 16.4±1.4 | 8.2±0.4 | 76.4±2.3 | 19.4±1.1 | 20.2±1.4 | 65.8±5.2 | 2.3±0±1.5 | 12.4±0.5 |
| A1BTL2     | 49.2±4.2 | 61.8±0.4 | 12.4±0.5 | 9.8±1.0 | 26.2±1.7 | 9.4±0.9 | 11.4±0.5 | 11.2±0.8 | 43.8±1.9 | 28.2±0.8 | 9.8±1.3 | 27.2±1.3 | 1.8±0±1.3 | 7.0±2.4 |
| A2BTL2     | 109.6±2.0 | 89.6±1.6 | 18.0±0.7 | 13.8±1.9 | 38.6±1.4 | 10.2±1.0 | 20.6±1.4 | 6.4±1.4 | 20.2±1.9 | 24.1±1.4 | 14.2±1.3 | 13.2±1.4 | 1.4±0±2.9 | 4.6±0.5 |
| A1SKH      | 49.6±2.4 | 58.8±1.9 | 11.0±0.7 | 12.2±1.0 | 24.6±1.1 | 7.4±1.6 | 12.4±0.5 | 10.2±0.4 | 45.4±2.3 | 30.0±1.5 | 11.4±1.4 | 29.2±1.3 | 1.8±0±0.9 | 8.8±2.1 |
| A1KRA      | 69.6±4.0 | 66.2±1.9 | 12.2±0.4 | 11.8±1.3 | 24.2±1.3 | 7.8±1.0 | 12.6±0.5 | 10.6±0.8 | 45.8±2.5 | 30.0±1.3 | 11.8±0.8 | 28.4±0.9 | 1.8±0±1.4 | 9.4±0.5 |
| A2KRA      | 109.2±1.9 | 90.8±0.5 | 18.6±0.5 | 14.2±1.0 | 38.2±0.8 | 12.6±1.7 | 20.8±0.8 | 6.6±1.1 | 47.2±2.7 | 23.6±1.4 | 15.2±3.0 | 11.8±1.6 | 0.8±0±1.3 | 4.8±1.3 |
| A1SRA      | 46.4±1.4 | 51.4±1.5 | 11.2±0.8 | 9.8±1.0 | 24.8±1.4 | 8.6±1.7 | 11.6±0.5 | 10.0±0.7 | 47.8±0.3 | 29.8±1.2 | 11.4±1.4 | 31.8±4.4 | 1.7±0±1.5 | 10.6±1.5 |
| A1TB1N     | 60.4±0.5 | 66.2±0.7 | 12.6±1.4 | 9.4±0.8 | 26.2±0.8 | 7.4±1.7 | 12.8±0.4 | 11.4±0.5 | 46.2±2.8 | 28.6±1.9 | 12.2±0.3 | 32.6±2.3 | 1.7±0±3.4 | 8.6±2.7 |
| A1TB1N2    | 52.2±3.8 | 69.1±1.8 | 11.8±0.4 | 11.8±1.0 | 25.8±1.4 | 9.0±1.4 | 13.0±1.0 | 11.6±0.5 | 48.4±1.6 | 30.2±1.3 | 12.4±1.4 | 28.2±1.6 | 1.6±0±1.1 | 8.8±1.0 |
| A1TB1N3    | 52.6±7.0 | 66.8±1.7 | 11.4±1.4 | 11.8±1.0 | 25.4±1.4 | 8.2±0.9 | 11.8±0.4 | 9.8±0.8 | 47.8±1.9 | 28.1±1.3 | 12.6±1.4 | 26.2±2.5 | 1.9±0±0.7 | 9.4±1.9 |

Note: * PIH: plant height (cm); CaD: canopy diameter (cm); StD: stem diameter (mm); NBr: number of branch; LrL: length of leaf rachis (cm); NLf: leaflets number per leaf rachis; LfL: leaflet length (mm); LFW: leaflet width (mm); IFl: inflorescence length (mm); NFf: number of flowers in an inflorescence; NFr: number of fruits in a raceme; FrL: fruits length (mm); FrW: fruits width (mm); NuS: number of seed in a pod. ** Values are means ± standard deviation. Different letters in the same column indicate significant differences at Duncan Multiple Range Test (p < 0.05)
Table 4. Eigenvectors, eigenvalues, individual and cumulative percentage of variation explained by the first two principal components after assessing 14 morphological characters of Indigofera accessions in Java

| Morphological characters | Principal Components (PC) |
|--------------------------|--------------------------|
|                          | PC 1                     | PC 2                     |
| Plant height             | 0.983                    | -0.053                  |
| Canopy diameter          | 0.901                    | 0.364                   |
| Stem diameter            | 0.968                    | -0.201                  |
| Number of branches       | 0.844                    | 0.385                   |
| Length of leaf rachis    | 0.924                    | 0.359                   |
| Leaflets number per leaf rachis | 0.813           | 0.208                   |
| Leaflet length           | 0.958                    | -0.238                  |
| Leaflet width            | -0.941                   | 0.157                   |
| Inflorescence length     | -0.310                   | 0.850                   |
| Number of flowers in an inflorescence | -0.886     | -0.393                  |
| Number of fruits in a raceme | 0.830            | 0.463                   |
| Fruits length            | -0.178                   | 0.961                   |
| Fruits width             | -0.503                   | 0.755                   |
| Number of seed in a pod  | -0.585                   | 0.767                   |
| Eigenvalue               | 8.948                    | 3.754                   |
| Individual percentage    | 63.91                    | 26.81                   |
| Cumulative percentage    | 63.91                    | 90.72                   |

Principal component (PC) analysis for the morphological characters data set revealed 2 components with Eigenvalues greater than 1 (Table 4). The first 2 principal components (PC) explained 90.72 % of the total variation. The first principal component (PC1), which accounted for 63.91 % of the total variation, was strongly and positively associated with plant height, canopy diameter, stem diameter, number of branch, length of leaf rachis, leaflets number per leaf rachis, leaflet length and number of fruits in a raceme but negatively associated with leaflet width, inflorescence length, number of flowers in an inflorescence, fruits length, fruits width and number of seed in a pod. The second principal component (PC2) explained only 26.81 % of the total variation, was strongly and positively associated with inflorescence length, fruits length, fruits width, and number of seed in a pod but negatively associated with plant height, stem diameter, leaflet length and number of flowers in an inflorescence.

Plotting of the accessions based on morphological characters across the first 2 PCs (PC1 and PC2), revealed a slight separation of groups across the PC1 axis (Figure 2). Accessions with higher values for PC1 (A2KRA, A2BTL1, A2KPG, A2BTL2) had a higher plant, larger stem diameter, and elongated leaflet while A3BTL1 had a longer leaf rachis, larger canopy diameter, more leaflets, branch and fruits than accessions with lower PC1 values such as A1SRA, A1SKH, A1KPG and A1TBN1. Similarly, accessions with higher values for PC2 (A1TBN3, A1SRA, A1KRA, A1TBN2, A1TBN1) had longer fruit and inflorescence, more seeds in a pod, and wider leaflet than accessions with lower PC2 values such as A2KRA, A2BTL1, A2KPG, A2BTL2.

A dendrogram of similarity among Indigofera accessions generated from the morphological character data is presented in Figure 3. The UPGMA cluster analysis revealed three clusters of Indigofera accessions. Cluster I only contained one accession from I. galegoides (A3BTL1) which characterized by longer leaf rachis, larger canopy diameter, more leaflets, branch and fruit. Cluster II included four accessions of I. suffruticosa (A2KPG, A2BTL2, A2BTL1, A2KRA) which differed from accessions in Cluster I and Cluster III, mainly due to their higher plant, larger stem diameter, and elongated leaflet. Cluster III included ten accessions of I. tinctoria (A1PNG, A1TBN2, A1TBN3, A1BTL1, A1SKH, A1KRA, A1TBN1, A1KPG, A1BTL2, A1SRA) which had longer fruit and inflorescence, more seeds in a pod, and wider leaflet than accessions in Cluster I and Cluster II. Therefore, cluster analysis based on measured morphological characters in this study classified accessions into three groups not completely match to their geographic origin. Accessions came from the same species had the tendency to cluster together.

Analysis of anatomical characters

The anatomical features of leaves of Indigofera accessions studied in Java are summarized in Table 5 and Table 6. The analysis of variance for anatomical characters revealed that there was highly significant variation (p < 0.05) for all the tested characters among Indigofera accessions. Stomatal density abaxial (329.9-625.46/mm²), stomatal density adaxial (299.76-599.98/mm²), trichome length adaxial (231.9-335.8 µm) and trichome length abaxial (209.2-317.6 µm) showed wide variation while stomatal length abaxial (20.4-28.06 µm), trichome width adaxial (18.8-29.4 µm), trichome width abaxial (18.3-28.2 µm), stomatal length adaxial (16.05-23.52 µm), stomatal width adaxial (12.3-16.28 µm), stomatal width adaxial (11.1-16.04 µm), trichome density adaxial (7.14-15.18 /mm²) and trichome density abaxial (7.18-13.96/mm²) showed a narrower range of phenotypic variation. The trichome density adaxial had the highest coefficient of variance (22.62%) then followed by stomatal density adaxial (22.28%), trichome density abaxial (21.37%), stomatal density adaxial (20.83%), stomatal length adaxial (12.38%), trichome length abaxial (12.34%), trichome width abaxial (11.37%), trichome width adaxial (11.26%), stomatal width adaxial (10.78%), trichome length adaxial (10.02%), stomatal length adaxial (8.58%) and stomatal width abaxial (8.46%).

Principal component (PC) analysis for the anatomical characters data set revealed 2 components with Eigenvalues greater than 1 (Table 7). The first 2 principal components (PC) explained 88.96 % of the total variation. In particular, the first principal component (PC1), which explained 61.07 % of the total variation, was strongly and positively associated with stomatal length abaxial, stomatal width abaxial, stomatal length adaxial, and stomatal width adaxial but negatively associated with stomatal density abaxial, trichomes density abaxial, stomatal density adaxial and trichomes density adaxial. The second PC (PC2), which explained 27.49% of the total variation, was strongly and positively associated with trichomes length abaxial, trichomes width abaxial, trichomes length adaxial, and trichomes width adaxial but negatively associated with...
stomatal length abaxial, stomatal width abaxial, stomatal length adaxial, and stomatal width adaxial.

Plotting of the accessions based on anatomical characters across the first 2 PCs (PC1 and PC2), revealed a slight separation of groups across the PC1 axis (Figure 4). Accessions with higher values for PC1 (A3BTL1) had larger size of stomata (length and width) on both surfaces (abaxial and adaxial) than accessions with lower PC1 values such as A1BTL2, A1TBN2, A1TBN1 and A1KPG. Similarly, accessions with higher values for PC2 (A2KPG, A2BTL1, A2KRA, A2BTL2) had larger size of trichomes (length and width) on both surfaces (abaxial and adaxial) than accessions with lower PC2 values such as A2KPG, A2BTL2, A2BTL1, A2KRA, A3BTL1.

A dendogram of similarity among Indigofera accessions generated from the anatomical character data is presented in Figure 5. The UPGMA cluster analysis revealed four clusters of Indigofera accessions. Cluster I included six accessions of I. tinctoria (A1KPG, A1TBN1, A1BTL1, A1TBN2, A1TBN3, A1BTL2) which differed from accessions in Cluster II, Cluster III and Cluster IV, mainly due to their lower density of stomata and trichome on both surfaces (abaxial and adaxial). Cluster II only contained one accession from I. galegoides (A3BTL1) which characterized by large size of stomata (length and width) on both surfaces (abaxial and adaxial). Cluster III included four accessions of I. suffrutcosa (A2KPG, A2BTL2, A2BTL1, A2KRA) which had larger size of trichomes (length and width) on both surfaces (abaxial and adaxial) than Cluster IV.

Cluster IV included four accessions of I. tinctoria (A1PNG, A1SRA, A1SKH, A1KRA) which characterized by small size of trichomes (length and width) on both surfaces (abaxial and adaxial). Therefore, cluster analysis based on measured anatomical characters in this study classified accessions into four groups not completely match their geographic origin. The grouping of the accession into four clusters shows that there was no correlation between ecological habitat origin and the anatomical character diversity expressed among Indigofera accession. However, accessions that came from the same species had the tendency to cluster together.

| Anatomical characters | Principal Components (PC) | PC 1 | PC 2 |
|-----------------------|----------------------------|------|------|
| Stomatal density abaxial | -0.841 | 0.158 |
| Stomatal length abaxial | 0.792 | -0.549 |
| Stomatal width abaxial | 0.884 | -0.338 |
| Trichomes density abaxial | -0.968 | 0.134 |
| Trichomes length abaxial | 0.609 | 0.761 |
| Trichomes width abaxial | 0.524 | 0.838 |
| Stomatal density adaxial | -0.805 | 0.204 |
| Stomatal length adaxial | 0.902 | -0.183 |
| Stomatal width adaxial | 0.834 | -0.309 |
| Trichomes density adaxial | -0.965 | 0.177 |
| Trichomes length adaxial | 0.567 | 0.803 |
| Trichomes width adaxial | 0.469 | 0.843 |

Eigenvalue
Individual percentage
Cumulative percentage

Table 5. Estimates of variability of anatomical characters among Indigofera accessions in Java, Indonesia

| Morphological characters | Mean | Min | Max | Standard deviation | Coefficient of variance (%) | P*(F-test) |
|--------------------------|------|-----|-----|-------------------|----------------------------|------------|
| Stomatal density abaxial (pore/mm²) | 469.23 | 329.9 | 625.46 | 97.76 | 20.83 | < 0.01 |
| Stomatal length abaxial (µm) | 22.47 | 20.4 | 28.06 | 1.93 | 8.58 | < 0.01 |
| Stomatal width abaxial (µm) | 13.56 | 12.3 | 16.28 | 1.14 | 8.46 | < 0.01 |
| Trichomes density abaxial (unit/mm²) | 11.63 | 7.18 | 13.96 | 2.48 | 21.37 | < 0.01 |
| Trichomes length abaxial (µm) | 265.52 | 209.2 | 317.6 | 32.77 | 12.34 | < 0.01 |
| Trichomes width abaxial (µm) | 23.93 | 18.3 | 28.2 | 2.72 | 11.37 | < 0.01 |
| Stomatal density adaxial (pore/mm²) | 499.78 | 299.76 | 599.98 | 100.21 | 22.28 | < 0.01 |
| Stomatal length adaxial (µm) | 19.18 | 16.05 | 23.52 | 2.37 | 12.38 | < 0.01 |
| Stomatal width adaxial (µm) | 12.98 | 11.1 | 16.04 | 1.39 | 10.78 | < 0.01 |
| Trichomes density adaxial (unit/mm²) | 12.65 | 7.14 | 15.18 | 2.86 | 22.62 | < 0.01 |
| Trichomes length adaxial (µm) | 289.47 | 231.9 | 335.8 | 29.01 | 10.02 | < 0.01 |
| Trichomes width adaxial (µm) | 24.66 | 18.8 | 29.4 | 2.77 | 11.26 | < 0.01 |

Note: *p < 0.01= highly significant
| Accessions | ABStd | ABStl | ABStd | ABTrd | ABTrw | ADStd | ADStl | ADStw | ADTrd | ADTrw |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| A1PNG      | 440.08±23.17b| 21.74±0.73abcd| 12.9±0.40abc| 13.38±0.53cd| 251.4±4.21d| 22.75±1.25b| 415.18±2.92bc| 17.75±1.30abc| 12.02±0.43bcd| 14.14±0.61cd| 289.9±1.34e| 24.8±1.2cd |
| A1KPG      | 602.75±16.57de| 20.98±1.58a| 13.48±0.72bc| 13.32±0.62cd| 244.6±4.15bc| 23.2±1.44b| 594.56±5.61de| 16.75±0.30ab| 13.25±0.60bcd| 14.16±0.61cd| 278.2±2.58bc| 25.4±1.71d |
| A2KPG      | 374.26±2.40a| 23.2±0.58bcde| 14.5±0.45de| 8.4±0.51b| 312.2±1.48b| 28.2±0.93c| 367.28±6.83bd| 21.25±1.25d| 14.02±1.70cde| 9.04±0.75b| 328.2±2.5fg| 29.4±0.97e |
| A1BTL1     | 566.14±22.85cd| 21.26±1.27abc| 13.14±0.47abc| 13.04±1.00cd| 251.8±4.2d| 23.9±1.09b| 560.0±8.1021de| 18.25±0.68abc| 13.02±0.56abcd| 14.88±0.67cd| 282.1±1.43d| 24.0±1.58bcd |
| A2BTL1     | 369.40±7.27a| 23.8±1.27de| 14.72±0.78e| 8.26±0.62b| 315.1±1.74hi| 27.8±1.71c| 347.2±16.22ab| 21.98±1.06def| 14.02±2.15cde| 9.5±0.79b| 332.7±2.19gh| 28.3±0.67e |
| A3BTL1     | 329.90±7.56a| 28.06±0.84f| 16.28±0.60f| 7.18±0.73a| 209.2±1.68a| 18.3±0.97a| 299.7±2.54a| 23.5±1.37f| 16.04±0.84f| 7.14±0.56a| 231.9±3.79a| 18.8±1.30a |
| A1BTL2     | 523.92±3.84c| 20.4±1.75b| 12.4±0.45a| 13.18±0.43cd| 252.2±4.1de| 22.2±1.14b| 512.7±3.87ad| 16.05±1.38a| 11.27±0.89ab| 14.5±0.91cd| 273.2±3.27b| 22.3±1.39bc |
| A2BTL2     | 371.60±5.66a| 24.42±2.13e| 14.6±0.73de| 8.7±0.48b| 312.2±2.65h| 27.5±1.56c| 363.6±4.3909ab| 22.5±1.7de| 14.52±3.8ef| 9.4±0.57b| 355.8±4.58b| 27.6±1.14de |
| A1SHK      | 446.52±29.23b| 21.98±1.62abcd| 12.5±0.59ab| 12.6±0.91c| 246.2±4.43c| 22.5±1.29b| 415.1±3.75bc| 19.02±1.04bc| 12.06±0.95abcd| 13.9±0.90c| 287.4±1.81e| 22.0±1.08b |
| A1KRA      | 459.22±2.22b| 20.6±1.67a| 13.7±0.85cd| 13.6±0.68cd| 240.4±4.87b| 23.8±1.14b| 399.9±3.21bc| 16.2±1.53a| 13.28±1.38cde| 14.76±0.95cd| 273.6±8.96b| 24.6±2.3cd |
| A2KRA      | 374.44±2.46a| 23.5±0.76cde| 14.5±0.95de| 8.96±0.32b| 317.6±2.07hi| 27.5±1.55c| 363.3±1.57ab| 21.25±1.42d| 14.1±0.61de| 9.08±0.85b| 327.3±1.6f| 27.9±1.94e |
| A1SRK      | 423.36±24.43b| 21.9±1.29abcd| 12.5±0.78ab| 13.5±0.89c| 263.2±4.1g| 21.8±1.2b| 409.5±4.15bc| 17.2±0.75abc| 11.28±0.89ab| 14.8±0.5cd| 275.1±7.82b| 24.0±1.22bcd |
| A1TB1N     | 625.46±30.42e| 21.92±1.59b| 12.8±0.59abc| 13.3±0.77abc| 256.8±3.11ef| 23.2±1.59b| 599.8±8.50f| 19.1±0.51c| 12.02±0.60abc| 14.2±0.39cd| 272.8±5.06b| 23.9±2.7bcd |
| A1TB1N2    | 559.56±68.94cd| 21.58±0.93abc| 12.3±0.25a| 13.02±0.68abcd| 251.2±4.48d| 23.2±0.07b| 549.1±8.041de| 18.0±0.71abc| 11.1±0.0f| 14.9±0.58cd| 280.2±1.92cd| 23.3±2.68bcd |
| A1TB1N3    | 571.9±89.39cd| 21.8±1.17abcd| 12.8±0.43abc| 13.9±0.49cd| 258.8±5.35gf| 23.1±2.67g| 549.1±10.32de| 18.7±0.88cd| 12.7±0.66abcde| 15.1±0.77d| 273.6±2.07b| 24.6±2.3cd |

Note: * ABStd: stomatal density abaxial ( pore/mm²); ABStl: stomatal length abaxial (µm); ABStd: stomatal width abaxial (µm); ABTrd: trichomes density abaxial (unit/mm²); ADTrd: trichomes length abaxial (µm); ADTrw: trichomes density adaxial ( unit/mm²); ADTrw: trichomes length adaxial (µm). ** Leaf anatomy comparison based on five samples for each Indigofera accessions. *** Values are means ± standard deviation. Different letters in the same column indicate significant differences at Duncan Multiple Range Test (p < 0.05)
Figure 2. Scatter diagram of 15 Indigofera accessions from Java, Indonesia and 14 morphological characteristics when plotted against the first two principal components of the correlation matrix (explaining 90.72% of the total variation). PIH: plant height, CaD: canopy diameter, StD: stem diameter; NBr: number of branch; LrL: length of leaf rachis; NLF: leaflets number per leaf rachis; LfL: leaflet length; LfW: leaflet width; IfL: inflorescence length; NF: number of flowers in an inflorescence; NFr: number of fruits in a raceme; FrL: fruits length; FrW: fruits width; NuS: number of seed in a pod.

Figure 3. Dendrogram of Indigofera accessions from Java, Indonesia derived by UPGMA from the similarity matrix of the morphological characters data.
Figure 4. Scatter diagram of 15 Indigofera accessions from Java, Indonesia and 12 anatomical characteristics when plotted against the first two principal components of the correlation matrix (explaining 88.96 of the total variation). ABStd: stomatal density abaxial; ABStl: stomatal length abaxial; ABStw: stomatal width abaxial; ABTrd: trichomes density abaxial; ABTrl: trichomes length abaxial; ABTrw: trichomes width abaxial; ADStd: stomatal density adaxial; ADStl: stomatal length adaxial; ADStw: stomatal width adaxial; ADTrd: trichomes density adaxial; ADTrl: trichomes length adaxial; ADTrw: trichomes width adaxial.

Figure 5. Dendrogram of Indigofera accessions from Java, Indonesia derived by UPGMA from the similarity matrix of the anatomical characters data.
Discussion

The variations of 15 Indigofera accessions from Java were studied using morphological and anatomical characters for description. Morphological characters include the external features of the plant parts used, including the particulars of their size, shape, and color. Most plants are classified based on external morphological structures, such as flowers and fruits (Awomukwu et al. 2015). Therefore, morphological character analysis can be used to measure phenotypic diversity (Sabaghnia et al. 2014; Suratman et al. 2016; Pitoyo et al. 2018). Leaf characters are the most varied anatomical features in Angiosperms. These features can be employed as useful taxonomic characters. Foliar micromorphological characters are considered as some of the primary diagnostic features in segregating the major groups of plants. In fact, the leaf characters are considered as second to those of flowers and fruits in taxonomic studies. Foliar epidermal characters are used successfully in the delimitation of a number of taxa (Prabhakar et al. 1985; Vijay-Kumar and Ramayya et al. 1987; Vijay-Kumar 1988; Chauhan and Daniel 2011).

The analysis of variance for the evaluated morphological and anatomical characters revealed highly significant differences among accessions for all of the tested characters suggesting that there was a high degree of phenotypic diversity among Indigofera accessions. Some morphological characters such as canopy diameter and plant height showed wide variation while the remained characters showed a narrower range of phenotypic variation. Indigofera accessions also showed wide variation of anatomical characters such as stomatal density and trichome length on both surfaces (abaxial and adaxial). This implies that these characters are indicative for distinguishing variability in the accessions could be employed in distinguishing the accessions (Osawaru et al. 2013).

Stomata of Indigofera accessions are generally paracytic and can be found on adaxial and abaxial surfaces although more abundant in lower (abaxial) surfaces. The highest stomatal density of abaxial surface was found in I. tinctoria accessions then followed by I. suffruticosa accessions and I. galegoïdes accession. The stomatal density or stomatal index has been found useful in diagnosis of some species (Aworinde et al. 2013). High values of stomata density generally on the abaxial surface of all the taxa are greater than that of the adaxial surface may be a strategy for water conservation for these species (Adegbite, 2008; Mbagwu et al., 2008). The diversity of stomata characteristics even on the same foliar surface has been reported for Indigofera species (Quesada 1997). Stomata characteristics such as frequency and dimensions can be affected by type of species and environmental factors. Although stomatal features can be affected by multiple ecological factors, as they are directly exposed to the environment, but stomatal differentiation and development are determined by genetic factors (Hetherington and Woodward 2003; Munir et al. 2011; Suratman et al. 2016; Pitoyo et al. 2018).

The trichomes are present on both surfaces but more abundant in upper (adaxial) surface. The longest trichome of adaxial surface was found in I. suffruticosa accessions then followed by I. tinctoria accessions and I. galegoïdes accession. However, trichome cells vary in size with taxonomic value. Therefore, trichomes can be used as a diagnostic character to distinguish each plant taxa because it can show differences qualitatively and quantitatively, besides its dispersion varies from one taxon to another, from one organ to another as well as from one surface to another from a particular organ (Leelavathi and Ramaya 1983). Since, trichomes have long been reported to play a significant role not only in plant biology including defense, pollination, dispersal of seeds, fruits, and propagules, but also in taxonomy (Prabhakar et al.1985). The morphological details found from this structure as diagnostic characters to delimitate species have been previously used within Neotropical Indigofera (Marquifáve et al. 2009).

Information of existing genetic variation between various morphological and anatomical characters, which is vital for any breeding program, will be obtained through multivariate analysis techniques (Hassen et al. 2006). Multivariate analysis is useful for characterization, evaluation, and classification of plant genetic resources when a number of accessions are assessed (Peeters and Martinelli 1989). Multivariate analysis such as principal component analysis and cluster analysis may assist plant breeders in the characterization of germplasm (Hassen et al. 2006). This multivariate analysis can be used to explore the presence of genetic variation (van de Wouw 1999), to identify valuable characteristics, which account for genetic variation (Veasey et al. 2001; Nunes and Smith 2003) and to find a limited number of highly differentiated populations for use in programs of crossing and selection (Veronesi and Falcinelli 1988). Multivariate analysis combines the capacity to provide synthetic summary of the most relevant traits and assessment of the relative importance of the different characters to the total difference for the study of morphologically complex samples (Camussi 1979; Abadie et al. 1998; Brandolini and Brandolini 2001; Osawaru et al. 2013). The use of statistical model and tests provide informative conclusion about a very large group of occurrences by observing a small representative (Osawaru et al. 2013). Therefore, multivariate analysis based on principal component analysis and hierarchical cluster analysis was also used in this study.

Principal Component Analysis (PCA) can be used to obtain ideas about how to identify groups of accessions that have desirable traits for breeding and enlightening the pattern of variation in a germplasm collection, to identify relationships among accessions and possible gaps (Camussi et al. 1985; Cowen and Frey 1987; Peeters and Martinelli 1989; Osawaru et al. 2013). PCA has the advantage of showing how distant each accession is from others and the variable most responsible for giving that pattern of relationship among the accessions (Adeniji et al. 2012).
Only principal component (PC) with eigenvalues greater than 1.00 which used in this study and cumulative proportion of variation explained was used to identify number of principal components (Thattil and Samita 2007). In this study, the variance accounted for by the first two PC of evaluated characters data sets was relatively high (>80%). The first two PC explained 90.72 % of the total variation of morphological characters while the first two PC explained 88.96 % of the total variation of anatomical characters. This result satisfactorily explains the variability manifested between individuals of accessions (Veasey et al. 2001; Hassen et al. 2006).

By the principal component analysis, it is possible to determine the relative contribution of each character to the total variation in accessions and to identify the most informative to represent the variability of the germplasm available (Arriel et al. 2007). Therefore, the importance of the principal component for each character is also evaluated by means of the percentage of the total variation it explains. The PC1 accounted for 63.91 % of the total variation for morphological characters while PC 2 explained only 26.81 %. For anatomical characters, PC1 explained 61.07 % of the total variation while PC2 only accounted for 27.49% of the total variation. These first two PC can explain the greatest part of the total variation found in the original data. If the first two PC accumulate a relatively high percentage of the total variation, generally determined as over 80%, they satisfactorily explain the variability expressed in the evaluated plants (Cruz and Carneiro 2003; Arriel et al. 2007).

Morphological characters such as plant height, canopy diameter, stem diameter, number of branches, length of leaf rachis, leaflets number per leaf rachis, leaflet length and number of fruits in a raceme, inflorescence length, fruits length, fruits width and number of seed in a pod had shown a strong contribution to PC1 and PC2 axis. Similarly, anatomical characters such as stomatal size (length and width) and trichomes size (length and width) on both surfaces (abaxial and adaxial) also strongly associated with PC1 and PC2 axis. Thus, improvement of Indigofera species is possible by selecting valuable morphological characteristics with anatomical characters significance (Hassen et al. 2006)

Cluster analysis is the partitioning of a set of objects into groups so that objects within a group are similar and objects in different groups are dissimilar. It is efficient in grouping objects with similar characters (Hodgkin et al., 1995). Cluster analysis using morphological characters data revealed three main clusters while four main clusters were then highlighted in cluster analysis of the anatomical character data. PC analysis helps to understand how the accessions of similar categories group together compared to dissimilar ones. The results of PC analysis partly confirmed the findings of cluster analysis. In PC analysis, the accessions grouped into three main clusters based on morphological character (Figure 2), while the cluster analysis of the accessions derived by UPGMA from the similarity matrix exhibited a similar dendrogram topology and cluster membership (Figure 3). PC analysis based on anatomical character revealed three main clusters (Figure 4), with only few differences between Cluster III and IV compared to cluster analysis (Figure 5). This demonstrates that the data obtained from this experiment were accurate, precise, and reliable. The groupings of hierarchical cluster analysis exhibited a similar dendrogram topology and cluster membership to that produced using PC analysis thereby confirming the accuracy of the constructed dendrogram. Therefore, the results of PC analysis clarify or verify the cluster analysis, if the results of one analysis support those of another, this confirms that the data are more precise and accurate (Tuhina-Khatun et al. 2015).

The existence of variation among accessions for the majority of the studied morphological and characters is a sign of the presence of genetic variation implying great potency for future breeding programs through selection (Nkansah et al. 2013; Roy et al. 2013; Sabaghnia et al. 2014; Suratman et al. 2016; Pitoyo et al. 2018). Genetic variability as reflected from variation of morphological and anatomical characters is the raw material of crop breeding. Thus, the higher amount of variations expressed for a character in the breeding material, then the scope for its improvement through selection is greater (Osawaru et al. 2013; Pitoyo et al. 2018). Subsequently, the grouping of Indigofera accessions by phenotypic diversity in the present study can be used to classify the accessions into distinct morphological and anatomical levels, which could be used for various breeding, collection, and conservation programs (Hassen et al. 2006).

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