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KARYOMORPHOLOGY OF GENUS *PINANGA* (ARECACEAE) IN JAVA AND BALI, INDONESIA

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Abstract

The karyomorphology on palm (Arecaceae) was infrequently reported, including the genus *Pinanga*. Our research aimed to observe the karyomorphology of *Pinanga* in Java and Bali, i.e., *Pinanga arinasae, P. javana*, and *P. coronata* and their relationships with morphology and molecular characters. Somatic chromosome preparation and observation were carried out using the aceto-orcein squash method. The chromosome number of the three species was 2n=32, however, the following differences were found: (1) karyotype formula of *P. arinasae* and *P. javana* is 12m+4sm, whereas *P. coronata* is 14m + 1sm + 1st; (2) the chromosome length of *P. arinasae*, *P. javana*, and *P. coronata* was 1.39-3.89 µm, 1.36-4.02 µm, and 3.05-6.03 µm, respectively. Our karyomorphological approach showed that *P. arinasae* was more closely related to *P. javana* than *P. coronata*. This is supported by ITS sequence data and ISSR genetic markers. In this paper, the karyomorphology of *Pinanga arinasae* and *P. javana* is reported for the first time.

Key words: Aceto-Orcein Method, Chromosome, Karyomorphology, *Pinanga*, Species Relationships.

Introduction

Pinanga belongs to the family Arecaceae, subfamily Arecoideae, tribe Areceae, subtribe Arecinae with two genera, i.e., *Areca* and *Nenga* (Dransfield *et al*., 2005). The genus *Pinanga* is composed of 141 accepted species (http://www.plantsoftheworldonline.org, 2020) and is distributed from tropical and subtropical Asia to the northwestern Pacific (Govaerts & Dransfield, 2005; http://www.plantsoftheworldonline.org, 2020). The greatest diversity of the genus is in the Sunda Shelf and it is very poorly represented in Papuasian region. In its natural habitat, *Pinanga* usually grows in the undergrowth of humid rainforests with rich soil from sea level to ca. 2,800 m above sea level. Some species may be associated with various rock types, including limestone and ultrabasics (Uhl & Dransfield, 1987).

There are three species of *Pinanga* native to Java and Bali, i.e., *P. coronata* (Blume ex Mart.) Blume, *P. javana* Blume, and *P. arinasae* Witono. *Pinanga coronata*, a clustered stem palm grows throughout Sumatra to Lesser Sunda Islands, whereas *P. javana* and *P. arinasae*, single-stemmed palms are endemic species to Java and Bali, respectively (Witono *et al*., 2002). The most recently discovered species was reported by Whitten *et al*., (1996) as *Pinanga* sp. with a massive, single-stem in the montane forest near Bedugul Bali, then published as a new species *P. arinasae* (Witono *et al*., 2002). Some current field observations reported that *P. arinasae* was distributed in Mt. Merbuk, Mt. Tapak, Mt. Pengelengan, Jatiluwih, and Pilan Customary Forest (Bali) (Yudaputra, 2018; Sutomo & Iryadi, 2020).

The karyomorphological studies within *Pinanga* were reported for the first time by Roser (1999) on *Pinanga coronata* and *P. subintegra*. A chromosome count of seven species of *Pinanga*, i.e., *P. coronata* (Eichorn, 1953, 1957; Read, 1966; Sarkar, 1970; Uhl & Dransfield, 1987; Roser *et al*., 1997; Roser, 1999; Witono, 2008), *P. disticha* (Uhl & Dransfield, 1987), *P. patula* (Uhl & Dransfield, 1987), *P. celebica* (Roser *et al*., 1997), *P. subintegra* (Roser *et al*., 1997; Roser, 1999), and *P. arinasae* and *P. javana* (Witono, 2008) has revealed the same chromosome number of 2n=32.

In general, studies that incorporate karyomorphology, morphology, and molecular characters provide much better informations and interpretations of biological diversity than those that focus on just one aspect (Moritz & Hillis, 1996). Species relationships among species of *Pinanga* based on those three characters have not been reported so far. Our research aimed to observe the karyomorphology of *Pinanga* in Java and Bali, i.e., *Pinanga arinasae, P. javana*, and *P. coronata* and their relationships with morphology and molecular characters.

Materials and Methods

Plant materials and treatments: *Pinanga coronata*, *P. arinasae*, and *P. javana* were collected from both wild and botanic garden collections (Fig. 1 and Table 1). The seeds were germinated in the greenhouse of the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University.

Table 1. Sample information used in this study.

Species	Distribution	Collected from
Pinanga coronata (Blume ex Mart.) Blume	Sumatra to Lesser Sunda Islands	Bogor Botanic Gardens
Pinanga arinasae Witono	Endemic to Bali	Eka Karya Botanic Gardens, Bali
Pinanga javana Blume	Endemic to Java	Mt. Pangrango, West Java

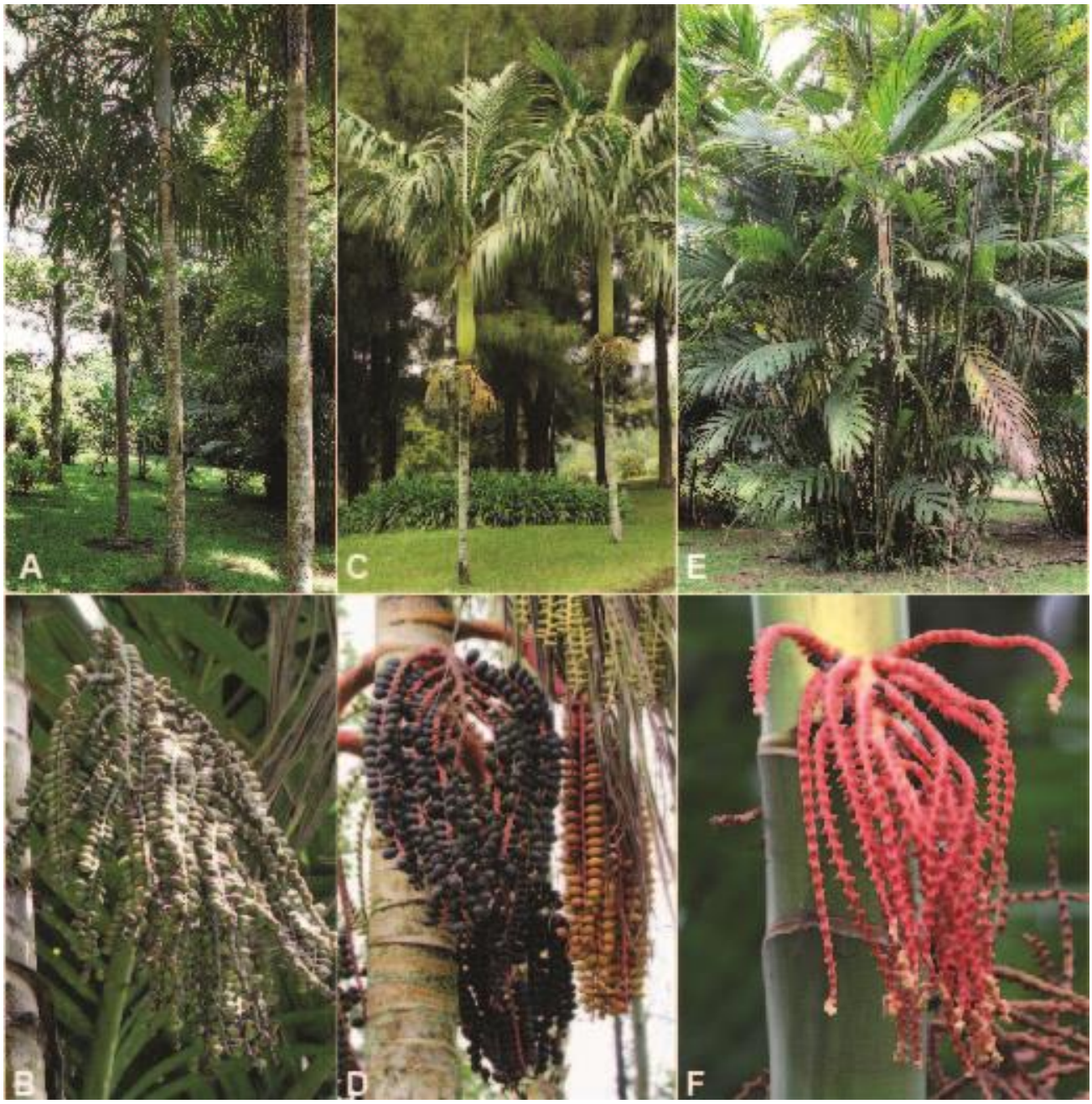

Fig. 1. The habit and inflorescences of three species of *Pinanga*. A-B, *P. arinasae*; C-D, *P. javana*; D-E *P. coronata.* Photograph by Deniek G. Sukarya.

Table 2. Chromosome shape classification based on long and short arms ratio.

Arm ratio (AR)	Sentomer position	Chromosome shape
$1.0 \leq AR < 1.7$	Median	Metasentric (m)
$1.7 \leq AR < 3.0$	Submedian	Submetasentric (sm)
$3.0 \leq AR < 7.0$	Subterminal	Subtelosentric (st)
$7.0 \leq AR < \infty$	Near terminal	Acrosentric (a)
∞	Terminal	Telosentric (t)

We followed the somatic chromosome preparation and observation methods using specifically the aceto-orcein squash method by Kondo *et al*., (1994) with modification by the authors. Young and fresh root tips 1−2 mm long were harvested, washed, and cut longitudinally into 2–4 pieces in 0.002 M 8-Hydroquinoline before being pre-treated with 0.002 M 8-Hydroxyquinoline at 18ºC for 1−2 hours. They were fixed in 45% acetic acid at 4ºC for 10−15 min before they were hydrolyzed in a 2:1 mixture of 1 N hydrochloric acid and glacial acetic acid at 60ºC for 7−15 sec. Then, they were stained with 1% aceto-orcein for 30 min and squashed for observation.

Karyotype analysis: Karyotype analysis was based on three mitotic metaphase cells from each species. Chromosome shape at metaphase was classified based on arm ratio (AR) (Levan *et al*., 1964) (see Table 2). Chromosome characteristics were measured using Ideokar 1.2 software (Mirzaghaderia & Marzangib, 2015).

Results

The karyotype of *P. arinasae* and *P. javana* is reported here for the first time, while the karyotype of *P. coronata* was published by Roser (1999) and Witono (2008). The three species of *Pinanga* disclosed the same chromosome number of 2n=32. Somatic chromosomes at interphase, prophase and metaphase and the idiogram of *P. arinasae*, *P. javana,* and *Pinanga coronata* are shown in Fig. 2. The chromosome shape of *P. arinasae* and *P javana* consisted of 12m + 4sm, whereas *P. coronata* is 14m + 1sm+ 1st (Table 3).

The chromosome length of *P. arinasae* and *P. javana* are around 1.39-3.89 µm and 1.36-4.02 µm, respectively. Whereas, the chromosome of *P. coronata* is around 3.05-6.03 µm (Table 3).

Discussion

Within the large species and diverse in morphological characters in subfamily Arecoideae, the chromosome number of the investigated genera usually presented a range of variation between 2n=28 and 2n=36, and the most frequent numbers in the subfamily seemed to be 2n=32 and 2n=34. Metaphase chromosomes in the subfamily were dominantly m to sm, more rarely st (Roser, 1994). The three species of *Pinanga* showed the same chromosome number of 2n=32. The chromosome shape of *P. arinasae* and *P. arinasae* were similar (12m + 4sm), and they had a different chromosome shape than *P. coronata* (14m + 1sm+ 1st). The chromosome length of *P. arinasae* and *P. javana* were almost similar, whereas the chromosome length of *P. coronata* was much longer than the other two species. The same chromosome number found in *Pinanga* is also present in other genera of the subfamily Arecoideae, such as *Areca* (2n=32) (Sharma & Sarkar, 1956; Uhl & Dransfield, 1987; Roser *et al*., 1997), *Hydriastele* (2n=32) (Roser, 1994; Uhl & Dransfield, 1987), *Butia* (2n=32) (Corrêa *et al*., 2009), and *Euterpe* (2n=36) (Oliveira *et al*., 2016). In the karyotype study of three species of *Euterpe* (*E. edulis*, *E. oleracea*, and *E. precatoria*), they found similar chromosome sizes, but different chromosome morphology and genome size (Oliveira *et al*., 2016). The subfamily Coryphoideae s.l. presents an extensive variation of karyotype characters in all parts of the mitotic cycle (interphase, prophase and metaphase), which distinguishes several groups within this subfamily. In most cases, karyological deviation is found to correspond with major alterations in reproductive or vegetative organization (Roser, 1993). Each organism has very different sets of chromosomes (karyotype) and the karyotype of closely related species usually compare similarly to their distantly related species. Changes in the size and morphology of chromosome are the evidence of the organism evolutionary process, and it is possible to present in numerous ways which chromosomes and whole genomes change during evolutionary process (Sumner, 2003).

Based on karyomorphological characters using chromosome shape and chromosome length, *P. arinasae* has shown to be closely related to *P. javana*, but very distant to *P. coronata*. Morphological and molecular characters support these results. Based on morphological characters, *P. arinasae* resembles *P. javana*. Those species have very different morphological characters to *P. coronata* (Fig. 1 and Table 4) (Witono *et al*., 2002).

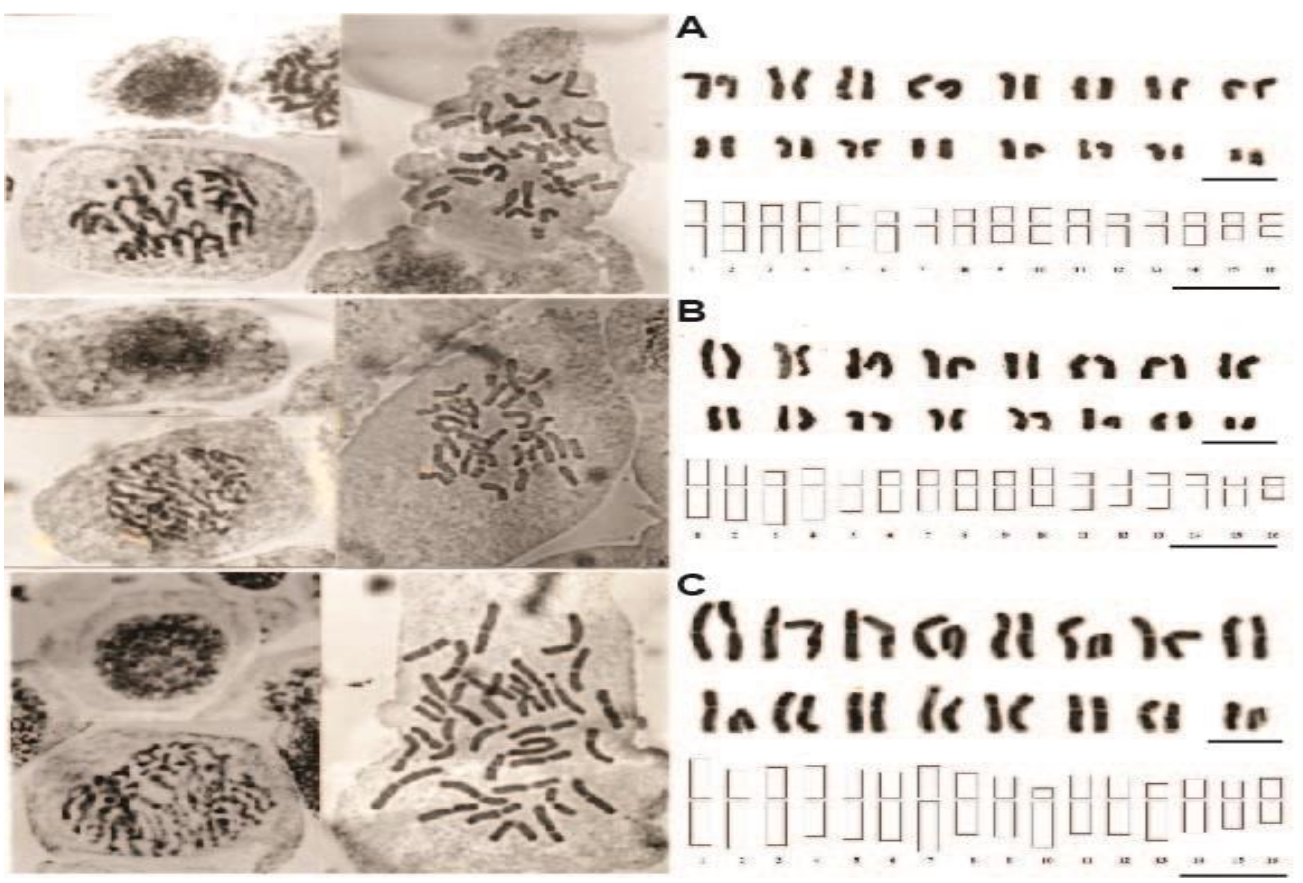

Fig. 2. Somatic chromosomes at interphase, prophase and metaphase and idiogram of A. *P. arinasae*, B. *P. javana*, and C. *P. coronata*. Scale bars = 5 µm.

Table 3. Karyotypic characters of three species of *Pinanga*.

C	*P. arinasae*					*P. javana*					*P. coronata*				
	LA		TL	AR	CS	LA		TL	AR	CS	LA		TL	AR	CS
	p	q	p+q	q/p		p	Q	p+q	q/p		p	Q	p+q	q/p	
1st	2.30	1.59	3.89	1.46	m	2.14	1.89	4.02	1.13	m	3.15	2.88	6.03	1.09	m
2nd	2.02	1.27	3.29	1.59	m	2.31	1.47	3.77	1.58	m	3.45	2.10	5.55	1.64	m
3rd	1.86	1.43	3.29	1.30	m	2.67	1.02	3.69	2.60	sm	3.16	2.25	5.41	1.42	m
4th	1.86	1.42	3.28	1.30	m	2.27	1.24	3.51	1.85	sm	2.54	2.39	4.93	1.06	m
5th	1.55	1.22	2.77	1.28	m	1.69	1.14	2.83	1.48	m	2.79	2.12	4.91	1.31	m
6th	1.90	0.79	2.69	2.40	sm	1.72	1.06	2.77	1.63	m	2.85	1.88	4.73	1.52	m
7th	1.37	0.97	2.34	1.42	m	1.65	1.11	2.76	1.47	m	2.35	2.06	4.40	1.14	m
8th	1.40	0.93	2.33	1.51	m	1.57	1.08	2.65	1.47	m	2.41	1.84	4.25	1.31	m
9th	1.23	1.10	2.33	1.10	m	1.56	1.02	2.58	1.51	m	2.61	1.56	4.17	1.69	m
10th	1.32	1.00	2.32	1.32	m	1.28	1.26	2.55	1.02	m	3.37	0.74	4.11	4.61	st
11th	1.39	0.91	2.30	1.53	m	1.57	0.94	2.51	1.68	m	2.38	1.69	4.07	1.41	m
12th	1.59	0.61	2.20	2.59	sm	1.24	1.16	2.40	1.06	m	2.41	1.58	3.99	1.54	m
13th	1.39	0.80	2.18	1.74	sm	1.43	0.92	2.35	1.58	m	2.83	1.07	3.90	2.67	sm
14th	1.42	0.69	2.11	2.10	sm	1.51	0.78	2.30	1.94	sm	2.30	1.40	3.71	1.65	m
15th	1.07	0.69	1.76	1.57	m	1.31	0.64	1.94	2.04	sm	2.01	1.25	3.26	1.63	m
16th	0.80	0.59	1.39	1.35	m	0.78	0.58	1.36	1.36	m	1.59	1.46	3.05	1.09	m

Abbreviations: C = Chromosome number; LA = The length of the arm; p = Long arm; q = Short arm; TL = Total arm length; AR = Arm ratio; CS = Chromosome shape (m = Metasentric; sm = Submetasentric; st = Subtelosentric)

Table 4. Morphological characters of *P. arinasae, P. javana*, and *P. coronata* (Witono *et al*., 2002).

Morphological characters	*P. arinasae*	*P. javana*	*P. coronate*
Stem	**Single stem**	**Single stem**	**Clustered stem**
Crownshaft	Purplish-green, covered with silvery indumentum	Brownish-green, covered with scaly brown indumentum	Green, yellowish- brownish green, covered with brown scales
Leaflets	35-45 on each side of rachis	10-15 on each side of rachis	6-30 on each side of rachis
Inflorescence	Silvery indumentose at the base	Glabrous	Glabrous
Rachillae	20-30, spirally arranged on the rachis, in same plane	8-14, arranged distichously on the rachis	5-22, spirally arranged on the rachis, not in same plane
Fruit	Obovoid	Ovoid to ellipsoid	Obovoid, ovoid to ellipsoid

Genetic diversity analysis of 13 species of *Pinanga* using ISSR (inter simple sequence repeat) genetic markers showed that *P. arinasae*, *P. javana*, and *P. coronata* were included in one cluster; but according to Jaccard's coefficient similarity between *P. arinasae* and *P. javana* (0.844) was higher than the coefficient similarity between *P. arinasae* and *P. coronata* (0.338), and also *P. javana* and *P. coronata* (0.328). Therefore, *P. arinasae* has a closer relationship with *P. javana*, than with *P. coronata*. Surprisingly, the cluster division does not depend on the habit, but it is correlated with their geographical distribution. The cluster of *Pinanga* species from the Sunda Shelf and the adjacent region was separated from the Philippines, Wallacea, and New Guinea regions (Witono & Kondo, 2006). The ISSR method is usually applied to investigate genetic relationships within species, but some studies show that this method is also useful to differentiate among species within a genus, such as *Oryza* (Joshi *et al*., 2000), *Morus* (Awasthi *et al*., 2004), and *Aethionema* (Sunar *et al*., 2016).

Analysis of ITS (internal transcribed spacers) sequence data of 52 species of *Pinanga* show that *P. javana* and *P. arinasae* are separated into a different clade from other solitary *Pinanga* species, such as *P. insignis* from the Philippines and *P. rumphiana* from New Guinea. However, *P. javana* and *P. arinasae* are supported by ITS sequence data and resolve in one clade. Two major lineages within the genus have been identified: first, *Pinanga* from West Malaysia (Sunda Shelf) and adjacent region clade and second, *Pinanga* from Wallacea, East Malaysia and an adjacent region clade. The clade separation strongly corresponds to the geographical distribution (Witono & Kondo, 2007). Based on the utility of the ITS sequence of nuclear ribosomal DNA (nrDNA) data in plants (Baldwin, 1992), it has been extensively used to distinguish among accessions within palm species, such as *Acrocomia aculeata* (Vieira *et al*., 2017) and *Phoenix dactylifera* (Maina *et al*., 2019).

Chromosome number and karyotype of certain species are stable characteristics that can reflect its basic genetic information (Sun *et al*., 2020). The Karyomorphological study of *Pinanga* in Java and Bali is supported by morphological, ISSR genetic markers, and ITS sequence data. Taxonomic relationships within the genus *Pinanga* are diverse and poorly understood. Only using a combination of morphological characteristics, pollen morphology, karyomorphology, nuclear genome size, molecular data based on genetic markers, and sequence data of all *Pinanga* species will we be able to provide a more accurate information of their relationships. These data will also afford the opportunity to gather insights into the evolutionary process in plant (Witono & Kondo, 2007).

Conclusions

The chromosome number of Pinanga in Java and Bali, i.e., *P. arinasae, P. javana*, and *P. coronata* is 2n=32. The karyomorphology of *Pinanga arinasae* and *P. javana* is here reported for the first time. Their karyotypes were different but yet similar to each other. The chromosome shapes show that *P. arinasae* and *P. javana* have a similar type as 12m + 4sm, whereas *P. coronata* is 14m + 1sm+ 1st. The chromosome length of *P. arinasae*, *P. javana*, and *P. coronata* is 1.39-3.89 µm, 1.36-4.02 µm, and 3.05-6.03 µm respectively. The present karyomorphological approach reveals that *P. arinasae* is more closely related to *P. javana* than to *P. coronata*.

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