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ภาคผนวก

ผลงานวิจัยที่ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ ฉบับที่ 1

ฉบับที่ 1 ตีพิมพ์ในวาราสารประเทศญี่ปุ่นซึ่งอยู่ในฐานข้อมูล ISI ดังรายละเอียดต่อไปนี้

ชื่อผู้วิจัย: Chaveerach, A., Sudmoon, R., Tanee, T. and Mokkamul, P. ชื่อเรื่อง: Notes on *Piper rubroglandulosum* (Piperaceae) in Thailand

ชื่อวารสาร: Acta Phytotaxonomica et Geobotanica

ปีที่: 60

ฉบับที่: 3

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ปีที่ตีพิมพ์: 2010

SHORT COMMUNICATION

Notes on Piper rubroglandulosum (Piperaceae) in Thailand

ARUNRAT CHAVEERACH^{1*}, RUNGLAWAN SUDMOON¹, TAWATCHAI TANEE²
AND PIYA MOKKAMUL¹

¹Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand. *raccha@kku.ac.th (author for correspondence); ²Faculty of Environment and Resource Studies, Mahasarakham University, Mahasarakham 44000, Thailand

Piper rubroglandulosum Chaveer. & Mokkamul was recently published from Thailand, but pistillate plants were unknown. In this paper the pistillate spike, pistillate flowers and fruit are described and illustrated based on pistillate plants found in the Khao Phra Thaeo Wildlife Conservation Development and Extension Center, Phuket Province, southern Thailand, in 2009.

Key words: Piper, Piper rubroglandulosum, Piperaceae, pisitllated flower, Thailand

Piper (Piperaceae) contains over 1,000 species (Tebbs 1993) distributed mainly ina tropical regions worldwide. Forty species have been reported from Thailand (Chaveerach et al. 2008). Among them, P. rubroglandulosum Chaveer. & Mokkamul, which was known only from staminate plants, has a staminate inflorescence similar to the one in P. betle L. On 23 March 2009, we found pistillate plants bearing flowers and fruits at Khao Phra Thaeo Wildlife Conservation Development and Extension Center, Phuket Province, southern Thailand. The pistillate spike, pistillate flowers and fruit are described and illustrated below (Figs. 1 & 2).

Piper rubroglandulosum Chaveer. & Mokkamul, Acta Phytotax. Geobot. 59(2): 142–145, figs. 26 & 27 (2008).

Pistillate plants resemble staminate plants, as described by Chaveerach *et al.* (2008). Pistillate spike pendulous, 1.5–2.5 cm long, 3–5 mm in diameter; peduncle 1–1.2 cm long; rachis hairy; bracts rounded, margin free, pedicel short, pubescent; stigmas (4)–5. Fruiting spike pendulous,

3-7 cm long, ca. 0.7-1.3 cm in diameter; peduncle 1-1.2 cm long; bracts pubescent. Fruits embedded in rachis, pubescent. Flowing and fruiting: March.

Specimens examined. THAILAND. Southern. Phuket Province: Khao Phra Thaeo Wildlife Conservation Development and Extension Center, 100–900 m alt., 23 March 2009, A Chaveerach 616 & 617 (BK). —Surat Thani Province: Khlong Phanom National Park, 100–200 m alt., A. Chaveerach 314 (BK). —Phang Nga Province, Sri Phang Nga National Park, 100–900 m alt., A. Chaveerach 317 (BK). Northeastern. Loei Province: Phu Luang Wildlife Sanctuary, 800–900 m alt., A. Chaveerach 318 (BK). Central. Suphan Buri Province: Phu Toei National Park, 800–900 m alt., A. Chaveerach 319 (BK).

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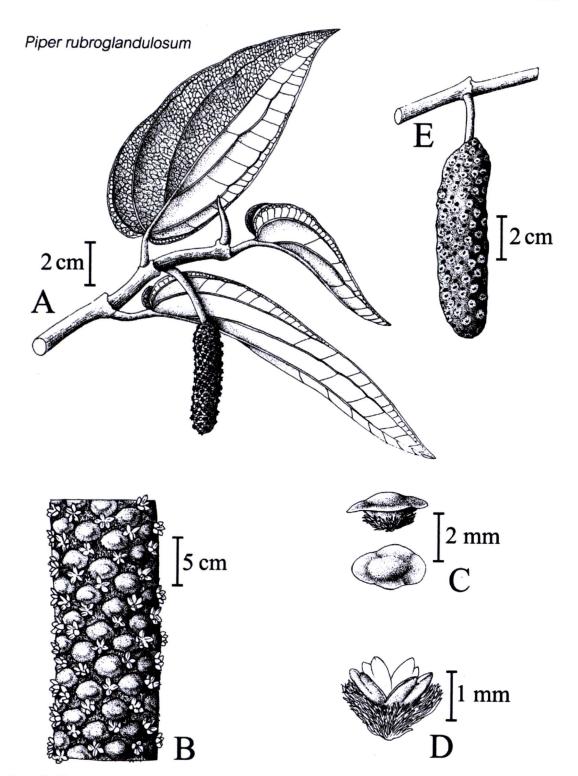


Fig. 1. Pistillate plants of *Piper rubroglandulosum* Chaveer. & Mokkamul. A: leaves and pistillate spike. B: enlarged pistillate spike. C: bract. D: four stigmas showing hairs on rachis. E: fruiting spike. [A–D: A. Chaveerach 616 (BK), E: A. Chaveerach 617 (BK).]

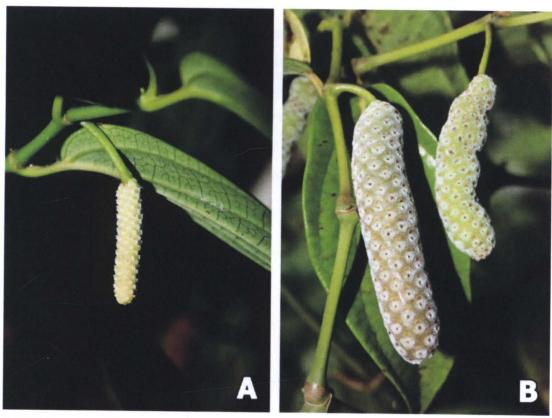


Fig. 2. Pistillate plants of *Piper rubroglandulosum* Chaveer. & Mokkamul. A: branch with pistillate spike [A. Chaveerach 616]. B: branch with fruiting spikes [A. Chaveerach 617].

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ผลงานวิจัยที่ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ ฉบับที่ 2

ฉบับที่ 2 ได้รับการยอมรับให้ตีพิมพ์ (accepted) ในวาราสารประเทศจีนซึ่งอยู่ในฐานข้อมูล ISI และมี ISI Impact Factor 2010 เป็น 1.295 ดังรายละเอียดต่อไปนี้

ชื่อผู้วิจัย: Sudmoon, R., Chaveerach, A. and Tanee, T.

ชื่อเรื่อง: *Piper protrusum* (Piperaceae), a new species from southern Thailand based on morphological and molecular evidence

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จดหมายตอบรับการตีพิมพ์และ Proof ของงานตีพิมพ์ฉบับที่ 2

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Dear Prof. Chaveerach:

It is a pleasure to accept your manuscript entitled "Piper protrusum (Piperaceae), a new species from southern Thailand based on morphological and molecular evidences" in its current form for publication in Journal of Systematics and Evolution. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

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Journal of Systematics and Evolution 00 (00): 1-8 (2011)

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Research Article

Piper protrusum (Piperaceae), a new species from southern Thailand based on morphological and molecular evidence

¹Runglawan SUDMOON ²Tawatchai TANEE ¹Arunrat CHAVEERACH*

¹(Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand)
²(Faculty of Environment and Resource Studies, Mahasarakham University, Mahasarakham 44000, Thailand)

Abstract Piper protrusum Chaveer. & Tanee, sp. nov. is described and illustrated. It dominantly comprises three branching types with three different types of leaf blades, bases, and apexes. The critical distinguishing character is the protruded receptacle having a bract and nine stamens. Individual plants have been discovered in areas of Southern Thailand since 2004 without reproductive parts. The investigated sites were revisited several times, and an individual with flowers was finally found in July 2009. Phylogenetic analysis of the new species and five similar species is carried out based on DNA fingerprinting. The genetic distances between the new species and five similar species range from 0.25 to 0.35, supporting new species designation. Molecular data conform to morphological data in validating that it is a new species. Additionally, its DNA barcodes have been provided for further identification in case the morphological data is unclear. The sequence data have been submitted to the GenBank database under accession numbers GU980898 (rpoB gene), GU980899 (rpoCl gene), and GU980900 (psbA-trnH region).

Key words DNA fingerprint, new species, Piperaceae, Piper protrusum, Thailand.

Species diversity and distribution of the genus Piper in Thailand have been investigated for over 10 years (Chaveerach et al., 2008). Taxonomic accounts of this genus are based on the stamen and stigma numbers and characters, floral bract morphology, leaf shape, and leaf venation (Tseng et al., 1999; Jaramillo & Manos, 2001). In taxonomic studies, species identification is commonly based on morphological characters. However, in some cases, it is difficult to determine all characters or to find all important plant parts. Therefore, molecular data have been introduced to solve this limitation. DNA fingerprinting, based on polymerase chain reaction, is the efficient and reliable technology used in many plant groups for several years (Wünsch & Hormaza, 2002), for example, apples (Koller et al., 1993; Tancred et al., 1994), pears (Oliveira et al., 1999; Monte-Corvo et al., 2000), chestnut (Galderisi et al., 1998; Oraguzie et al., 1998), and Curcuma species (Chaveerach et al., 2007). Recently, DNA barcodes, comprising short sequences of standard regions, have been evaluated for systematics. Some plant groups such as Dendrobium species (Asahina et al., 2010), Liparis species (Lee et al., 2010), mosses (Liu et al., 2010), and Nyholmiella (Sawicki et al., 2010) have had their

barcodes recorded for further identification and other applications.

In this research *Piper* sp. 2, recently reported by Chaveerach et al. (2008) without flowering details, has been discovered with male spikes. It was examined and identified as a new species based on morphological and molecular data. The new species is described and illustrated.

1 Material and methods

1.1 Field study and plant collection

During exploration of the genus *Piper* in Thailand, *Piper* sp. 2 (Chaveerach et al., 2008) was seen without flower in Southern Thailand. The authors revisited the investigated sites, speculating that it was a new species. Plant use information was recorded through observation, surveys of markets, and interviews with traditional healers and local people.

Individuals of *Piper* sp. 2 mentioned in Chaveerach et al. (2008) were taken from Southern Thailand, namely: Sri Phang Nga National Park and Khao Luk-Lumru National Park, Phang Nga Province; Khao Sok National Park, Surat Thani Province; and Nam Tok Ngao National Park, Ranong Province. An individual with male spikes was taken from Sri Phang Nga National Park in July 2009. For molecular studies, leaves of two

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* Author for correspondence. E-mail: raccha@kku.ac.th; Tel.: 66-4334-2908; Fax: 66-4334-4190

Species	Distribution	Habit				Characters			
	in Thailand			Leaf	Leaf, on free branch			Male spike	
			Shape	Base	Apex	Venation	Inflorescence	Stamen	Bract
Piper sp. 2	Southern	Climber	Elliptic	Subcuncate- cuneate	Acuminate- cuspidate	Veins 7, apical pair arising 2.5–5 cm apart from base, alternate, reaching apex	Pendulous; flowers sparsely stick on protruded receptacle	Stamens 9, filament flat	Sessile, semi-circle, margin ciliated, one margin adnate to protruded rachis the
P. dominantinervium Southern	Southern	Climber	Ovate or narrowly elliptic to elliptic	Oblique with unequally sides	Cuspidate	Veins many, all arising from midrib at regular intervals of base to apex reaching near margin and toining with each other	Pendulous; flowers densely embedded in rachis	Stamens 2	Sessile, elliptic, margin ciliated
um	Central, Northeastern	Climber	Narrowly elliptic to Oblique or elliptic or rounded narrowly ovate to broadly ovate	Oblique or rounded	Acuminate	Veins 7–9, abaxially prominent, 2–3 pairs basal, I pair arising 1.5–3 cm apart from base		Not seen	
	Throughout, cultivated	Climber	Ovate to elliptic	Rounded to oblique	Acuminate	Veins 7, 2 pairs basal, 1 pair arising 1–2 cm apart from base, reaching anex	Pendulous; flowers short-stalked	Stamens 2 (mo- noecious)	Sessile, elliptic, base adnate to rachis tip free
Polysyphonum	Southern	Climber	Ovate to elliptic	Rounded, subcuneate or oblique	Short acuminate	Veins 7-9, abaxially often reddish when dry, apical pair arising 1.6-2 cm apart from base, alternate, reaching apex, others basal or arising near base, reticulate veins transverse.	Pendulous; flowers sparsely scattered on rachis	Stamens 3 (monoccious)	
mson	Southern	Climber	Ovate or elliptic	Shallowly cordate, often oblique	Acute or cuspidate	Veins 7, adaxially prominent, apical pair arising 0,5-1,5 cm apart from base, opposite or alternate reaching apex, reticulate weins conspicuous	Pendulous; flowers densely embedded on rachis	Stamens 2	Stalk short, pubescent, subrounded, margin free
P. umbellatum	Northern, Southern	Small shrub	Southern Small shrub Ovate or reniform to rounded	Shallowly- oblique cordate	Acute	Veins 11–13, apical pair arising 1–2 cm apart from base, opposite or alternate	Straight up, compound umbel; flowers densely embedded on rachis	Stamens 2–3 (monoc- cious)	Stalked, triangular, margin ciliated

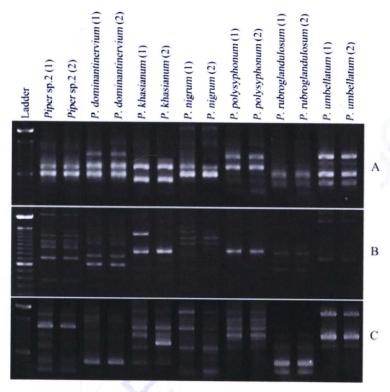


Fig. 1. Example of distinguishable DNA fingerprints for *Piper* sp. 2 (*P. protrusum*) and five similar species with one dissimilar species, *P. umbellatum*, from different primers. Two individuals (1, 2) from each species were included in the analysis. A, Primer 5'-CAGGCCCTTC-3'; B, Primer 5'-CAAACGTCGG-3'; C, Primer 5'-GGTGGTCAAG-3'.

individuals of *Piper* sp. 2 and other similar species were collected. The five similar species were *P. dominantinervium* Chaveer. & Mokkamul, *P. khasianum* C. DC., *P. nigrum* L., *P. polysyphonum* C. DC., and *P. rubroglandulosum* Chaveer. & Mokkamul. The most dissimilar species, *P. umbellatum* L., was also included to be an outgroup in phylogenetic analysis.

Table 2 Sequences of arbitrary primers. Thirty-six primers from three primer sets (sets P, C, and R) were screened, and 16 primers successfully produced clear and distinguishable DNA fingerprints for the identification of *Piper* species

Primer	Sequence (5' to 3')	Primer	Sequence (5' to 3')
PI	CAGGCCCTTC	C5	GTCCCGACGA
P2	TGCCGAGCTG	C6	AAGCCTCGTC
P3	AATCGGGCTG	C7	TTATCGCCCC
P4	GGGTAACGCC	R8	CTACTGCCGT
P5	CAATCGCCGT	R9	GGTGGTCAAG
P6	GTTGCGATCC	R10	GACCTACCAC
P7	CAAACGTCGG	R11	TCAGTCCGGG
C4	GGACCCTTAC	R12	CACCATCCGT

1.2 Morphological identification

The plant materials were identified following evidence cited in Chaveerach et al. (2008) and the taxo-

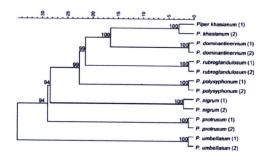


Fig. 2. Dendrogram constructed using Fingerprinting II software (Bio-Rad) based on DNA fingerprints from 16 random amplified polymorphic DNA primers showing genetic distances of *Piper* sp. 2 (*P. protrusum*) and other species. Bootstrap values are based on 1000 recalculates of the dataset and are indicated as a percentage on the tree branches.

nomic identifications were investigated based on references available from Roxburgh (1820), Hooker (1885), Candolle (1910a, 1910b), Merrill (1912), Quisumbing (1930), Henderson (1959), Backer & Bakhuizen van den Brink (1963), Ridley (1967), Chew (1972, 2003), Yuncker (1972), Aghard (1981), Royen (1982), Tebbs (1982, 1993), Long (1984), Heyne (1987), Huber (1988), Keng (1990), Lin & Lu (1996), Gilbert & Xia (1999), Ho (1999), Tseng et al. (1999), Tawan et al. (2002), and Gardner (2003).

Morphological characters of *Piper* sp. 2 and the five similar species were compared. Morphological diagnosis of the new species was prepared. The scientific name was established followed the International Code of Botanical Nomenclature. Type specimens were kept at BK, BKK, PE, and Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

1.3 DNA extraction and amplification

Total genomic DNAs from two individuals of each species, 14 individuals in total, were extracted using the Plant Genomic DNA Extraction Kit (RBC Bioscience, Xindian City, Taiwan). The extracted DNAs were used in fingerprinting and barcoding analyses.

For DNA fingerprinting, polymerase chain reaction was carried out based on the random amplified polymorphic DNA (RAPD) technique. Amplification was carried out in 25 µL reactions consisting of GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.5 μmol/L primer and 10 ng DNA template. The reaction mixture was incubated at 94 °C for 3 min and the amplification was carried out with the following thermal cycles: 35 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 30 s, extension at 72 °C for 2 min, and a final extension for 5 min at 72 °C. Thirty-six arbitrary primers were screened for producing distinguishable fingerprints. The fingerprints were analyzed using Fingerprinting II software (Bio-Rad, Philadelphia, PA, USA). Dendrogram and genetic distance matrix were generated.

For DNA barcoding, polymerase chain reaction was carried out with primer pairs: 5'-ATGCAACGTCAAGCAGTTCC-3' and 5'-GATCCC AGCATCACAATTCC-3' for the rpoB region; 5'-GTG GATACACTTCTTGATAATGG-3' and 5'-TGAG AAAACATAAGTAAACGGGC-3' for the rpoC1 region; and 5'-GTTATGCATGAACGTAATGCTC-3' and 5'-CGCGCATGGTGGATTCACAATCC-3' for the psbA-trnH region (http://www.kew. org/barcoding/update.html; accessed 8 August 2009). The reaction mixture was done in 50 μ L consisting of

Table 3 Genetic distances	listances be	between Pipe	er species based of	n 16 random an	Ther species based on 16 random amplified polymorphic DNA fingerprinting analysis using Eingerprinting II software (1	hic DNA fingern	rinting analysis	ancino Finant	mining	II coffin	odoid) out	17		
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	_	7		2	_	,	-		-	, -		,		
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r. Khushahimi 2	0.03	0.00	0.16	0.17	0.24	0.23	0.22	0.23	0.23	0.25	96 0	26.0	0.36	0 37
F. dominantinerrum	0.17	91.0	0.00	0.01	0.22	0.21	0.33	0 33	0 37	200	200		0.00	100
P dominantinerrium ?	91.0	0.17	100	000		170	77.0	0.23	0.27	67.0	0.26	0.25	0.38	0.39
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r morogramamosum .	0.21	0.24	0.22	0.21	0.00	0.01	0.24	0.23	0.35	0.35	0 34	0 11	0.34	0 13
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P nalvsvahomm 1	100	0.33	0 33	0 33		O. C.	0.43	0.24	0.36	0.36	0.35	0.34	0.33	0.34
The state of the s	9 0	27.0	27.0	0.23	0.24	0.23	0.00	0.01	0.27	0.27	0.28	0.29	0.28	0 27
r panysyphonam 2	07.0	0.23	0.23	0.22	0.23	0.24	0.01	000	0.28	80.0	0.00	0.20	0 33	200
P. nigrum	0.24	0.23	0.27	86.0	0.35	0.35	-	000	07.0	0.50	67.0	0.30	0.57	0.70
P mionum 7	0.34	30.00	900	02.0	0.33	0.36	0.27	0.28	0.00	0.05	0.31	0.32	0.39	0.38
D. C.	170	67.0	67.0	0.26	0.35	0.36	0.27	0.28	0.02	0.00	0.29	0.30	0.37	98 0
F. prograssim 1	0.25	0.26	0.26	0.27	0.34	0.35	0.28	0.00	0.31	0.00	000	100	0.36	20.0
P. protrusum 2	0.24	0.25	0.25	90 0	0.33	0.34	00.00	0.00		-	0.00	10.0	0.30	0.35
P umbellettum 1	0.35	0.36	30	0.00	0.00	100	67.0	0.30	0.32	0.30	0.01	0.00	0.37	0.36
D. T. C.	0.33	0.30	0.38	0.39	0.34	0.33	0.28	0.27	0.39	0.37	0.36	0.37	0.00	100
C. Innocuation 2	0.30	0.37	0.39	0.40	0.33	0.34	0.27	0.26	0.38	0.36	0.36		100	000
													0.0	0000

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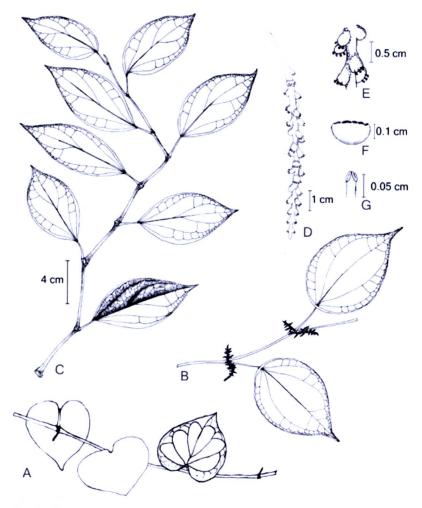


Fig. 3. Piper protrusum (from holotype). A, Creeping branch; B, Epiphytic branch; C, Free branch; D, Pendulous male spike; E, Enlarged male spike; F, Bract; G, Stamen.

GoTaq Green Master mix (Promega), $0.25~\mu$ mol/L each primer, and 20 ng DNA template. The reaction mixture was incubated at 94 °C for 1 min and amplification was carried out with the following thermal cycles: 35 cycles of 30 s denaturation at 94 °C, 40 s annealing at 53 °C, 40 s extension at 72 °C. Then the final extension at 72 °C was done for 5 min. The amplified specific fragments were sequenced and the sequences were annotated by the BLAST tool (http://blast.ncbi.nlm.nih.gov/; accessed 24 June 2011) and submitted to the GenBank database (http://www.ncbi.nlm.nih.gov; accessed 25 July 2010).

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2 Results and discussions

2.1 Data from field studies and evaluation of a new species

Plant leaves from *Piper* sp. 2 have been used for folk medicine in southern Thailand. At Amphur Kuraburi, Pung Nga Province, leaves are ground with water and sprayed onto the skin to treat herpes zoster and urticaria.

Morphological characters of *Piper* sp. 2 were carefully observed. There is no closely related species to *Piper* sp. 2. A morphological comparison of *Piper* sp.



Fig. 4. Piper protrusum (from holotype and paratype). A, Creeping branch; B, Epiphytic branch; C, Free branch; D, Free branch with a pendulous male spike; E, Enlarged spike from stereo microscope illustrating protruded rachis, ciliated bract margin, and stamens.

2 and five similar species (according with Chaveerach et al., 2008) is shown in Table 1.

Genetic distance of the new species and similar species was evaluated using RAPD analysis. Thirty-six arbitrary primers were screened; of these, 16 primers produced a distinguishable fingerprinting pattern. Figure 1 shows examples of distinguishable fingerprints. The successful primer sequences are listed in Table 2. A dendrogram (Fig. 2) shows that P. umbellatum is separated out as an outgroup where Piper sp. 2 is not closely related to any similar species. Genetic distances (Table 3) within a species range from 0.01 to 0.03, whereas those between species range from 0.16 to 0.39. Interestingly, Piper sp. shows genetic distances ranging from 0.25 (with P. khasianum) to 0.34 (with P. rubroglandulosum) which are higher than the lowest value. The genetic distance value supports Piper sp. 2 as a separated species.

According to morphological and molecular data, Piper sp. 2 is identified as a new species and named Piper protrusum Chaveer. & Tanee. DNA barcodes of the new species was provided with GenBank accession numbers GU980898 (RNA polymerase beta subunit (rpoB), 451bp), GU980899 (RNA polymerase C (rpoC1), 499bp), and GU980900 (psbA-like (psbA) gene, psbA-trnH intergenic spacer, tRNA-His (trnH) gene, 265bp). The DNA barcode markers can be used for rapid, accurate, and automatable species identification by botanists if morphological data is incomplete.

2.2 Taxonomic treatment

Piper protrusum Chaveer. & Tanee, sp. nov. Figures 3, 4

Latin diagnosis: Species *P. polysyphonum* C.DC. affinis, sed lamina crassa elliptica, apice acuminato-cuspidato, basi subcuneata vel cuneata distinguenda, receptaculum protrusum bracteo et 9 staminibus instructum differt.

Thailand, Phang Nga Province: Sri Phang Nga National Park, alt. ca. 100 m, evergreen forest, 2009-07-12, *A. Chaveerach 615* (holotype, BK).

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Fig. 5. Distribution of *Piper protrusum* (▲) and all other *Piper* species (♦) throughout Thailand. A, Ranong Province; B, C, Phang Nga Province; D, Surat Thani Province.

Description: Climbers, large creeping, glabrous, polymorphic leaf shapes. Creeping branch: slender, but looking plump, often pinkish-dotted, densely hairy; petiole densely hairy, 3.5-4 cm long; leaf blade thick coriaceous, brownish-yellow and pellucid glands, broadly ovate, 7-8.5 cm long, 6-7 cm wide; apex cuspidate; base shallowly or deeply cordate with lobes divergent; veins 7, sparsely hairy or glabrous, apical pair arising 0.5-0.7 cm apart from base, opposite, reaching leaf apex, others basal, reticulate veins inconspicuous. Epiphytic branch: slender, glabrous; petiole 3-3.2 cm long; leaf blade broadly elliptic, 11 cm long, 7-7.5 cm wide; apex cuspidate; base rounded; veins 7, apical pair looks like arising from midrib, but basal and parallel of midrib 0.5-1 cm apart from base then separate out reaching leaf apex, others basal, reticulate veins inconspicuous. Free branch: slender, glabrous, node joint, swollen; petiole 1-1.5 cm long; leaf blade thick, elliptic, 9.5-11 cm long, 4-6 cm wide; base subcuneate-cuneate; apex acuminatecuspidate; veins 7, apical pair arising 2.5-5 cm apart

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from base, alternate, reaching leaf apex. Male spike: pendulous, 8–10 cm long, ca. 0.2 cm in diam.; peduncle 1–1.7 cm long; flowers sparsely stick on protruded receptacle of a spike; bract sessile, semicircle, margin ciliated, one margin adheres to protruded rachis, the other margin free; stamens 9, filament flat.

Distribution and ecology: This plant is distributed in Southern Thailand (Fig. 5) from Ranong Province (Nam Tok Ngao National Park), Phang Nga Province (Sri Phang Nga National Park and Khao Luk-Lumru National Park), and Surat Thani Province (Khao Sok National Park). The plant grows in moist evergreen forest. It occurs at altitude of 100 m in light-moist area, creeping on ground and climbing on trees.

Note: The epithet protrusum refers to its dominant character, the protruded rachis. No specific Piper species is closely related. It looks similar to other dicotyledonous plants. Only creeping leaves and spikes, which are flowered once in a very long while, show its Piper characters. Female spike and fruit of this species has not yet been discovered. Its vernacular names are Sa-kan look yai or Sa-kan thin tai. It has been locally used for relief of dizziness.

Selected specimens examined: Thailand. Ranong Province: Nam Tok Ngao National Park, 2004, A. Chaveerach 100 (paratype, BK).

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