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Source: Willdenowia, 54(2-3) : 121-149

Published By: Botanic Garden and Botanical Museum Berlin (BGBM)

URL: https://doi.org/10.3372/wi.54.54201

Willdenowia
Annals of the Botanic Garden and Botanical Museum Berlin

Botanischer Garten Berlin

Cytotaxonomy of *Kaempferia* subg. *Protanthium* (*Zingiberaceae*) supports a new limestone species endemic to Thailand

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Version of record first published online on 28 June 2024 ahead of inclusion in August 2024 issue.

Abstract: Thailand is the biodiversity hotspot of genus *Kaempferia* (*Zingiberaceae*) and harbours 14 species belonging to *K.* subgenus *Protanthium*. To better understand the taxonomic circumscription and verify the taxonomic status, the present characterization of cytogenetic characters included all recognized and one undescribed species of *K.* subg. *Protanthium* from Thailand. Overall, 88 accessions of plant materials were analysed cytogenetically: 84 and 42 accessions were subjected to flow cytometry and karyology, respectively. Based on genome size and mitotic chromosome numbers, 74 accessions from all species investigated were putative diploid, whereas the others were putative polyploid: triploid (three accessions) and tetraploid (11 accessions). The cytogenetic evidence indicates that $2n = 2x = 22$ is the diploid number and $x = 11$ is the base chromosome number for *K.* subg. *Protanthium*. The genome sizes among the diploid accessions ranged from 3.687 to 6.412 pg while high intraspecific variation in genome size was observed with up to 19.4%. Two species included accessions with different ploidy levels: *K. rotunda* L. (diploid, triploid and tetraploid) and *K. takensis* Boonma & Seansouk (diploid and tetraploid). The increase in genome size of tetraploid *K. rotunda* is nearly in correlation to the increase in ploidy level, whereas the triploid plants represent genome expansion with an approximately 11% larger than expected genome. Interestingly, tetraploid *K. takensis* displays genome downsizing of 15.3% compared to their diploids. The cytogenetic characteristics, together with morphology, unequivocally clarify the taxonomic status of a new species, named *Kaempferia calcicola* Nopporn. A revised identification key to species of *K.* subg. *Protanthium* is provided.

Keywords: chromosome number, cytogenetics, cytotaxonomy, genome size, *Kaempferia*, *Kaempferia* subg. *Protanthium*, Khon Kaen province, new species, polyploidy, species circumscription, taxonomy, Thailand, *Zingiberaceae*

Article history: Received 29 November 2023; peer-review completed 18 March 2024; received in revised form 7 May 2024; accepted for publication 14 May 2024.

Citation: Nopporncharoenkul N., Sukseansri W., Nopun P., Meewasana J., Jenjittikul T., Chuenboonngarm N., Viboonjun U. & Umpunjun P. 2024: Cytotaxonomy of *Kaempferia* subg. *Protanthium* (*Zingiberaceae*) supports a new limestone species endemic to Thailand. – Willdenowia 54: 121–149. https://doi.org/10.3372/wi.54.54201

Introduction

Kaempferia L. (1753) is a genus of small, rhizomatous perennial herbs belonging to the family *Zingiberaceae*, subfamily *Zingiberoideae*, tribe *Zingibereae* (Kress & al. 2002). Several species are recognized for their medicinal and economic values (Saokaew & al. 2017; Paramee & al. 2018; Pham & al. 2021), due to being sources of bioactive compounds such as essential oils, diterpenoids and flavonoids (Mekjaruskul & al. 2012; Atun & al. 2013; Atun & Arianingrum 2015; Kaewkroek & al. 2013, 2017; Muthachan & Tewtrakul 2019), as well as having the potential to be developed as attractive ornamental plants (Picheansoonthon & Koonterm 2008; Leong-Škorničková & Newman 2015). Currently, POWO lists 63 accepted species, but according to the most recent taxonomic studies, the genus comprises approximately 55 accepted species mainly throughout monsoonal tropical Asia (Mabberley 2017; Jenjittikul & al. 2023; Nopporncharoenkul & al. 2024). Thailand, which is situated in the Indo-Chinese biodiversity hotspot, is regarded as one of the centres of distribution of the genus (Larsen & Larsen 2006; Leong-Škorničková & Newman 2015) and with about 40 recognized species including 20 strictly endemic provides the richest species diversity (Jenjittikul & al. 2023). Taxonomically, *Kaempferia* is subdivided into two subgenera according to the inflorescence position (Horaninow 1862; Baker 1890; Kam 1980; Insisiengmay & al. 2018), namely *K.* subg. *Kaempferia* and *K.* subg. *Protanthium* (Horan.) Baker. The species in *K.* subg. *Kaempferia* typically produce central inflorescences terminating leafy shoots, usually enclosed by the inner-

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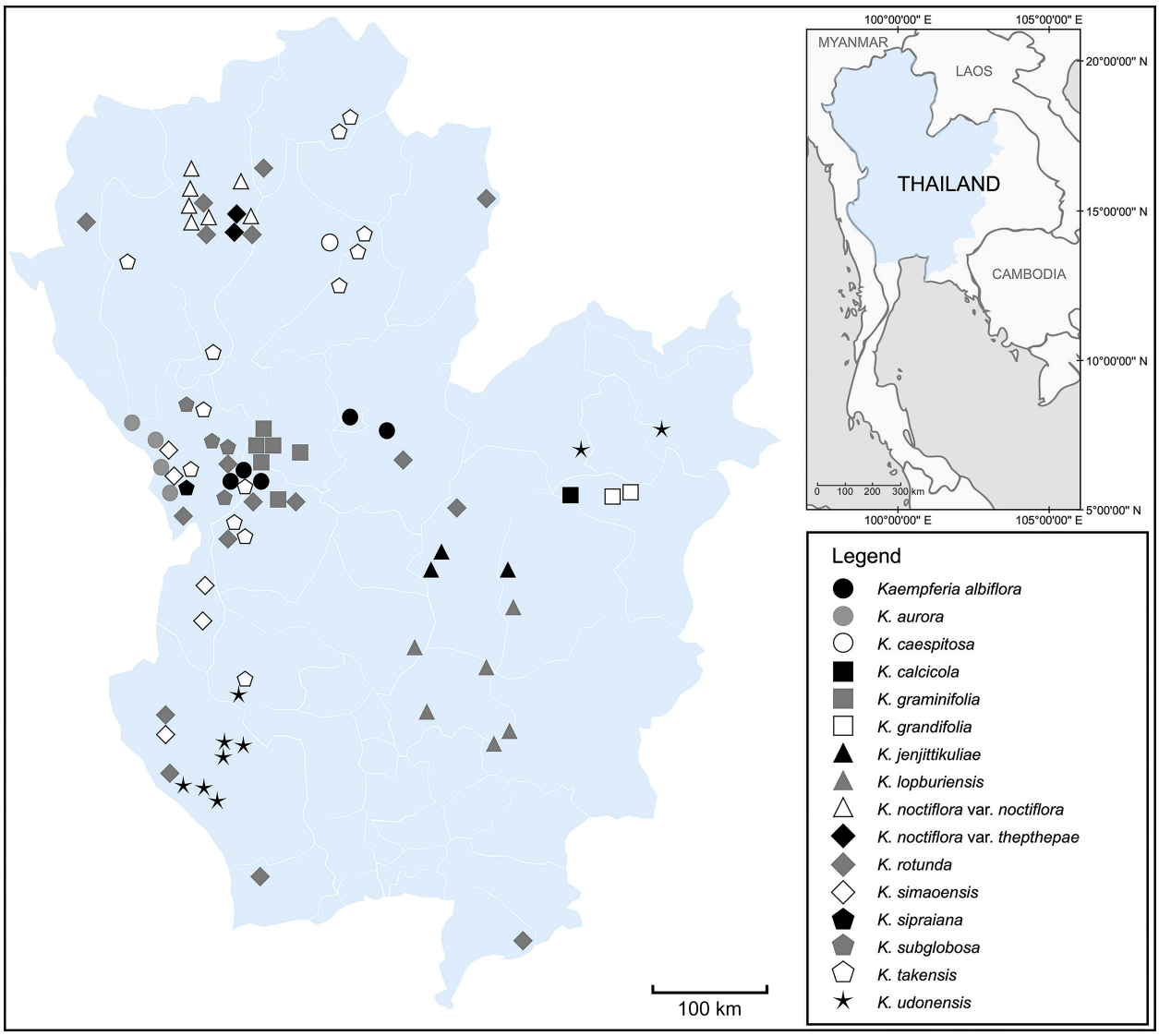

Fig. 1. Map showing distribution of accessions of *Kaempferia* subg. *Protanthium* in Thailand included in this study. Each point represents an individual collecting site of accession. Two accessions of *K. rotunda* (accession *NNSB-600–1* and *NNSB-600–2*) introduced from Laos were not included in a map.

most leaf sheaths or embedded in the pseudostems. By contrast, the species belonging to *K.* subg. *Protanthium* produce lateral inflorescences arising on bladeless shoots directly from the rhizomes, usually before, or sometimes concurrently with, separate leafy shoots. The racemes of *K.* subg. *Protanthium* are mostly embedded in the soil and emerge before the leafy shoot arises (Nopporncharoenkul & al. 2021). According to the previous studies of Techaprasan & al. (2010) and Nopporncharoenkul & al. (2016), the species belonging to *K.* subg. *Protanthium* included in the molecular phylogenetic analyses are strongly clustered in a single clade based on sequences of nuclear and plastid DNA regions. Currently, Thailand harbours 14 species and one variety of the subgenus in accordance with the taxonomic revision in the Flora of Thailand (Jenjittikul & al. 2023) and the most recent publications (Nopporncharoenkul & Jenjittikul 2024; Nopporncharoenkul & al. 2024), namely: *K. albiflora* Jenjitt. & Ruchis., *K. aurora* Nopporncharoenkul & Jenjitt., *K. caespitosa* Nopporncharoenkul & Jenjitt., *K. graminifolia* Nopporncharoenkul & Jenjitt., *K. grandifolia* Saensouk & Jenjitt., *K. jenjittikuliae* Nopporncharoenkul., *K. lopburiensis* Picheans., *K. noctiflora* Nopporncharoenkul & Jenjitt. var. *noctiflora*, *K. noctiflora* var. *thepthepae* Nopporncharoenkul & Somnoo, *K. rotunda* L., *K. simaoensis* Y. Y. Qian, *K. sipraiana* Boonma & Saensouk, *K. subglobosa* Nopporncharoenkul & Jenjitt., *K. takensis* Boonma & Saensouk and *K. udonensis* Picheans. & Phokham. Biogeographically, another species of the subgenus, *K. xiengkhouangensis* Picheans. & Phokham, is strictly endemic to Xiangkhouang province of Laos (Phokham & al. 2013; Insisiengmay & al. 2019). Interestingly, during recent field observation and specimen collection throughout Thailand we found an undescribed species occurring on the limestone hills in Khon Kaen province, NE Thailand. Consequently, the present study includes all 14 recognized species, one variety, and one

undescribed species from Thailand, except for *K. xiengkhouangensis* only.

Cytogenetic insights are not only important for plant improvement programs and for the management and conservation of plant genetic resources, but also have extensively proven useful in plant taxonomic identification and in the interpretation of species evolution and speciation, especially in native species and natural hybrids (Guerra 2008, 2012). Chromosome numbers ($2n$), base chromosome number (x) and ploidy level, either together or separately, are considered as effective classification criteria in the same manner as morphological characters (Guerra 2008, 2012). Genome size, which is another cytogenetic character commonly known as the nuclear DNA content or 2C value, provides more precise information and effectively supports plant taxonomic classification and identification (Šlenker & al. 2018; Španiel & al. 2018). The estimation of genome size in plants determined by flow cytometry is a rapid, convenient and relatively uncomplicated technique and often applied for putative ploidy level determination in flowering plants (Schutte & al. 1985; Doležel & al. 2007).

Over the past decade, cytogenetic approaches have been widely applied in taxonomic classification and species-specific identification of several genera in *Zingiberaceae*, such as *Curcuma* L. (Leong-Škorničková & al. 2007; Puangpairote & al. 2015; Anamthawat-Jónsson & Umpunjun 2020; Nopporncharoenkul & al. 2020a), *Gagnepainia* K. Schum. (Moonkaew & al. 2020) and *Kaempferia* (Nopporncharoenkul & al. 2017; Saensouk & Saensouk 2021a). Regarding the genus *Kaempferia*, most of the species are diploid having $2n = 2x = 22$ with the base number $x = 11$. However, polyploids were documented within the genus, including triploidy ($2n = 33$), tetraploidy ($2n = 44$), and pentaploidy ($2n = 55$) (Raghavan & Venkatasubban 1943; Chakravorti 1948; Ramachandran 1969; Mahanty 1970; Eksomtramage & Boontum 1995; Eksomtramage & al. 1996; Saensouk & Jenjittikul 2001; Eksomtramage & al. 2002; Saensouk & Saensouk 2004, 2021; Nopporncharoenkul & al. 2017). Interestingly, intraspecific ploidy variation was often observed within the species (Nopporncharoenkul & al. 2017): for example, *K. angustifolia* Roscoe ($2n = 22$ and 33), *K. elegans* (Wall.) Baker ($2n = 22$, 33 and 44) and *K. galanga* L. ($2n = 22$, 44 and 55). In addition, the study of Nopporncharoenkul & al. (2017) revealed that aneuploid numbers were occasionally occurred in the genus, such as *K. galanga* ($2n = 54$), *K. parviflora* Wall. ex Baker ($2n = 24$), *K. rotunda* ($2n = 30$, 54) and *K. siamensis* Sirirugsa ($2n = 40$).

For the *Kaempferia* subg. *Protanthium* to date, $2n$ chromosome numbers of only two species were published, for *K. grandifolia* ($2n = 22$: Saensouk & Jenjittikul 2001), and one for *K. rotunda* ($2n = 22$, 30, 33, 44 and 54: Nopporncharoenkul & al. 2017; Saenprom & al. 2018). Additionally, only the genome size of *K. rotunda* is available to date (Chandrmai & al. 2012; Sadhu & al. 2016; Basak & al. 2018; Záveská & al. 2024). In the present research we therefore aimed (1) to complete the information of the $2n$ chromosome numbers of all species belonging to *K.* subg. *Protanthium* in Thailand, including both recognized and undescribed species, (2) to estimate the genome sizes of a large collection of 84 accessions from natural habitats and in cultivation by flow cytometry, (3) to infer the putative ploidy levels of the accessions lacking chromosome information by comparing the genome sizes to those of the accessions obtained with both chromosome numbers and genome sizes, and (4) to better understand the taxonomic circumscription and verify the taxonomic status of the species using combined data of morphological and cytogenetic characters, excluding discussion with the phylogenetic relationship.

Material and methods

Plant material and plant identification

Field studies and plant sample collections were carried out throughout Thailand from April 2014 to April 2024. A large collection of 88 accessions covering 14 recognized species, one variety, and one undescribed species belonging to *Kaempferia* subg. *Protanthium* were included in the present study (Table 1), representing 14 of the 15 recognized species worldwide. An accession number was assigned to refer to the geographical location (district and province names) of the individual population sampled. Two accessions of *K. rotunda* (accession NNSB600–1 and NNSB600–2) were obtained from the Chatuchak plant market in Bangkok, although these plants were initially introduced from their natural habitat in S Laos. One cultivated accession of *K. rotunda* (accession NNSB602) was collected from a private preservation area of the fifth author. Rhizomes of all samples were collected when the plants were blooming. During the present study, the living plant samples have been maintained at the nurseries of Mahidol University in Nakhon Pathom, Thailand and the National Science Museum Arboretum (NSM Arboretum) in Pathum Thani, Thailand. Some replicates of the living specimens have been grown at the Ginger collection nursery in Queen Sirikit Botanic Garden (QSBG), Chiang Mai, Thailand. The species identification was based on the identification key to species according to the taxonomic revision in the Flora of Thailand (Jenjittikul & al. 2023) and the most recent publication (Nopporncharoenkul & Jenjittikul 2024; Nopporncharoenkul & al. 2024), using both floral and vegetative morphological characters, phenological character and distribution information. Protologues and herbarium specimens of species in the subgenus held at BK, BKF, QBG, SING and SLR herbaria (for herbarium abbreviations see Thiers 2023+) and several online herbarium specimen databases, especially the Kew Herbarium Catalogue (http://apps.kew.org/herbcat/navigator.do) and the Chinese Virtual Herbarium (CVH; https://www.cvh.ac.cn/) were also extensively ex-

amined. An undescribed species is provisionally named as *Kaempferia* sp. A distribution map of the accessions included in the present study is displayed as Fig. 1. The flowers of all species investigated are shown in Fig. 2. The specimens were prepared and deposited in the BKF, QBG or SLR herbaria (see Appendix S1 in Supplemental content online).

Mitotic chromosome study

Of the overall 88 accessions, 42 accessions representing 14 recognized species, one variety, and one undescribed species of *Kaempferia* subg. *Protanthium* were subjected to chromosome number investigation. The 2*n* chromosome number of each accession was analysed from at least 20 cells per plant, and three to five individual plants per accession. Mitotic chromosome preparation was performed using the enzymatic squash technique, according to the protocols of Mandáková & Lysak (2016) and Chow & al. (2020) with modifications. Actively growing root tips were excised from living plants, which were grown from rhizomes originally collected from their natural habitats and in cultivation. The root samples were immediately pre-treated with saturated *para*-dichlorobenzene solution at 4 °C for 16–18 hours in darkness. To stop all cellular activities and reactions, the samples were fixed in Farmer's fluid (3: 1 v/v of absolute ethanol: glacial acetic acid) at 4 °C for 10 min, then transferred and preserved in 70% ethanol at 4 °C until further use. To soften the root tips, the fixed roots were rinsed in a citrate buffer (4 mM citric acid monohydrate and 6 mM trisodium citrate dihydrate) at room temperature twice for 10 min, and then incubated in an enzyme mixture (citrate buffer with added 10% w/v of cellulase [Onozuka R10, Saint Louis, USA] and 12% v/v of pectinase [Sigma P-4716, Saint Louis, USA]) at 37 °C for 15–20 min. Afterward, each softened root tip was carefully rinsed with 45% acetic acid on a clean microscopic slide at room temperature twice for 2 min. The meristematic cells were gently separated in a drop of 45% acetic acid using dissecting needles and smeared in 2% w/v of aceto-orcein stain. Finally, the fine cell suspension was covered with a coverslip and tapped vertically with dissecting needles to squash the cells flat. The chromosomes were investigated at 1000× magnification under an Olympus CX23 light microscope (Tokyo, Japan). The spread chromosomes were photographed with an Olympus DP73 digital camera (Tokyo, Japan) attached to the microscope. The somatic chromosome number was determined from the well-spread chromosomes in metaphase cells.

Genome size estimation

Of the overall 88 accessions, 84 accessions (276 individual plants) representing 14 recognized species, one variety and one unidentified taxon were included in the present genome size analysis. In order to obtain the intraspecific variation of genome size, the samples analysed were obtained from at least three accessions (different geographical locations) per species, and one to five individual plants were subjected and analysed for each accession. Each individual plant was re-analysed three times on different days. However, data for species with a small population distributed in a restricted area, including *Kaempferia caespitosa*, *K. grandifolia*, *K. sipraiana* and *Kaempferia* sp., were obtained from only one to two populations (Table 1). Leaf samples were harvested from living plants and immediately used for analysis on the same day. The fresh young, unfolded leaves without diseases and pests were selected for analyses. In this study, *Glycine max* (L.) Merr. cv. Polanka (obtained from the Institute of Experimental Botany, Olomouc, Czech Republic, 2C = 2.5 pg: Doležel & al. 2007) and *Musa serpentina* Swangpol & Somana 'SS&JS 246 clone' (2C = 1.36 pg: Rotchanapreeda & al. 2016) were used as the reference standards (Moonkaew & al. 2020).

Genome size (2C value) was estimated using propidium iodide flow cytometry, according to the two-step protocol described by Doležel & al. (2007), with minor modifications. For nuclei extraction, the leaves of both sample and standard were concurrently chopped using a new sharp razor blade in a petri dish with 1 ml of fresh ice-cold Otto's nuclear-isolation buffer I (0.1 M citric acid and 0.5% Tween 20). The homogenate nuclei suspension was mixed by pipetting and then filtered through a 42-µm nylon mesh. The nuclei were pelleted by centrifugation at 3500 rpm for 5 min, and the supernatant was carefully removed. Afterward, the nuclear pellet was resuspended in 200 µl of ice-cold Otto's buffer I by gentle shaking. Thereafter, 400 µl of Otto II solution (0.4 M disodium hydrogenphosphate) supplemented with 50 µg/ml of propidium iodide (PI), 50 µg/ml of RNase A and 2 µl/ml of β-mercaptoethanol was applied to each sample tube with nuclei suspension in Otto's buffer I. The nuclei suspension was subsequently incubated at room temperature for 30 min in the dark. Each sample was analysed using the BD FACSCalibur Flow Cytometer (BD Biosciences, California, USA). Histograms of the relation between PI fluorescence intensity (PI-A, X-axis) and number of nuclei (event, Y-axis) were generated and 2C peaks of sample and standard were gated with a coefficient of variation lower than 3% using the BD FACSDiva version 6.1.1 software (BD Biosciences, California, USA). The estimated genome size was calculated using the linear relationship between the fluorescent intensity from stained nuclei of sample and internal standard, according to the following formula: Genome size of sample (pg) = (sample G0/G1 mean peak/reference standard G0/G1 mean peak) × Standard genome size (pg). In addition, the putative ploidy levels of the accessions which were excluded in chromosome analysis were inferred based on comparison of the genome sizes to those of the accessions obtained with both genome sizes and chromosome counts.

Fig. 2. Floral morphology of *Kaempferia* subg. *Protanthium* in Thailand – A: *K. albiflora* (*NNSB-546*); B: *K. aurora* (*NNSB-713*); C: *K. caespitosa* (*NNSB-733*); D: *K. calcicola* (*NNSB-903*); E: *K. graminifolia* (*NNSB-686*); F: *K. grandifolia* (*NNSB-519*); G: *K. jenjittikuliae* (*NNSB-760*); H: *K. lopburiensis* (*NNSB-541*); I: *K. noctiflora* var. *noctiflora* (*NNSB-554*); J: *K. noctiflora* var. *thepthepae* (*NNSB-928*); K: *K. rotunda* (*NNSB-534*); L: *K. simaoensis* (*NNSB-676*); M: *K. sipraiana* (*NNSB-656*); N: *K. subglobosa* (*NNSB-749*); O: *K. takensis* (*NNSB-697*); P: *K. udonensis* (*NNSB-508*). – All photographs by N. Nopporncharoenkul.

Species description

The morphological and phenological characters of an undescribed species were investigated, measured, photographed and described from the living specimens in its natural habitat and from material cultivated in the nursery of NSM Arboretum. The morphological terminology used in the species description followed Beentje (2016). The diagnostic characters were discussed in relation to the morphologically closest similar species. The conservation status was assessed following the guidance to the IUCN Red List Categories and Criteria, version 15.1 (IUCN Standards and Petitions Subcommittee 2022). The extent of occurrence (EOO) and area of occupancy (AOO) were calculated using GeoCAT (Bachman & al. 2011). Type specimens with duplicates were prepared and will be deposited in the BK, BKF, QBG, SLR and SING herbaria.

Statistical analysis

The genome size data were analysed using descriptive statistics (mean ± standard deviation), Kolmogorov-Smirnov normality test (K-S test), and non-parametric statistical test, Kruskal-Wallis ANOVA, with the software IBM SPSS Statistics for Windows Version 21.0 (IBM Corp., New York, USA) (Ostertagová & al. 2014). Pairwise comparisons of species were also conducted using Kruskal-Wallis one-way ANOVA test at a significant level of p-value < 0.05 to test the difference in genome size among the species. In addition, box and dot plots of genome size variation in *Kaempferia* subg. *Protanthium* were also created using IBM SPSS Statistics version 21.0 (Spriestersbach & al. 2009; Sen & Yildirim 2022).

Results

Chromosome numbers

The mitotic chromosomes of all 14 recognized and one undescribed species belonging to *Kaempferia* subg. *Protanthium* from Thailand were successfully investigated. The 2*n* chromosome numbers were found to be 22, 33, and 44 (Table 1; Fig. 3). The chromosome results unequivocally clarified $x = 11$ as the base chromosome number of *K.* subg. *Protanthium*. All species investigated, including *Kaempferia* sp., were diploid ($2n = 2x = 22$) or included diploid accessions. Two species, *K. rotunda* and *K. takensis*, provided included with different ploidy levels. *Kaempferia rotunda* included diploid (Fig. 3K), triploid ($2n = 3x = 33$: Fig. 3L) and tetraploid ($2n = 4x = 44$: Fig. 3M–N) accessions, whereas *K. takensis* included diploid (Fig. 3R) together with tetraploid (Fig. 3S) accessions. None of the species examined in the present chromosome study substantiated presence of aneuploid.

Genome size variation

In the present genome size study, we thoroughly examined 84 accessions of the species belonging to the *Kaempferia* subg. *Protanthium* collected from Thailand and Laos. The mean genome size with the standard deviation (S.D.) of each accession were summarized in Table 1, while those of individual plants analysed were reported in Appendix S2. The putative ploidy levels of the accessions without chromosome number information were inferred based on comparison of the genome sizes with those of the accessions which were successfully clarified both genome sizes and *2n* chromosome numbers. Among the diploid accessions, the genome sizes of the subgenus were found to range from 3.687 ± 0.052 pg in *K. simaoensis* accession NNSB676 to 6.412 ± 0.070 pg in *K. albiflora* accession NNSB741. Surprisingly, *Kaempferia* sp. had the highest mean genome size of 6.255 ± 0.097 pg, although it was not statistically different from that of *K. albiflora* (Table 1). However, it should be noted that genome size of *Kaempferia* sp. was analysed in only five individual plants from a single accession only. The range of genome sizes of all species was analysed and performed in the box and dot plots as shown in Fig. 4, 5, respectively.

After initially testing the normal distribution of genome sizes using the K-S test, the genome sizes of several species were not normally distributed (see Appendix S3). Therefore, a non-parametric statistical test using Kruskal-Wallis one-way ANOVA was performed to analyse the differences between genome sizes of the studied species. The statistical result indicated significant difference between means of genome sizes of the studied species. Moreover, statistical pairwise comparisons were also reported to categorized group between the analysed species based on the estimated genome sizes (Fig. 4; Appendix S3).

Regarding the species representing accessions with different ploidy levels, the genome sizes of 17 accessions of *Kaempferia rotunda* collected from different geographic localities were extensively examined. The results displayed as the box and dot plots clearly revealed the significant difference in three ranges of genome size (Fig. 4, 5A). Consequently, three ploidy level ranges were assigned: diploid (range 4.071–4.296 pg: 7 accessions), triploid (range 6.787–7.156 pg: 3 accessions) and tetraploid (range 8.165–9.172 pg: 7 accessions). The mean genome sizes of diploids, triploids and tetraploids were 4.193, 6.983 and 8.543 pg, respectively. The mean genome size values of triploid and tetraploid were 1.67 and 2.04 times the diploid mean value. The increase in genome size of the tetraploid *K. rotunda* is linearly proportionate to the increase in ploidy level, while the triploids had an approximately 11% larger than expected genome.

Kaempferia takensis is another species representing different ploidy levels among the accessions investigated, and the box and dot plot analyses revealed discontinuous

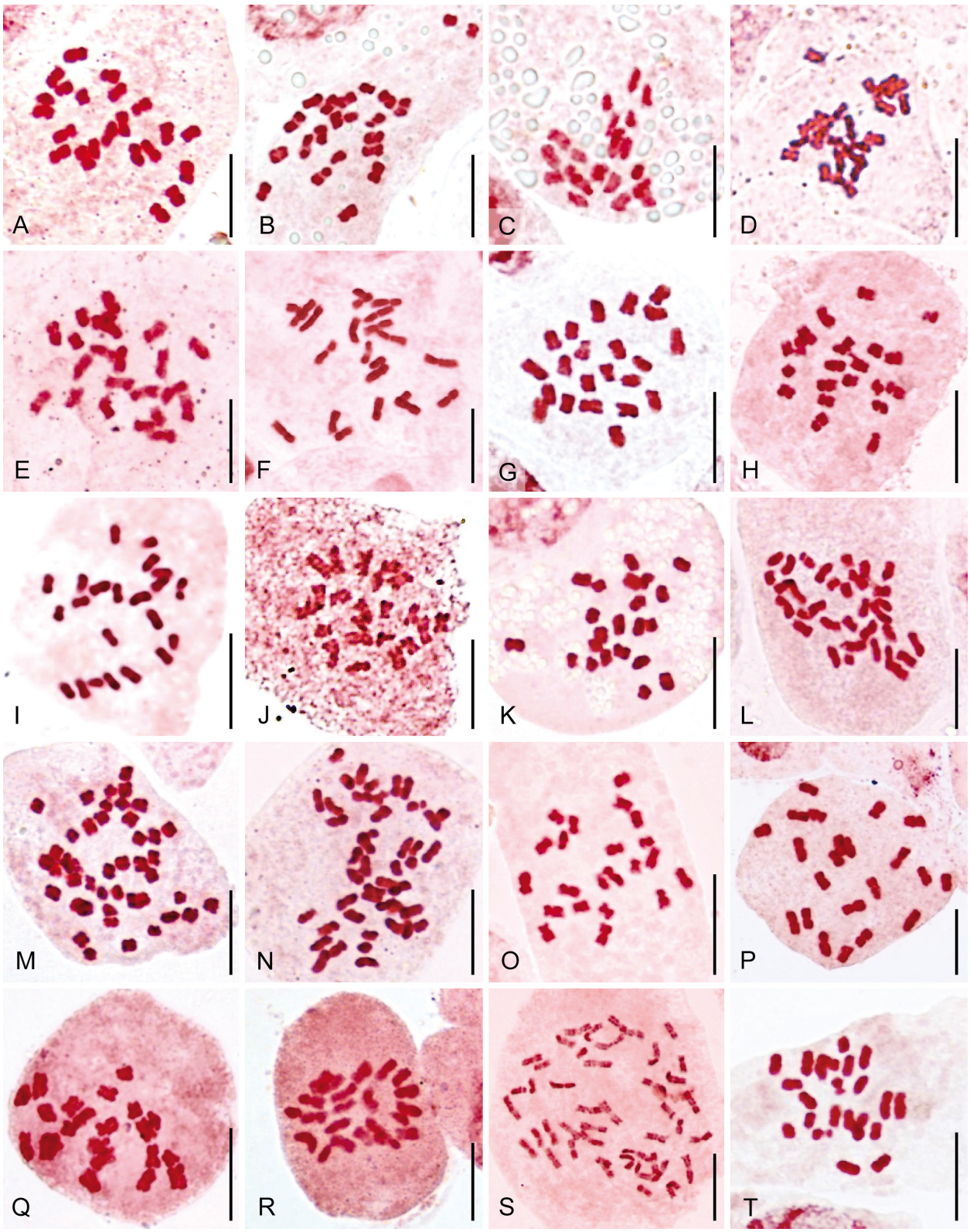

Fig. 3. Mitotic metaphase chromosomes of selected accessions of *Kaempferia* subg. *Protanthium* in Thailand – A: *K. albiflora*, 2*n* = 22 (*NNSB-634*); B: *K. aurora*, 2*n* = 22 (*NNSB-713*); C: *K. caespitosa*, 2*n* = 22 (*NNSB-733*); D: *K. calcicola*, 2*n* = 22 (*NNSB-903*); E: *K. graminifolia*, 2*n* = 22 (*NNSB-684*); F: *K. grandifolia*, 2*n* = 22 (*NNSB-519*); G: *K. jenjittikuliae*, 2*n* = 22 (*NNSB-836*); H: *K. lopburiensis*, 2*n* = 22 (*NNSB-335*); I: *K. noctiflora* var. *noctiflora*, 2*n* = 22 (*NNSB-640*); J: *K. noctiflora* var. *thepthepae*, 2*n* = 22 (*NNSB-928*); K: *K. rotunda*, 2*n* = 22 (*NNSB-567*); L: *K. rotunda*, 2*n* = 33 (*NNSB-629*); M: *K. rotunda*, 2*n* = 44 (*NNSB-642*); N: *K. rotunda*, 2*n* = 44 (*NNSB-703*); O: *K. simaoensis*, 2*n* = 22 (*NNSB-535*); P: *K. sipraiana*, 2*n* = 22 (*NNSB-656*); Q: *K. subglobosa*, 2*n* = 22 (*NNSB-749*); R: *K. takensis*, 2*n* = 22 (*NNSB-697*); S: *K. takensis*, 2*n* = 44 (*NNSB-524*); T: *K. udonensis*, 2*n* = 22 (*NNSB-752*). – Scale bars: A–T = 10 µm. – Photographs: A–C, E–I, K–T by N. Nopporncharoenkul; D, J by W. Sukseansri.

ranges of genome sizes (Fig. 4, 5C). The diploids had a range of 4.579–5.100 pg (9 accessions) and tetraploids exhibited a range of 7.959–8.341 pg (4 accessions). The mean genome size of the tetraploids was up to 1.7-fold larger than that of a diploid. The increase in genome size of tetraploid *K. takensis* is not in linearly proportionate to the increase in ploidy level, but intermediate between 1.5 times the theoretical diploid size in triploids and 2 times in tetraploids.

Identification of undescribed species

An undescribed species, *Kaempferia* sp., was found on the top of limestone hills and cliffs in Khon Kaen province, NE Thailand (Fig. 6). After intensive morphological study and comparison with protologues and herbarium specimens were conducted, we could not taxonomically identify this plant with any of the existing species belonging to *K.* subg. *Protanthium*. However, an undescribed species can be recognized and differentiated from the others by the combination of the following diagnostic characters: (1) well-developed pseudostems above ground, (2) elliptic, elliptic-oblong to lanceolate-oblong leaf blade with a long petiole, (3) flat type floral plane, consisting of horizontal to slightly arcuate lateral staminodes and labellum, which laid in the same plane and parallel to the ground, (4) bilobed labellum with an incision c. 3/5 of its length, (5) an involute labellum base, loosely enclosing the anther, (6) a subsessile stamen with an extremely short filament and 3–4(–5) mm long anther thecae, (7) an obreniform, broadly ovate, obovate to obdeltoid anther crest with an irregular undulate to crenate apex and (8) the anther crest extends backward and positioned nearly perpendicular (c. 90 degree) to the anther connective.

Discussion and Conclusions

Taxonomy of *Kaempferia* subg. *Protanthium* in Thailand

Thailand is regarded as the biodiversity hotspot of the genus *Kaempferia* (Jenjittikul & al. 2023). The recent taxonomic studies revealed 14 accepted species and one variety belonging to *K.* subg. *Protanthium* distributed throughout Thailand (except the peninsular region), including 10 strictly endemic taxa (Nopporncharoenkul & Jenjittikul 2024; Nopporncharoenkul & al. 2024). However, the taxonomic circumscription of several recognized and unidentified taxa still remains unclear, leading to the problems with delimitation of *Kaempferia* species. Taxonomically, the presence of both inflorescences and leafy shoots is extremely necessary for accurate species-specific identification in the genus (Sirirugsa 1989). However, the reproductive and vegetative parts of the plants in *K.* subg. *Protanthium* cannot be observed at the same time (see introduction). Although the flowering period of the subgenus is generally from March to June, it is very short with only two to three weeks observed in each population. In addition, after the end of the flowering period, growth of the leafy shoot mostly coincides with the beginning of the rainy season, and the shoot dies off and goes into dormancy for several months during the dry season. Moreover, the vegetative part of *Kaempferia* is highly variable within the species, especially in the length of the petiole, leaf blade size and shape, the presence of the variegated patterns on the adaxial side of leaf blade, and the presence of indumentum. Furthermore, several species of *K.* subg. *Protanthium* imply morphological similarities of vegetative and reproductive parts among the species, even with other genera in *Zingiberaceae*, particularly *Boesenbergia* Kuntze and *Curcuma* L. (Larsen & Larsen 2006; Techaprasan & al. 2010). For example, the leafy shoot (up to 80 cm tall) of *K. simaoensis*, consisting of a prominent, well-developed pseudostem, long petioles (up to 15 cm long) and lanceolate, elliptic to ovate leaf blades (up to 50 by 30 cm), usually slightly plicate and sometimes with a red to purplish red patch along the midrib adaxially (Jenjittikul & al. 2023), is morphologically similar to that of several species of *Boesenbergia* and *Curcuma*. Regarding within *K.* subg. *Protanthium*, *K. simaoensis* collected in Thailand had previously been recognized as a variation of *K. rotunda* (referred to *K. rotunda* accession TT15732 and TT16426, Techaprasan & al. 2010) owing to sharing the similarity in both leafy shoot and inflorescence characters. The morphological diversifications distinguishing between the species can be found in the anther crest and the presence of two prominent yellow bands on the labellum base toward incision. However, the taxonomic status of *K. simaoensis* was clarified and subsequently recognized as another species based on molecular phylogenetic analysis of ITS2 sequences (Nopporncharoenkul & al. 2016). According to the ambiguity in morphological and phenological variations, it is extremely difficult to identify or differentiate the species based on the investigation of morphological characters alone, especially in the absence of inflorescences.

The predominantly floral morphological characteristics for accurate identification of the species in *Kaempferia* subg. *Protanthium* include the floral plane type, the colouration and incision depth of the labellum, the length of the filament, and anther crest shape and size. Regarding the floral plane, two main types are classified for the genus *Kaempferia*, namely perpendicular type and horizontal (flat) type (Nopporncharoenkul & al. 2021). The flowers representing the perpendicular type are characterized by having upright to slightly arcuate lateral staminodes and a deflexed in distal half labellum. The labellum base is often flat and an incision is around or less than 1/2 of the labellum length. A filament is noticeable and flat. On the other hand, the flowers with the flat floral plane type (T shape formed) are characterized by having horizontal to slightly arcuate lateral stamin-

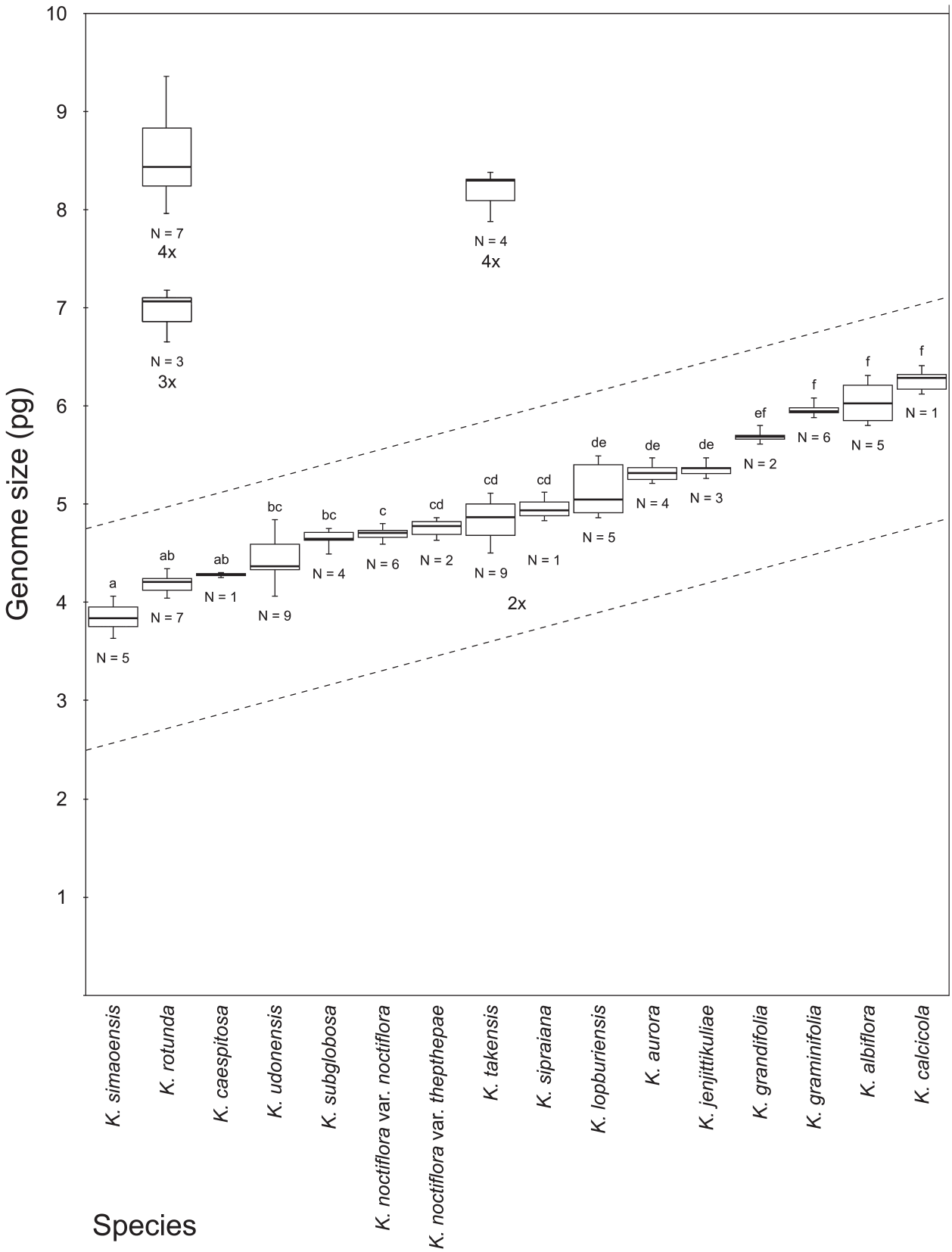

Fig. 4. Boxplot representing range of genome sizes (in picograms) of species of *Kaempferia* subg. *Protanthium* in Thailand based on 84 accessions (276 individual plants) from 15 species and one variety. Lines extending from boxes (whiskers) indicate variability outside upper and lower quartiles. Different letters above each box indicate statistically significant difference between means of genome sizes ($p < 0.05$). – N = number of accessions included in flow cytometry analysis. – 2*x* = diploid; 3*x* = triploid; 4*x* = tetraploid. Mean genome size with standard deviation data of individual plants follows Appendix S2.

odes and labellum, which are arranged in the same plane and usually parallel to the ground. The labellum base is conspicuously involute, tightly enclosing the anther connective and thecae. A labellum incision is around or more than 2/3 of its length. The stamen of the flat floral type is mostly subsessile with an extremely short filament (Nopporncharoenkul & al. 2021). Additionally, the presence of glandular hairs on the filament and anther connective is another floral characteristic, supporting taxonomic differentiation among the species sharing the close similarity in the flowers (Nopporncharoenkul & al. 2024). Also, anthesis time, referred to the period during which a flower is fully open and functional, is a phenological characteristic classifying *Kaempferia* into two distinct groups (Nopporncharoenkul & Jenjittikul 2017; Jenjittikul & Ruchisansakun 2020): nyctanthous (nocturnal anthesis) and hemeranthous (diurnal anthesis). Although anthesis time is considered as a species-specific phenological character, the most recent study unveiled that *K. noctiflora* can produce both nocturnal and diurnal flowers (Nopporncharoenkul & al. 2024). Biogeographically, *K. noctiflora* var. *noctiflora* and *K. noctiflora* var. *thepthepae* are endemic to Chiang Mai province of N Thailand, but their populations are distributed allopatrically. However, both varieties can be differentiated based on anthesis time and the colouration on the labellum. Remarkably, *K. noctiflora* var. *noctiflora* produces the nocturnal (night-blooming) flowers with a white labellum having a pale yellow patch on the labellum base toward incision. Conversely, *K. noctiflora* var. *thepthepae* represents the diurnal flowers, which start to open in the morning (around 6 a.m.) and wither around noon. The labellum of *K. noctiflora* var. *thepthepae* is white to pale light purple labellum with the central white to cream white patch basally surrounded by two light purple stripes from base toward centre of lobes (Nopporncharoenkul & al. 2024).

In this study, an undescribed species (*Kaempferia* sp.) was found in Khon Kaen province, NE Thailand. It was morphologically classified into the species producing the diurnal flowers with the flat type floral plane. The flowers consist of white to pale light pink lateral staminodes and a white to pale light pink labellum with the central white to cream white patch basally surrounded by two light pink to pale purple stripes from base toward centre of lobes, resembling those of *K. lopburiensis* and *K. takensis* (Picheansoonthon 2010; Boonma & al. 2020; Jenjittikul & al. 2023). The anther connective and filament are glabrous dorsally and laterally, as also observed in *K. rotunda* and *K. takensis* (Jenjittikul & al. 2023; Nopporncharoenkul & al. 2024). The anther crest is remarkable large, obreniform, broadly ovate, obovate to obdeltoid in shape with an irregular undulate to crenate apex, extending backward and positioned nearly perpendicular to the connective, which is similar to that of *K. lopburiensis* and *K. udonensis* (Picheansoonthon 2010; Phokham & al. 2013; Jenjittikul & al. 2023). Considering the leafy shoots, an undescribed species has a prominent, well-developed pseudostem, long petioles and elliptic, elliptic-oblong to lanceolate-oblong leaf blades that resembled those of *K. rotunda* and *K. takensis* (Boonma & al. 2020; Jenjittikul & al. 2023). As mentioned above, the species having the flat type floral plane represent the labellum with an incision around or more than 2/3 of its length. Interestingly, the labellum incision of an undescribed species is approximately 3/5 of the labellum length. In addition, the labellum base of an undescribed species is slightly involute and loosely encloses the anther connective and thecae. According to the morphological characters, we could not taxonomically identify it with any of the existing species, suggesting that it could be recognized as another species new to science. The morphological characters of an undescribed species are clearly compared and discussed below with the morphologically closest alliances, *K. lopburiensis*, *K. rotunda* and *K. takensis* and also shown in Table 2.

Chromosome number and genome size variation

In this study, we conducted extensive characterization of cytogenetic characters, including the 2*n* chromosome number and genome size, in order to better understand the species circumscription and support the taxonomic status of the species belonging to *Kaempferia* subg. *Protanthium*. The study included 88 accessions belonging to 14 recognized species, one variety, and one undescribed species from various regions throughout Thailand, except the peninsular part. Regarding the accessions analysed, 84 and 42 accessions were subjected to flow cytometry and chromosome investigation, respectively. The 2*n* chromosome numbers of the species in *K.* subg. *Protanthium* apart from *K. grandifolia* and *K. rotunda* were reported here for the first time, varying from 2*n* = 22, 33 to 44. In those species for which chromosome numbers had been determined previously, in the present cytogenetic analyses we obtained chromosome numbers in plant materials from other geographical locations. According to the mitotic chromosome results, the somatic chromosome 2*n* = 22 was observed in all species analysed, including *K. grandifolia* which is congruent with the number reported in the previous study (Saensouk & Jenjittikul 2001). Regarding *K. rotunda*, the 2*n* chromosome numbers revealed in the present mitotic analysis well agreed with those from the previous studies: 2*n* = 22 (Saensouk & al. 1999; Chandrmai & al. 2012; Nopporncharoenkul & al. 2017; Saensouk & Saensouk 2021b; Saensouk & al. 2023), 2*n* = 33 (Chakravorti 1948; Mahanty 1970; Eksomtramage & Boontum 1995; Nopporncharoenkul & al. 2017), and 2*n* = 44 (Ramachandran 1969; Omanakumari & Mathew 1984; Sadhu & al. 2016). However, we did not detect aneuploid number among 42 accessions included in the present chromosome analysis, but other previous studies published 2*n* = 30 and 2*n* = 54 from materials collected from Thailand (Saenprom & al. 2018) and India (Raghavan & Venkatasubban 1943), respectively. The

unusual chromosome numbers may have originated from unbalanced gametes through irregular chromosome segregation during unequal meiotic division of the odd ploidy levels. However, some unbalanced gametes can take part in fertilization to produce aneuploid progeny (Wang & al. 2017). With the present and previous cytogenetic results, it is possible to hypothesize that the somatic chromosome numbers $2n = 30$ and 54 have possibly arisen from the triploid ($2n = 3x = 33$) and pentaploid ($2n = 5x = 55$) ancestors with some chromosome eliminations, suggesting $3x - 3$ and $5x - 1$ respectively.

During the mitotic chromosome analysis, we did not measure the chromosome length because high fluctuation in chromosome size was observed from the cells of root tips collected from the individual plant. Variation in chromosome size may have resulted from the patterns of chromatin condensation, varying from the different responses of the meristematic tissue in each root material during the pretreated step (Pitaktharm & al. 2024). As the length of chromosome depends on several uncontrollable factors, we therefore extensively analysed the genome sizes of the samples, which reflect the correlation with the chromosome numbers and morphology and ploidy levels over evolutionary time, using flow cytometry. In addition, the putative ploidy levels of the accessions were inferred based on comparison of the genome sizes to those of the accessions obtained with both genome sizes and chromosome numbers. In the present study, we uncovered the genome sizes of the species of *Kaempferia* subg. *Protanthium*, except *K. rotunda*, for the first time. The combined results from mitotic and genome size analyses indicate that 74 accessions from all analysed species are putative diploid, whereas the others are putative polyploid, including putative triploid (three accessions) and putative tetraploid (11 accessions). The cytogenetic evidence clearly indicates that diploids with $2n = 2x = 22$ predominate as the most common in *K.* subg. *Protanthium*, which is congruent with the previous cytogenetic analyses in *K.* subg. *Kaempferia* (Nopporncharoenkul & al. 2017). The genome sizes among the diploid accessions ranged from 3.687 ± 0.052 pg in *K. simaoensis* accession NNSB676 to 6.412 ± 0.070 pg in *K. albiflora* accession NNSB741. A 1.74-fold range in genome size was observed among the diploid species having $2n = 22$ of *K.* subg. *Protanthium* analysed here.

Interestingly, high intraspecific variation in genome size among the diploid accessions was obtained from several species, especially in *Kaempferia albiflora*, *K. lopburiensis*, *K. takensis* and *K. udonensis* (Fig. 4). Regarding *K. takensis*, the genome sizes of the species were found to range from 4.579–5.100 pg with an approximately 11.4% variation. According to the geographic distribution, *K. takensis* could be classified into two populations: Chiang Rai province population and other provinces (type) population (Fig. 5F). The plants imply morphological overlap among the populations. Notably, the accessions belonging to the type population generally produce pale light pink flowers with two deep pink to light reddish spots at the centre of the labellum (see Fig. 2O), which is the same colour as the flowers of the plants in the type locality (Boonma & al. 2020). Although the plants collected from Chiang Rai province also produce pale pink to pale light purple flowers, but they have two purple to deep purple marks at the centre of the labellum which resemble the flowers of *K. xiengkhouangensis* in Laos (Phokham & al. 2013). Interestingly, the collecting sites of accessions in Chiang Rai province are the same latitude as the type locality of *K. xiengkhouangensis*, but they are c. 550 km apart. The morphological differences between *K. takensis* and *K. xiengkhouangensis* are found only in the length of petiole and the presence of variegated patterns on the leaf blade adaxially. *Kaempferia takensis* has the leaves with the prominent petioles (up to 5 cm long) and usually represents the variegated patterns on the leaf blade adaxially (Boonma & al. 2020; Jenjittikul & al. 2023), whereas *K. xiengkhouangensis* has sessile green leaves (Phokham & al. 2013). As the plants distributed in Chiang Rai province represent the petiolate leaves while *K. xiengkhouangensis* has not been reported in Thailand, we therefore provisionally identified the accessions collected from Chiang Rai province as *K. takensis* (referred to accessions NNSB531 and NNSB696, Table 1). Cytogenetically, the genome sizes (4.579–4.649 pg) of *K. takensis* accessions collected from Chiang Rai population were smaller, but not statistically significant, than those of the type population (4.742–5.100 pg) (Fig. 5E). With the present genome size and geographic distribution data it is possible to postulate that intraspecific variation in genome size between the geographically distant populations of *K. takensis* may have resulted from either (1) the difference in genome structure through the divergent evolutionary processes, such as mutations, natural selection, genetic drift, genetic hitchhiking and/or gene flow, in each individual population or (2) the cryptic species may be included within the accessions in the present study. However, *K. xiengkhouangensis* from the type locality in Laos was not included in the present cytogenetic analyses. The species is therefore extremely necessary and will be subjected to further studies in order to clarify the species circumscription of the *K. takensis* complex.

Besides *Kaempferia takensis*, *K. udonensis* also provided high variation in genome size with an approximately 19.4% (range 4.057–4.844 pg). The species could be classified into two populations: a northeastern (NE) population and a southwestern (SW) population, based on the distinct collecting sites (Fig. 5H). Although the plants from both populations are distributed allopatrically, the plants do not only grow in the same habitat type of a mixed deciduous forest usually with bamboos, but also represent the same morphological characters. Interestingly, *K. udonensis* collected from NE Thailand displayed the larger genome sizes with no significant than those of the accessions from SW Thailand (Fig. 5G).

According to intraspecific genome size variation observed in *K. udonensis*, we imply that the plants which are distributed in severely fragmented areas have been precluded opportunities for gene flow between genetically distant populations by geographic discontinuities, contributing to high genetic difference between the populations (Choudhuri 2014). Consequently, the reason can also explain for the occurrences of intraspecific variation in genome sizes of *K. albiflora* and *K. lopburiensis* as the populations have been observed in severely fragmented localities geographically.

Although the underlying evolutionary mechanisms involving intraspecific variation in genome size of genus *Kaempferia* remain unknown, we propose that it may be influenced by variation in heterochromatin levels and chromosome sizes via chromosomal rearrangements, duplications, deletions or translocations through retrotransposon or repetitive DNA element expansion, which play an important role in plant adaptation (Ortiz-Barrientos & al. 2016). Actually, variation in chromatin levels generally exists at the diploid level in several plants, for example, 1.7-fold in *Cirsium* Mill. (Bureš & al. 2004), 2.8-fold in *Streptocarpus* Lindl. (Möller 2018), and 4-fold in *Lactuca* L. (Doležalová & al. 2002) and *Trifolium* L. (Vižintin & Bohanec 2008). In addition, genome size variation within the ploidy level is also associated with evolutionary constraints on plant development, phenology or ecological performance (Vesely & al. 2012; Greilhuber & Leitch 2013). The recent study on the correlation between genome size and habitat type of the plants belonging to subfamily *Zingiberoideae* with dormancy period revealed that the species having small genome sizes tend to be more frequent in dry habitats since they enable faster growth, which is important especially at the beginning of rainy season. Conversely, the species which exist in shady habitats have significantly larger genome sizes than those occurring in full sun to partial shaded areas (Záveská & al. 2024). Furthermore, a sexual reproduction through seed production has been recognized as the mechanism for maintaining high genetic diversity within the species. In family *Zingiberaceae*, Záveská & al. (2011) revealed that diversity of Nei's gene in sexually-reproducing diploid *Curcuma* is significantly greater than in vegetatively-reproducing taxa. During the present study, we found all diploid accessions analysed were fully fertile as good seed sets have been observed in natural habitats and/or in cultivation. Consequently, the viable seed producing evidence is considered as one of the factors resulting intraspecific morphological and genome size variation in several *Kaempferia* species.

Polyploidy

Polyploidy plays a crucial role in plant evolution and speciation (De Storme & Mason 2014). In genus *Kaempferia*, polyploidy has been continually reported (Chakravorti 1948; Ramachandran 1969; Mahanty 1970; Omanakumari & Mathew 1984; Eksomtramage & Boontum 1995; Sadhu & al. 2016; Nopporncharoenkul & al. 2017; Saenprom & al. 2018; Záveská & al. 2024) while the first chromosome number evidence of polyploids, *K. galanga* ($2n = 54$) and *K. rotunda* ($2n = 54$) from India, was published by Raghavan & Venkatasubban (1943). A comprehensive chromosome number investigation of *K.* subg. *Kaempferia* from Thailand and Laos revealed ploidy variation within the species, ranged from diploid ($2x$), triploid ($3x$), tetraploid ($4x$) to pentaploid ($5x$) (Nopporncharoenkul & al. 2017). The present cytogenetic study of *K.* subg. *Protanthium* also unveiled polyploidies in two species, namely *K. rotunda* and *K. takensis*, whereas other species investigated were diploidy. Regarding *K. rotunda*, the investigated accessions can be classified into three ploidies based on the $2n$ chromosome numbers: diploid ($2n = 22$), triploid ($2n = 33$) and tetraploid ($2n = 44$), which are congruent with the number reported in the previous cytogenetic study (Nopporncharoenkul & al. 2017). However, we did not encounter any pentaploid *K. rotunda*, which was previously reported by Raghavan & Venkatasubban (1943) based on plant samples from India. The present genome sizes of *K. rotunda* are mostly consistent with those of the previous studies, although some discrepancies occur. In particular, the estimated genome sizes of diploid *K. rotunda* (4.071–4.296 pg) fit well into the previous diploid range of 3.468–4.43 pg reported by Chandrmai & al. (2012) and Basak & al. (2018), as well as the genome sizes of triploid *K. rotunda* (6.787–7.156 pg) which fully support the range (6.307–7.291 pg) revealed in the most recent study of Záveská & al. (2024). However, genome sizes of tetraploid *K. rotunda* accessions (8.165–9.172 pg) obtained in the present study were greater than those of tetraploid materials in the previous genome size report (7.45 pg, $2n = 44$: Sadhu & al. 2016). Although the evolutionary causes involving in high intraspecific variation in genome size of *K. rotunda* are the topic of ongoing debate and still remain unclear, we propose that the difference in genome size at the same ploidy level (1) may result from disturbing effects of phenolic compounds from the leaf samples, such as deep purple pigments from the leaf blades abaxially (Jenjittikul & al. 2023) or secondary metabolites of plant materials with potential seasonal fluctuation (Walker & al. 2006); (2) may be influenced by the differences in measurements among different laboratories and protocols, and/or errors of instruments and methodologies (Doležel & al. 1998); (3) may potentially refer to chromosomal heterogeneity (aneuploidy) and/or variation in repetitive elements (non-coding regions) through evolutionary time of the plants distributed in geographic discontinuity as discussed before; or (4) may be mistaken from the taxonomic heterogeneity of plant materials analysed because *K. rotunda* provides a high degree of intraspecific morphological variation and also implies morphological overlap among *Kaempferia* species (Jenjittikul & al. 2023), possibly contributing to misidentification between *K. rotunda* and the cryptic species.

In triploid *Kaempferia rotunda*, three analysed accessions having the same chromosome number of $2n$ = 33 represented genome size ranged of 6.787–7.156 pg with c. 5.4% intraspecific variation. Interestingly, the triploid plants had an approximately 11% larger than expected genome due to genome increasing of 1.67-fold compared to the diploids. The genome size expansion in polyploids can be mostly explained by duplications of repetitive elements, such as heterochromatin, microsatellites and retrotransposon expansion, which are less likely to cause phenotypic changes (Blommaert 2020). Morphologically, the leafy shoots and inflorescences of both diploid and triploid *K. rotunda* are very similar to each other. Notably, the diploid *K. rotunda* generally produces a short, ovoid to subglobose rhizome with a single leafy shoot, whereas the triploid plants colonize via a clump of large moniliform-like rhizomes usually with several leafy shoots. Plausibly, genome expansion and intraspecific genome size variation in triploids may be related to a long-term cultivation, due to the plant improvement purposes, such as targeted selection of desirable external features and massive production of high chemical amounts in their rhizomes (Leong-Škorničková & al. 2007). Actually, the triploid *K. rotunda* has been cultivated commercially and commonly sold as traditional herb and attractive ornamental plant in plant markets throughout Thailand, referring to accession NNSB166 in Nopporncharoenkul & al. (2017) and accession NNSB602 in the present study originally obtained from the plant markets in Thailand. Moreover, the plants have been widely cultivated and used in several countries in S and SE Asia, which are concordant with the triploid materials obtained from India and Laos detected in the previous genome and/or chromosome studies (Chakravorti 1948; Mahanty 1970; Záveská & al. 2024). Unsurprisingly, no fertile seed was found in all triploid *K. rotunda* accessions during the present study, implying that triploid plants are not expected to be sexually fertile. Fundamentally, triploid *K. rotunda* having chromosome number $2n$ = 33 tends to generate unbalanced gametes owing to abnormalities in meiotic chromosome pairing. In *Zingiberaceae*, the irregularities in meiotic configuration comprising an assortment of univalents, bivalents and/or trivalents were observed in several previous meiotic studies of triploid *Kaempferia* (i.e. *K. elegans*, n = 11III: Nopporncharoenkul & al. 2017) and *Curcuma* (i.e. *C. comosa* Roxb., n = 21III and *C. latifolia* Roscoe, nearly regular synapsis: Puangpairote & al. 2015). The meiotic figure evidences most likely indicate that the triploid plants in family *Zingiberaceae* scarcely produce a fertile seed. However, they predominantly reproduce vegetatively by expansion and fragmentation of rhizomes. The producing of bigger rhizomes of triploid plants may indicate that they can store more nutrient, water and secondary metabolites, allowing more effective survival during a dormant period of dry season (Leong-Škorničková & al. 2007; Puangpairote & al. 2015).

Regarding tetraploid *Kaempferia rotunda* with chromosome number $2n$ = 44, the analysed accessions provided an approximately 12.3% variation in genome size (range 8.165–9.172 pg). The mean genome size was 2.04-fold compared the diploid mean, indicating that the increase in genome size is linearly proportionate to the increase in ploidy level. Accordingly, the present cytogenetic evidences imply that analysed plants are recent autotetraploids, displaying the complete whole genome duplication through polyploidization process, without genome downsizing observed (Möller 2018). The autotetraploid could be generated by three potential pathways (Ramsey & Schemske 1998): (1) the union of unreduced gametes ($n = 2x$) of diploid progenitors, (2) the union of reduced gametes ($n = x$) of diploids followed by chromosome doubling, and (3) the union of reduced and unreduced gametes to generate triploids ($2n = 3x$) and subsequently backcrossing to diploids or crossing to triploids. However, the pathway passing the triploid bridge seems to rarely occur in nature as the coexistence between triploid and tetraploid *K. rotunda* within the same populations was not encountered during the study. Geographically, tetraploid plants have been found only in N Thailand, whereas the diploids are widespread throughout SW and C Thailand (Fig. 5B). Consequently, it can be explained that after polyploidization, tetraploid *K. rotunda* has proceeded the physiological adaptation, survived in cooler and drier habitats, and distributed covering the entire areas in N Thailand. The adaptation in polyploid *K. rotunda* well agree with the previous reports that polyploid plants display a better adaptability to different ecological niches, increasing their chance for successful establishment through natural selection (Pelé & al. 2018; Van de Peer & al. 2021; Islam & al. 2022).

In tetraploid *Kaempferia takensis*, geographic distribution of tetraploid plants relates to distribution of the diploid populations, as discussed before (Fig. 5D). Accordingly, we classified tetraploid *K. takensis* into two populations: western and northern populations. Regarding the western population, the tetraploid *K. takensis* accession NNSB526–2 was collected from Kamphaeng Phet province which is the same locality as the diploid *K. takensis* accession NNSB526–1. The genome size of this tetraploid accession (range 8.315–8.362 pg, mean 8.331 ± 0.018 pg) was an approximately 1.7-fold compared the diploid *K. takensis* accessions from the type population (4.742–5.100 pg; Fig. 5F), indicating c. 15.3% genome downsizing. In the northern population, three accessions of tetraploid *K. takensis* were obtained from Phrae province of N Thailand. The genome size of tetraploid accessions from the northern population (range 7.883–8.387 pg, mean 8.152 ± 0.165 pg) was an approximately 1.77-fold compared the diploid accessions from the Chiang Rai population (4.579–4.649 pg; Fig. 5F), displaying an approximately 11.7% genome downsizing. Consequently, tetraploid accessions of *K. takensis* represent genome downsizing with 11.7–15.3% compared to their diploid

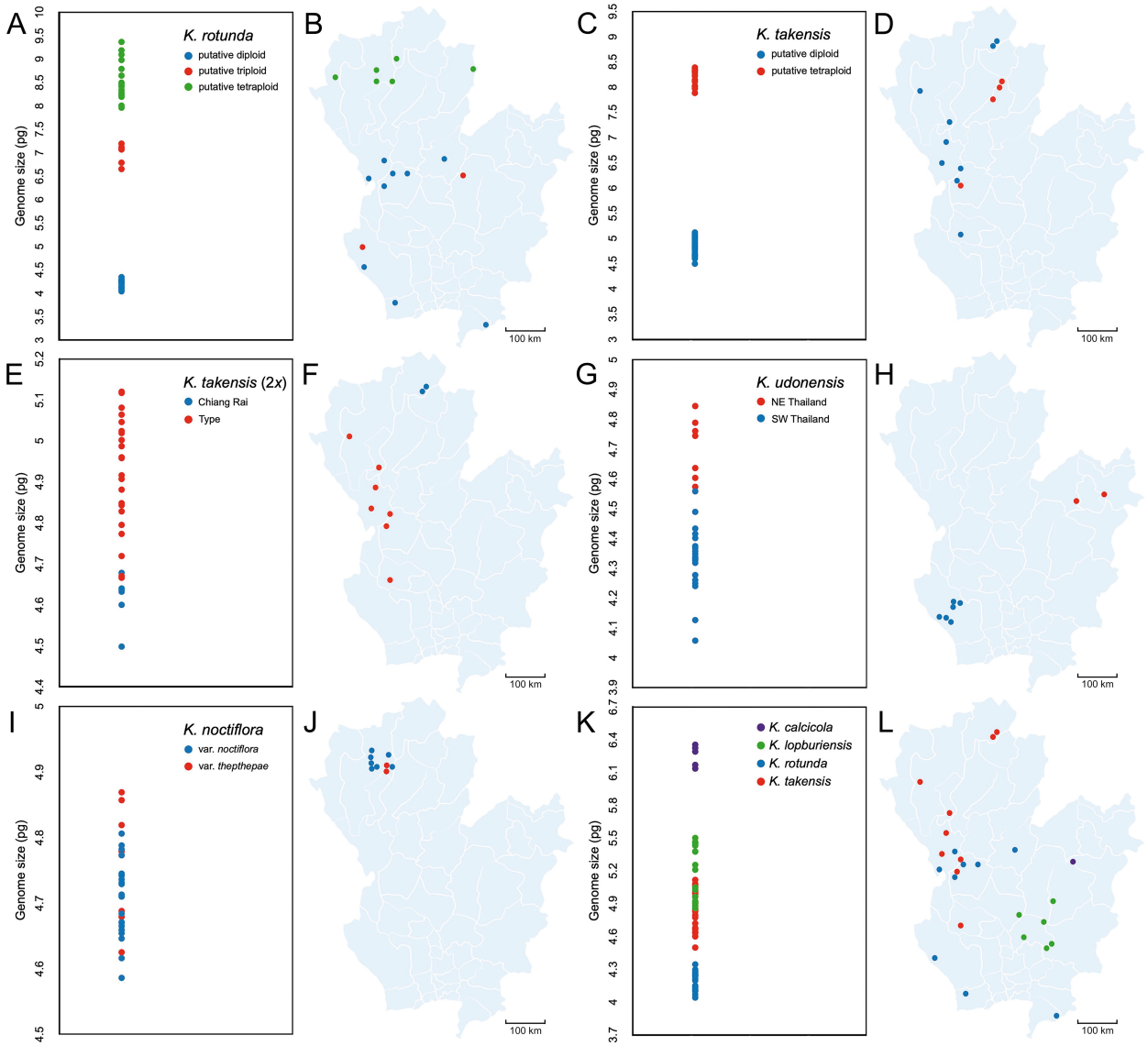

Fig. 5. Dot plots showing intra- or interspecific variation in genome sizes (A, C, E, G, I, K) and distribution maps of plant samples (B, D, F, H, J, L) – A, B: intraspecific variation of *Kaempferia rotunda*: diploid (blue dots), triploid (red dots) and tetraploid (green dots); C, D: intraspecific variation of *K. takensis*: diploid (blue dots) and tetraploid (red dots); E, F: intraspecific variation of diploid *K. takensis*: Chiang Rai population (blue dots) and Type population (red dots); G, H: intraspecific variation of *K. udonensis*: SW Thailand population (blue dots) and NE Thailand population (red dots); I, J: intraspecific variation of *K. noctiflora*: *K. noctiflora* var. *noctiflora* (blue dots) and *K. noctiflora* var. *thepthepae* (red dots); K, L: interspecific variation of *K. calcicola* and morphologically similar species: *K. rotunda* (blue dots), *K. takensis* (red dots), *K. lopburiensis* (green dots) and *K. calcicola* (purple dots). – A, C, E, G, I, K: each dot represents mean genome size of individual plant based on re-analysis three times. – B, D, F, H, J, L: each dot represents collecting site of individual accession.

relatives. During the study, all tetraploid accessions of *K. takensis* produce numerous viable seeds in both natural habitats and in cultivation, suggesting high fertility and productivity. Based on genome downsizing and viable seed setting evidences, tetraploid *K. takensis* could be postulated as either autotetraploid or allotetraploid which has been long evolutionary history and proceeded genome reorganization through the diploidization process. Genome downsizing in autopolyploids occurs rapidly at or in early polyploid generations after the polyploidization owing to genetic instability resulted from additivity of DNA amounts of the diploid progenitors (Eilam & al. 2010; Wang & al. 2021). In general, the recent autopolyploids usually show a high frequency of multivalent meiotic configuration and represent some degree of sterility, especially in unbalanced gametes (Parisod & al. 2010). After polyploidization, polyploid plants have proceeded genome reorganization through DNA elimination over evolutionary time until complete restoration of diploid-like behaviour, representing a high percentage of bivalents, via the diploidization process, contributing to genome downsizing in diploidized autopolyploids and allopolyploids (Eilam & al. 2010; Song & Chen 2015). Alternatively, massive DNA losses are more continu-

ous process over evolutionary time, such as limiting the damaging activity of repetitive DNA (Wang & al. 2021). The effect of genome downsizing is well established in numerous species of flowering plants. For example, a decrease in gene number and a 10–25% reduction in genome size were observed in *Triticum* spp. (Feldman & al. 1997), *Brassica napus* L. (Gaeta & al. 2007) and *Tragopogon* L. (Buggs & al. 2012), while a remarkable genome downsizing of up to 44.4% was detected in polyploid *Streptocarpus* Lindl. (Möller 2018).

However, the underlying evolutionary mechanisms involving intraspecific variation in genome size and polyploidization of *Kaempferia rotunda* and *K. takensis* still remain unclear. Further studies of meiotic configuration, male gamete chromosome and karyotype are necessary, as well as accessing additional polyploid materials throughout Thailand. These efforts will unveil and fully understand the mechanisms of genome origin and evolution in polyploids.

Cytogenetic characters support species circumscription

In the present study, the combined data of 2*n* chromosome number and genome size for taxonomic purposes seems to be rather limited owing to much overlap between the species (Fig. 4). Moreover, the limitation in plant materials (only one to three individual plants analysed for each accession or species) might be inexact intraspecific genome size variation in some species, particularly in *Kaempferia caespitosa* (only three plants analysed) and *K. sipraiana* (only three plants analysed). However, we reveal the difference in the range of genome sizes, together with 2*n* chromosome numbers, can be used as supportive taxonomic markers for understanding species circumscription and elucidating differences between some species. Notable cases are observed in the species differentiation among *K. rotunda* and other closely morphologically similar species. The study of Nopporncharoenkul & Jenjittikul (2018) described *K. graminifolia*, distinguishing it from *K. rotunda* by having linear grass-like to narrowly lanceolate-oblong leaf blades, usually less than 5 cm wide. Moreover, the floral morphological diversification is found in the anther crest shape. Remarkable, *K. graminifolia* has broadly obdeltoid to broadly obovate anther crest with a bifid to crenate-bifid apex, whereas *K. rotunda* represents ovate-oblong anther crest with a bilobed apex, usually with 2–3 small teeth between lobes (Nopporncharoenkul & al. 2024). According to the results obtained from the present cytogenetic analyses, *K. graminifolia* is diploid with 2*n* = 22 and represents the genome size ranged 5.927–6.084 pg, which is significantly higher than that of *K. rotunda* at the diploid level (range 4.071–4.296 pg). The cytogenetic evidence clarifies and strongly supports the taxonomic status of *K. graminifolia* that it is not a morphologically variation of *K. rotunda*.

Besides *Kaempferia graminifolia*, *K. aurora* also shares the morphological characters of both leafy shoot and inflorescence with *K. rotunda*. The obvious differences are the presence of the anther crest with a tridentate to undulate-truncate apex and 6–12 mm long epigynous glands in *K. aurora* (Nopporncharoenkul & al. 2020b), whereas *K. rotunda* has the anther crest with a bilobed apex and shorter epigynous glands (2–5 mm long). As mentioned previously in Nopporncharoenkul & al. (2020b), we applied the genome size data as a taxonomic marker for supporting the differentiation of *K. aurora* from *K. rotunda*. In this study, the genome size range of *K. aurora* (5.205–5.402 pg) with 2*n* = 22 is significantly higher than that measured in the diploid *K. rotunda*, supporting the taxonomic status and species circumscription of *K. aurora*.

Regarding *Kaempferia grandifolia*, it is endemic species to the area surrounded by the Phu Wiang mountains of Khon Kaen province, NE Thailand (Jenjittikul & al. 2023). Due to the previous molecular phylogenetic analyses of Techaprasan & al. (2010) and Nopporncharoenkul & al. (2016), the accessions of *K. grandifolia* were clustered among the *K. rotunda* accessions, suggesting both species are phylogenetically closely related. However, *K. grandifolia* and *K. rotunda* show morphological diversifications in both leafy shoot and flower characters. Apparently, *K. grandifolia* has orbicular, suborbicular to ovate leaf blades, often appressed to the ground, and produces nocturnal flowers with the flat type floral plane (Saensouk & Jenjittikul 2001). Conversely, *K. rotunda* has upright lanceolate-oblong, elliptic to ovate leaf blades and represents diurnal flowers with the perpendicular floral plane type (Jenjittikul & al. 2023). Our cytogenetic results unveil that the genome size (range 5.634–5.731 pg) of *K. grandifolia* is larger than that of diploid *K. rotunda* (range 4.071–4.296 pg), well supporting the taxonomic status of *K. grandifolia* which was differentiated from *K. rotunda* (Saensouk & Jenjittikul 2001).

As discussed previously, based on morphological and phenological characters, *Kaempferia noctiflora* is taxonomic classified into two varieties, namely *K. noctiflora* var. *noctiflora* and *K. noctiflora* var. *thepthepae* (Nopporncharoenkul & al. 2024). The present mitotic chromosome investigation uncovers that both varieties are diploid with 2*n* = 22. Moreover, the range of genome size of *K. noctiflora* var. *noctiflora* (4.616–4.777 pg) mostly overlaps with that observed in *K. noctiflora* var. *thepthepae* (4.625–4.869 pg) (Fig. 5I), possibly implying the closely related genomes. Consequently, these cytogenetic evidences support the taxonomic status of *K. noctiflora* var. *thepthepae* that it cannot be recognized as an individual species differentiating from *K. noctiflora* despite the morphological and phenological diversifications.

Interestingly, another notable case using cytogenetic characters for supporting the taxonomic status is found in an undescribed species. *Kaempferia* sp. is a diploid species with the somatic chromosome number 2*n* = 22,

which is the same as that obtained from the other diploid *Kaempferia* species (Nopporncharoenkul & al. 2017). Remarkably, the plants represent the highest mean genome size among the diploid accessions of the subgenus. Moreover, the range of genome size of this species (6.136–6.354 pg) does not overlap and is also higher than that of morphologically similar species: *K. rotunda* (4.071–4.296 pg), *K. takensis* (4.579–5.100 pg) and *K. lopburiensis* (4.884–5.445 pg) (Fig. 5K), supporting that it belongs to another species. On the basis of these findings, morphological characters (as discussed previously) together with cytogenetic evidence unequivocally clarify the taxonomic status of an undescribed species that it deserves recognition as a species new to science, which is taxonomically described below as *K. calcicola* Nopporch.

The present study emphasizes that characterization of cytogenetic characters, 2*n* chromosome number and genome size, is not crucial only in species discrimination between morphologically similar species but can also support the taxonomic description of new species. However, further studies focusing on integration of karyotype, genome size and molecular systematic analyses will be conducted, as well as accessing additional plant materials and populations covering geographic distribution throughout Asia in order to clearly understand the mechanisms involving in chromosomal and genome evolution and relationship of the species within *Kaempferia* subg. *Protanthium*.

Taxonomic treatment

Kaempferia calcicola Nopporch., **sp. nov.** – Fig. 6, 7.
Holotype: Thailand, Khon Kaen province, Phu Pha Man district, Na Fai, 347 m elevation, 27 Apr 2023 (in flower), *N. Nopporncharoenkul NNSB-903* (QBG including flowers preserved in spirit as part of a single specimen; isotypes: BK, BKF, SING, SLR).

Diagnosis — *Kaempferia calcicola* Nopporch. is similar to *K. takensis* Boonma & Seansouk in overall habit, inflorescence and floral plane, but differs by anther crest obreniform, broadly ovate, obovate to obdeltoid (vs anther crest oblong to ovate in *K. takensis*) with irregular undulate to crenate apex (vs bilobed to irregularly tridentate apex, usually with 2–3 small teeth between lobes in *K. takensis*) and labellum with an incision c. 3/5 of its length (vs labellum with an incision more than 2/3 of its length in *K. takensis*). It is also similar to *K. lopburiensis* Picheans. in inflorescence, floral plane and flower colour, but differs by prominent pseudostem upright above ground (vs pseudostem short and completely embedded in soil in *K. lopburiensis*), leaf blades elliptic, elliptic-oblong to lanceolate-oblong and longer petiole 3–18(–30) cm long (vs leaf blades suborbicular to ovate, adpressed on ground and subsessile petiole in *K. lopburiensis*).

Description — Rhizomatous herb, up to 75 cm tall. Rhizome ovoid to subglobose, 1.5–2.5(–3) cm long, 1–1.5(–2.3) cm in diam., brown externally, cream white internally, with short fascicled storage roots and terminal tubers; root stalk swollen, 0.4–1.8(–3.5) cm long, 0.2–0.3 cm in diam.; tubers fusiform to narrowly fusiform, 2–5.8(–9.5) cm long, 0.6–1.5 cm in diam. Leafy shoot with (4–)6–8 leaves; pseudostem upright, up to 25 cm tall; bladeless sheaths 2–3, up to 10 cm long, plain green to dull reddish, apex obtuse to mucronate, mucro c. 1 mm long, glabrous to sparsely villous; leaf sheaths plain green to dull reddish, glabrous to sparsely villous; ligule bilobed, partly overlapping when young, lobes rounded to triangular with rounded to obtuse apex, 4–7(–12) mm long, semi-translucent, white to reddish, glabrous to sparsely villous; petiole 3–18(–30) cm long (lower leaves shorter), plain green, glabrous to sparsely villous; leaf blade elliptic (usually found in first two leaves), elliptic-oblong to lanceolate-oblong, 20–35(–40) × (5–)6.5–15 cm, adaxially plain green to grey-green, sometimes with 1–2 layers of white to silvery patches arranged parallel along leaf edges (between midrib and both sides of edges), glabrous, abaxially plain green, sometimes deep purplish red, sparsely villous, base attenuate, obtuse to rounded, slightly oblique, margin entire, slightly undulate, apex mucronate, mucro c. 1 mm long. Inflorescence lateral, emerging from rhizome before leafy shoot, sheathing bracts 2, deltoid-ovate, 0.5–0.8 × 0.5–0.8 cm, apex mucronate to acute with densely villous, light green to reddish sparsely villous; peduncle (1.5–)3.8–7(–10) cm long, glabrous to sparsely villous; raceme fusiform-ovoid, 4–5.5 cm long, 0.8–2 cm in diam., composed of up to 30 bracts each supporting a single flower; bracts deltoid-ovate to lanceolate-ovate, 1.5–5 × 0.6–4 cm (outer bracts larger), apex mucronate to acute with densely villous, light green, reddish to purplish red, sparsely villous; bracteoles lanceolate-ovate, 1.5–2.8 × 0.6–1.2 cm, irregularly bifid with an incision 1–1.5 mm, apex mucronate to acute, hyaline, sparsely villous. Flowers 8–11 cm long, diurnal anthesis; floral plane flat type, consisting of horizontal to slightly arcuate lateral staminodes and labellum, which are laid in same plane and parallel to ground; calyx 3.5–5.8(–6.5) cm long, 0.4–0.6 cm in diam., with unilateral incision 1–1.8 cm long from apex, apex truncate to shallowly trilobed-crenate with 2 mucro-teeth, semi-translucent light green, sometimes with several reddish spots, sometimes densely villous at apex; flo-

Fig. 6 (right page). *Kaempferia calcicola*. – A: habitat with plants in flowering period; B: plant habit in flowering period; C: inflorescences and flowers in top view; D: habitat with plants in rainy season; E. plant habit in rainy season. – All photographs taken at type locality: Thailand, Khon Kaen Province, Phu Pha Man District, A: 27 Apr 2023; B, C: 6 Apr 2024; D, E: 29 Aug 2023, by N. Nopporncharoenkul.

ral tube 5.5–8(–9) cm long, 2–3 mm in diam., narrowly cylindric at base above ovary, narrowly funnel-shaped distally, white, glabrous externally and internally; dorsal corolla lobe lanceolate-oblong, 2.4–3.8 × (0.5–)0.6–0.8 cm, apex hooded, mucronate, mucro 2–3 mm long, concave, sometimes cochleate, white, glabrous; lateral corolla lobes lanceolate-oblong, 2–3.5 × 0.5–0.6(–0.7) cm, apex mucronate, mucro c. 1 mm long, concave, sometimes arcuate, white, glabrous; lateral staminodes obovate to elliptic-oblong, 2.6–3.7 × 1.3–2 cm, apex acute, obtuse to rounded, sometimes crenate to incised at apex, arcuate, white to pale light pink; labellum obdeltoid to obovate, 2.4–3.8 × (2–)2.4–3.2 cm, bilobed, with an incision c. 3/5 of labellum length, base slightly involute, loosely enclosing anther, lobes obovate, elliptic-oblong to suborbicular, 1.6–2.3 × 1.2–2 cm, apex acute, obtuse to obcordate, sometimes crenate to incised at apex, partly overlapping, arcuate, white to pale light pink with central white to cream white patch basally surrounded by two light pink to light purple stripes from base toward centre of lobes; stamen 7–9 mm long, subsessile; filament short, up to 1 mm long; anther 6–8 mm long including nearly perpendicular anther crest, connective tissue white, glabrous; anther crest extends backward with nearly perpendicular to anther, obreniform, broadly ovate, obovate to obdeltoid, 5–7.5(–11) × (6–)8–10 mm, apex irregular undulate to crenate; anther thecae 3–4(–5) × 1–1.2 mm, cream white, dehiscing along their entire length, pollen white to cream white; ovary cylindric, (4–)5–6 mm long, 2–3(–4) mm in diam., trilocular, cream white, glabrous, ovules numerous, placentation axile; epigynous glands 2, subulate, 6–7 mm long, cream white; style 5.8–8.5 cm long; stigma crateriform, 1.5–1.8 × 0.5 mm, ostiole ciliate. Fruit ovoid to subglobose, 1.8–2 cm long, 1–1.4 cm in diam., trilocular, light greenish yellow to purplish red, usually with several reddish to purplish red spots, glabrous, rarely sparsely villous at apex, with 9–22 seeds; seeds subglobose, prolate, obovoid to ovoid, 4.2–5.8 mm long, 2.6–3.5(–4.5) mm in diam., yellowish cream to light brown with reddish spots, enclosed in a fleshy semi-translucent white, laciniate aril.

Chromosome number — $2n = 22$.

Phenology — Flowering period starts in late March and lasts until April (diurnal anthesis). Fruit and seeds mature in April to May. Leafy shoots usually emerge in May. The plants enter dormancy in November.

Distribution and ecology — *Kaempferia calcicola* is distributed in Phu Pha Man District, Khon Kaen province, NE Thailand. It grows on the top of limestone hills or cliffs, in the pockets filled with loamy soil and covered with leaf debris, full sun to partial shaded, usually with *Euphorbia lacei* Craib and *Dracaena* sp., at 340–355 m elevation.

Conservation status — The species is strictly endemic to the limestone hills in Phu Pha Man District of Khon Kaen province. The EOO and AOO, which are considered from the overall area of limestone hills in the type locality and surrounding areas observed in Google Earth (https://www.google.com/earth/), are estimated to be less than 50 km^2 and 4 km^2 respectively, with approximately 200 mature individuals. Currently, the suitable habitats of the species, especially the limestone area in Khon Kaen and adjacent provinces in NE Thailand, are severely fragmented geographically and continue to decline in the area due to quarrying for the construction industry (limestone and cement materials). Moreover, the type locality is not under any legal protection while the plants have been collected to sell as a rare, ornamental plant. The current information on the EOO and AOO leads us to propose that *Kaempferia calcicola* be treated as Critically Endangered (CR) B1ab(i,iii,v)+B2ab(ii,iii,v) in accordance with guidelines to the IUCN Red List Categories and Criteria, version 15.1 (IUCN Standards and Petitions Subcommittee 2022).

Etymology — The specific epithet *calcicola* refers to the limestone habitat of the species.

Vernacular name — We propose the common name ดอกดินเขาหินปูน (Dok Din Khao Hin Poon) in Thai language. “Dok Din” is the flower which occurs on the ground (well-known as earth flower), and “Khao Hin Poon” means limestone hill. This common name refers to the earth flowers occur on the limestone hill.

Additional specimens examined (paratypes) — Thailand, Khon Kaen province, Phu Pha Man district, 350 m elevation, 29 Aug 2023, *N. Nopporncharoenkul NNSB-944* (QBG, SLR); ibid., 340 m elevation, 6 Apr 2024, *N. Nopporncharoenkul NNSB-969* (QBG, SLR).

Remarks — The leafy shoot of *Kaempferia calcicola* consists of a remarkable upright pseudostem, a long petiole and elliptic, elliptic-oblong to lanceolate-oblong leaf blades, which are extremely similar to those of *K. rotunda* morphologically. The differences of both spe-

Fig. 7 (left page). *Kaempferia calcicola* – A: Inflorescence and rhizome; B: habit; C: pseudostem with ligules; D: rhizome with fascicled storage roots and numerous fusiform tubers; E: flower in front and side view; F: calyx (inset: detail of calyx apex); G: floral tube with ovary and stamen in front and side view; H: flower dissection (dc: dorsal corolla lobe; l: labellum; lc: lateral corolla lobe; ls: lateral staminode); I: detail of stamens and anther crests in front and side view; J: detail of pistil (from left to right: pistil; stigma and style in front and side view; ovary with epigynous glands); K: fruit; L: seeds. – All from *Nattapon Nopporncharoenkul NNSB-903* (QBG). – All photographs by N. Nopporncharoenkul.

cies can be found mainly in flower morphology (Table 2; Fig. 2). The flower of *K. calcicola* is the flat type floral plane (Nopporncharoenkul & al. 2021), consisting of lateral staminodes and labellum horizontal to slightly arcuate, which laid in the same plane and parallel to the ground, and represents an obreniform, broadly ovate, obovate to obdeltoid anther crest with an irregular undulate to crenate apex, the anther crest extends backward and positioned nearly perpendicular (c. 90 degree) to the anther connective, a labellum with an incision c. 3/5 of its length, a labellum base involute loosely enclosing the anther, and an extremely short filament. In contrast, the flower of *K. rotunda* is the perpendicular type consisting of upright to slightly arcuate lateral staminodes and a deflexed in distal half labellum (Nopporncharoenkul & al. 2021) and represents an oblong to ovate anther crest with a bilobed apex, usually with 2–3 small teeth between lobes, the anther crest extends upward with an angle nearly of 180 degree to the connective, a labellum with an incision c. 1/2 of its length, a flat labellum base, and a prominent, flat filament. However, the flowers and inflorescences of *K. calcicola* are entirely similar to those of *K. lopburiensis* and *K. takensis*. The distinct characteristics between *K. calcicola* and *K. lopburiensis* are found in the leafy shoots (Table 2). *Kaempferia lopburiensis* is easily distinguished from *K. calcicola* by having a short pseudostem completely embedded in the soil, suborbicular to ovate leaf blades flat on the ground with very short petioles, and a deeply bilobed labellum with an incision more than 2/3 of its length. *Kaempferia takensis* can also be differentiated from *K. calcicola* by having an oblong to ovate anther crest with a bilobed to irregularly tridentate apex, usually with 2–3 small teeth between the lobes and a deeply bilobed labellum with an incision more than 2/3 of its length. Geographically, *K. calcicola* occurs only on the tops of limestone hills or cliffs without a coexistence of any *Kaempferia* species. We found only *K. udonensis* in deciduous forest with bamboos in the foothills, without the occurrence of *K. calcicola* sympatrically.

Key to the taxa of *Kaempferia* subg. *Protanthium* of Thailand

(Revised from Nopporncharoenkul & Jenjittikul 2024 and Nopporncharoenkul & al. 2024)

1. Floral plane perpendicular to ground; lateral staminodes upright to slightly arcuate; labellum deflexed in distal half with an incision around or less than 1/2 of its length **2**
– Floral plane flat, parallel to ground; lateral staminodes and labellum horizontal to slightly arcuate, arranged in same plane; labellum with an incision around or more than 3/5 of its length **10**
2. Anther connective tissue and filament puberulent with very short glandular hairs dorsally and laterally **3**
– Anther connective tissue and filament glabrous . . . **6**
3. Leaves adpressed to ground, suborbicular to ovate; ligule 1.5–3 cm long . . . . . ***K. jenjittikuliae***
– Leaves semi-adpressed to upright, ovate, elliptic, to lanceolate-oblong; ligule less than 1 cm long **4**
4. Labellum with two conspicuous yellow bands from base toward centre; anther crest with obtuse to trilobed-undulate apex, middle lobe more elongated than side lobes . . . . . ***K. simaoensis***
– Labellum with central white, cream white to pale yellow patch basally; anther crest with bifid to bilobed, sometimes with 1–3 small teeth between main lobes **5**
5. Labellum with central pale yellow patch basally; nocturnal anthesis . . . . . ***K. noctiflora* var. *noctiflora***
– Labellum with central white to cream white patch basally surrounded by two light purple stripes from base toward centre of lobes; diurnal anthesis . . . . . ***K. noctiflora* var. *thepthepae***
6. Leaves linear grass-like to narrowly lanceolate-oblong, less than 5 cm wide . . . . . ***K. graminifolia***
– Leaves lanceolate-oblong, elliptic to ovate; more than 5 cm wide **7**
7. Labellum narrowly obovate with an incision c. 1/3 of its length; ligule opaque, 7–14 mm long; storage roots branched with numerous, lateral and terminal subglobose to ovoid tubers; tubers 0.5–1 by 0.5–0.8 cm . . . . . ***K. subglobosa***
– Labellum obdeltoid to broadly obovate with an incision c. 1/2 of its length; ligule translucent, 1–5 mm long; storage roots unbranched with a single, terminal fusiform, ellipsoid to ovoid tuber; tuber 1.2–3.5 by 1–2 cm **8**
8. Anther crest with bifid to bilobed apex, usually with 1–3 small teeth between main lobes; epigynous glands 2–6 mm long . . . . . ***K. rotunda***
– Anther crest with tridentate to undulate-truncate apex; epigynous glands 6–12 mm long **9**
9. Bracts and bracteole sparsely villous; epigynous gland 8–12 mm long . . . . . ***K. aurora***
– Bracts and bracteole glabrous; epigynous gland 6–7 mm long . . . . . ***K. sipraiana***
10. Anther connective tissue and filament puberulent with very short glandular hairs dorsally and laterally **11**
– Anther connective tissue and filament glabrous . . **12**
11. Labellum with white to cream white patch basally toward centre . . . . . ***K. lopburiensis***
– Labellum with deep pink to deep purple patch basally toward centre . . . . . ***K. udonensis***
12. Pseudostem buried in ground; leaves adpressed to semi-adpressed to ground, orbicular, suborbicular to ovate . . . . . ***K. grandifolia***
– Pseudostem upright and outstanding above ground; leaves lanceolate-oblong, elliptic to broadly ovate **13**
13. Lateral staminodes white; labellum white with pale yellow patch basally toward centre **14**

– Lateral staminodes white, light pink to deep pink; labellum white, light pink to purple with two deep reddish to deep purple spots at centre **15**

14. Petiole subsessile to 2.5 cm long; nocturnal anthesis . . . . . ***K. albiflora***
– Petiole more than 10 cm long; diurnal anthesis . . . . . ***K. caespitosa***

15. Anther crest oblong to ovate with bifid to bilobed apex, usually with 2–3 small teeth between lobes; labellum with an incision more than 2/3 of its length . . . . . ***K. takensis***
– Anther crest obreniform, broadly ovate, obovate to obdeltoid with irregular undulate to crenate apex; labellum with an incision c. 3/5 of its length . . . . . ***K. calcicola***

Author contributions

NN designed the research, obtained the research grant, conducted the field surveys and sample collections, performed cytogenetic analyses and morphological investigations, described a new species, prepared the first draft of the manuscript and figure plates; WS performed cytogenetic analyses; PN performed statistical analyses and discussed the results; JM conducted the field surveys and sample collections; TJ conducted the field surveys and sample collections, examined and identified the plant samples, studied and prepared the herbarium specimens, provided the crucial comments on plant taxonomy; NC and UV supervised and coordinated the study; PU designed the research, supervised and coordinated the study and provided the crucial comments on plant cytogenetics. All authors contributed to the concept and implementation of the study and took part in the final revision, discussing and approving of the manuscript.

Acknowledgements

The research project is financially supported by the Thailand Science Research and Innovation: TSRI (Grant number TSRI-4696906). The authors thank the Office of Natural Science Research and NSM Arboretum, National Science Museum, Pathum Thani, Thailand, and the Department of Plant Science, Faculty of Science, Mahidol University for facilities and support during the study. We gratefully acknowledge the directors and curators of BK, BKF, QBG, SING and SLR for specimen assessments. We are also grateful to Dr. Piyakaset Suksathan and Dr. Kittiyut Punchay for access to living collections in the *Zingiberaceae* nursery at Queen Sirikit Botanic Garden (QSBG), Chiang Mai, Thailand. We would like to thank Mrs. Darat Tanomaied, Mr. Withit Phumsaringkharn, Ms. Thunchanok Somnoo, Dr. Puttamon Pongkai, Ms. Weenussa Eakjamnong, members of Mahidol Plant Cytogenetics Laboratory and QSBG taxonomists for their assistance during our field trips, to Mr. Sirapope Wongniam for valuable comments on flow cytometry technique and to Ms. Pichsinee Meethong for coordination of the research project. We are grateful to Jana Leong-Skornickova (SING), another, anonymous reviewer and the editor for helpful comments.

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Supplemental content online

See https://doi.org/10.3372/wi.54.54201

Appendix S1 (Fig. S1, Table S1). List of accessions, localities and specimen references included in genome size and mitotic chromosome analyses.

Appendix S2 (Table S2). Means with standard deviations (S.D.) of estimated genome sizes of individual plants included in genome size analysis. Each individual plant was re-analysed three times (three replicates) on different days.

Appendix S3 (Table S3, S4). Statistical data of genome sizes based on 276 analysed plants from 15 *Kaempferia* species: normality test and pairwise comparison using Kolmogorov-Smirnov test and Kruskal-Wallis one-way analysis of variance at a significance level of $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics version 21.0.

Willdenowia
Open-access online edition bioone.org/journals/willdenowia
Online ISSN 1868-6397 · Print ISSN 0511-9618 · 2022 Journal Impact Factor 1.900
Published by the Botanic Garden and Botanical Museum Berlin, Freie Universität Berlin

Table 1. Genome size (2C value), chromosome number (2*n*) and putative ploidy level variation of species belonging to *Kaempferia* subg. *Protanthium*. Estimated genome sizes with standard deviations (S.D.) were calculated based on three replicates (analysed on different days) of each individual plant, and one to five plants for each accession. 2*n* chromosome numbers were clarified based on at least 20 metaphase cells from one plant and three to five plants per accession. Putative ploidy levels of accessions were inferred based on comparison of genome sizes to those of accessions obtained with both genome sizes and chromosome numbers.

Species	Accession numbers	Locality	2C value (pg) ± S.D.	Mean 2C value (pg) ± S.D.	2C value range	2*n*	Putative ploidy level	Plate no.
Kaempferia albiflora Jenjitt. & Ruchis.	NNSB634 (QBG)	Thailand, Kamphaeng Phet, Phran Kratai	5.851 ± 0.173	6.045 ± 0.239	5.838–6.412	22	2×	Fig. 3A
	NNSB741 (SLR)	Thailand, Phitsanulok, Wat Bot	6.412 ± 0.070				2×	
	NNSB546 (QBG)	Thailand, Tak, Amphoe Mueang	5.838 ± 0.057			22	2×	
	NNSB716 (QBG)	Thailand, Tak, Amphoe Mueang	6.110 ± 0.093			22	2×	
	NNSB532 (SLR)	Thailand, Uttaradit, Phichai	6.115 ± 0.114				2×	
K. aurora Noppornch. & Jenjitt.	NNSB855 (SLR)	Thailand, Tak, Mae Ramat	5.205 ± 0.082	5.314 ± 0.103	5.205–5.402		2×	
	NNSB545 (SLR)	Thailand, Tak, Mae Sot	5.333 ± 0.025				2×	
	NNSB713 (QBG)	Thailand, Tak, Mae Sot	5.402 ± 0.089			22	2×	Fig. 3B
	NNSB757 (SLR)	Thailand, Tak, Tha Song Yang	5.319 ± 0.127				2×	
K. caespitosa Noppornch. & Jenjitt.	NNSB733 (QBG)	Thailand, Lampang, Ngao	4.286 ± 0.032	4.286 ± 0.032	2.251–4.313	22	2×	Fig. 3C
K. calcicola Noppornch.	NNSB903 (QBG)	Thailand, Khon Kaen, Phu Pha Man	6.255 ± 0.097	6.255 ± 0.097	6.136–6.354	22	2×	Fig. 3D
K. graminifolia Noppornch. & Jenjitt.	NNSB686 (QBG)	Thailand, Kamphaeng Phet, Phran Kratai	5.927 ± 0.006	5.972 ± 0.072	5.927–6.084	22	2×	
	NNSB516 (SLR)	Thailand, Sukhothai, Amphoe Mueang	5.990 ± 0.043				2×	
	NNSB499 (QBG)	Thailand, Sukhothai, Ban Dan Lan Hoi	6.084 ± 0.022				2×	
	NNSB594 (SLR)	Thailand, Sukhothai, Ban Dan Lan Hoi	5.990 ± 0.078				2×	
	NNSB657 (SLR)	Thailand, Sukhothai, Ban Dan Lan Hoi	5.936 ± 0.029				2×	
	NNSB684 (SLR)	Thailand, Sukhothai, Ban Dan Lan Hoi	6.006 ± 0.110			22	2×	Fig. 3E
K. grandifolia Saensouk & Jenjitt.	NNSB320 (SLR)	Thailand, Khon Kaen, Phu Wiang	5.731 ± 0.027	5.677 ± 0.079	5.634–5.731	22	2×	
	NNSB519 (SLR)	Thailand, Khon Kaen, Phu Wiang	5.634 ± 0.081			22	2×	Fig. 3F
K. jenjittikuliae Noppornch.	NNSB802 (SLR)	Thailand, Chaiyaphum, Phakdi Chumphon	5.415 ± 0.068	5.364 ± 0.077	5.333–5.415		2×	
	NNSB760 (QBG)	Thailand, Phetchabun, Chon Daen	5.333 ± 0.119			22	2×	
	NNSB836 (QBG)	Thailand, Phetchabun, Chon Daen	5.348 ± 0.037			22	2×	Fig. 3G
K. lopburiensis Picheans.	NNSB755 (SLR)	Thailand, Chaiyaphum, Thep Sathit	4.930 ± 0.030	5.147 ± 0.250	4.884–5.445		2×	
	NNSB541 (QBG)	Thailand, Lopburi, Amphoe Mueang	5.445 ± 0.046			22	2×	
	NNSB335 (SLR)	Thailand, Lopburi, Lam Sonthi	–			22	2×	Fig. 3H
	NNSB544 (SLR)	Thailand, Nakhon Sawan, Phaisali	4.884 ± 0.020				2×	

	NNSB652 (SLR)	Thailand, Saraburi, Muak Lek	5.222 ± 0.190				2×	
	NNSB665 (SLR)	Thailand, Saraburi, Muak Lek	5.254 ± 0.013				2×	
K. noctiflora Noppornch. & Jenjitt. var. *noctiflora*	NNSB640 (SLR)	Thailand, Chiang Mai, Amphoe Mueang	4.682 ± 0.043	4.703 ± 0.058	4.616–4.777	22	2×	Fig. 3I
	NNSB725 (SLR)	Thailand, Chiang Mai, Amphoe Mueang	4.751 ± 0.019				2×	
	NNSB468 (SLR)	Thailand, Chiang Mai, Amphoe Mueang	–			22	2×	
	NNSB554 (BKF)	Thailand, Chiang Mai, Mae On	4.703 ± 0.056			22	2×	
	NNSB625 (SLR)	Thailand, Chiang Mai, Mae Rim	4.688 ± 0.028				2×	
	NNSB708 (SLR)	Thailand, Chiang Mai, Mae Taeng	4.777 ± 0.032				2×	
	NNSB653 (SLR)	Thailand, Chiang Mai, Phrao	4.644 ± 0.055			22	2×	
K. noctiflora var. *thepthepae* Noppornch. & Somnoo	NNSB928 (QBG)	Thailand, Chiang Mai, Doi Saket	4.664 ± 0.034	4.761 ± 0.089	4.625–4.869	22	2×	Fig. 3J
	NNSB953 (SLR)	Thailand, Chiang Mai, San Kamphaeng	4.819 ± 0.044				2×	
K. rotunda L.	NNSB623 (SLR)	Thailand, Chonburi, Bo Thong	4.213 ± 0.018	4.193 ± 0.087	4.071–4.296		2×	
	NNSB664 (SLR)	Thailand, Kamphaeng Phet, Khlong Lan	4.243 ± 0.034				2×	
	NNSB698 (SLR)	Thailand, Kamphaeng Phet, Kosamphi Nakhon	4.071 ± 0.033				2×	
	NNSB718 (SLR)	Thailand, Kamphaeng Phet, Phran Kratai	4.117 ± 0.028				2×	
	NNSB567 (SLR)	Thailand, Kanchanaburi, Sai Yok	4.296 ± 0.049			22	2×	Fig. 3K
	NNSB660 (SLR)	Thailand, Phitsanulok, Wang Thong	4.264 ± 0.024				2×	
	NNSB282 (SLR)	Thailand, Ratchaburi, Suan Phueng	–			22	2×	
	NNSB534 (SLR)	Thailand, Tak, Amphoe Mueang	4.166 ± 0.060			22	2×	
	NNSB163 (SLR)	Thailand, Tak, Phop Phra	–			22	2×	
	NNSB629 (SLR)	Thailand, Kanchanaburi, Thong Pha Phum	6.934 ± 0.244	6.983 ± 0.213	6.787–7.156	33	3×	Fig. 3L
	NNSB602 (SLR)	Thailand, Phetchabun, Khao Kho	7.156 ± 0.054			33	3×	
	NNSB600–1 (SLR)	Laos, Champasak	6.787 ± 0.035			33	3×	
	NNSB642 (SLR)	Thailand, Chiang Mai, Amphoe Mueang	8.242 ± 0.245	8.543 ± 0.427	8.165–9.172	44	4×	Fig. 3M
	NNSB722 (SLR)	Thailand, Chiang Mai, Mae On	8.165 ± 0.191				4×	
	NNSB703 (SLR)	Thailand, Chiang Mai, Mae Rim	8.355 ± 0.158			44	4×	Fig. 3N
	NNSB540 (SLR)	Thailand, Chiang Rai, Wiang Pa Pao	8.348 ± 0.094				4×	
	NNSB588 (SLR)	Thailand, Mae Hong Son, Khun Yuam	9.140 ± 0.065				4×	
	NNSB522 (SLR)	Thailand, Nan, Bo Kluea	8.718 ± 0.100				4×	
	NNSB600–2 (SLR)	Laos, Champasak	9.172 ± 0.273				4×	

Species	Accession numbers	Locality	2C value (pg) ± S.D.	Mean 2C value (pg) ± S.D.	2C value range	2*n*	Putative ploidy level	Plate no.
Kaempferia simaoensis Y. Y. Qian	NNSB535 (QBG)	Thailand, Kanchanaburi, Thong Pha Phum	4.043 ± 0.027	3.852 ± 0.136	3.687–4.043	22	2×	Fig. 3O
	NNSB655 (SLR)	Thailand, Tak, Mae Ramat	3.838 ± 0.053				2×	
	NNSB613 (QBG)	Thailand, Tak, Mae Sot	3.915 ± 0.060			22	2×	
	NNSB676 (SLR)	Thailand, Tak, Umphang	3.687 ± 0.052			22	2×	
	NNSB678 (SLR)	Thailand, Tak, Umphang	3.771 ± 0.031				2×	
K. sipraiana Boonma & Seansouk	NNSB656 (SLR)	Thailand, Tak, Mae Sot	4.956 ± 0.158	4.956 ± 0.158	4.807–5.121	22	2×	Fig. 3P
K. subglobosa Nopporncharoen. & Jenjitt.	NNSB749 (QBG)	Thailand, Tak, Ban Tak	4.619 ± 0.096	4.655 ± 0.073	4.489–4.754	22	2×	Fig. 3Q
	NNSB746 (SLR)	Thailand, Tak, Sam Ngao	4.725 ± 0.013				2×	
	NNSB662 (SLR)	Thailand, Tak, Wang Chao	4.675 ± 0.054				2×	
	NNSB751 (SLR)	Thailand, Tak, Unknown	4.622 ± 0.036				2×	
K. takensis Boonma & Saensouk	NNSB737 (SLR)	Thailand, Chiang Mai, Mae Cham	5.049 ± 0.031	4.854 ± 0.178	4.579–5.100		2×	
	NNSB531 (SLR)	Thailand, Chiang Rai, Thoeng	4.579 ± 0.073				2×	
	NNSB696 (SLR)	Thailand, Chiang Rai, Thoeng	4.649 ± 0.025				2×	
	NNSB526–1 (QBG)	Thailand, Kamphaeng Phet, Khlong Lan	4.890 ± 0.037				2×	
	NNSB697 (QBG)	Thailand, Kamphaeng Phet, Kosamphi Nakhon	4.822 ± 0.025			22	2×	Fig. 3R
	NNSB827 (SLR)	Thailand, Lamphun, Li	4.968 ± 0.016			22	2×	
	NNSB595 (SLR)	Thailand, Tak, Mae Sot	4.742 ± 0.089			22	2×	
	NNSB754 (SLR)	Thailand, Tak, Sam Ngao	5.100 ± 0.032				2×	
	NNSB598 (SLR)	Thailand, Uthai Thani, Ban Rai	5.013 ± 0.016				2×	
	NNSB526–2 (SLR)	Thailand, Kamphaeng Phet, Khlong Lan	8.331 ± 0.018	8.208 ± 0.160	7.959–8.341		4×	
	NNSB690 (SLR)	Thailand, Phrae, Long	7.959 ± 0.071				4×	
	NNSB524 (QBG)	Thailand, Phrae, Song	8.153 ± 0.102			44	4×	Fig. 3S
	NNSB692 (SLR)	Thailand, Phrae, Song	8.341 ± 0.040			44	4×	
K. udonensis Picheans. & Phokham	NNSB508 (QBG)	Thailand, Kanchanaburi, Sai Yok	4.357 ± 0.031	4.448 ± 0.210	4.057–4.844	22	2×	
	NNSB633 (SLR)	Thailand, Kanchanaburi, Sai Yok	4.363 ± 0.013				2×	
	NNSB736 (SLR)	Thailand, Kanchanaburi, Sai Yok	4.250 ± 0.010				2×	
	NNSB707 (SLR)	Thailand, Kanchanaburi, Si Sawat	4.360 ± 0.048			22	2×	
	NNSB747 (SLR)	Thailand, Kanchanaburi, Si Sawat	4.092 ± 0.049				2×	
	NNSB753 (SLR)	Thailand, Kanchanaburi, Si Sawat	4.307 ± 0.027				2×	

NNSB687 (SLR)	Thailand, Loei, Pha Khao	4.788 ± 0.035	22	2×	
NNSB599 (SLR)	Thailand, Udon Thani, Nong Wua So	4.639 ± 0.075		2×	
NNSB752 (SLR)	Thailand, Uthai Thani, Ban Rai	4.493 ± 0.063	22	2×	Fig. 3T

Table 2. Diagnostic characters between *Kaempferia calcicola* and morphological similar species.

Character	*K. calcicola*	*K. takensis*	*K. lopburiensis*	*K. rotunda*
Leafy shoot	up to 75 cm tall with (4–)6–8 leaves	up to 65 cm tall with 5–7 leaves	adpressed to ground, 3–4 leaves	up to 70 cm tall with 4–7 leaves
Pseudostem	well-developed above ground, up to 25 cm tall	well-developed above ground, up to 30 cm tall	buried in ground, 6–7(–10.5) cm tall	well-developed above ground, 7–30 cm tall
Leaf shape	elliptic, elliptic-oblong to lanceolate-oblong, 20–35(–40) × (5–)6.5–15 cm	lanceolate-oblong, elliptic to broadly ovate, 20–55 × 10–26 cm	suborbicular to ovate, 19–35 × 18–33 cm	lanceolate-oblong, elliptic to ovate, 16–48(–60) × 6–19 cm
Petiole	3–18(–30) cm long	up to 5 cm long	subsessile	subsessile to 9 cm long
Ligule	rounded to triangular, 0.4–0.7(–1.2) cm long, semi-translucent	broadly triangular, 0.4–1 cm long, semi-translucent	broadly triangular, 1–1.8 cm long, opaque	broadly triangular, 0.1–0.3(–0.6) cm long, translucent
Floral plane	parallel type	parallel type	parallel type	perpendicular type
Labellum incision	c. 3/5 of labellum length	more than 2/3 of labellum length	more than 2/3 of labellum length	c. 1/2 of labellum length
Labellum base	slightly involute, loosely enclosing anther	involute, enclosing anther	involute, enclosing anther	flat
Anther connective	glabrous	glabrous	puberulent with very short glandular hairs dorsally and laterally	glabrous
Anther crest position	extends backward with c. 90 degree to connective	extends backward with 90–135 degree to connective	extends backward with 45–90 degree to connective	extends upward with c. 180 degree to connective
Anther crest shape	obreniform, broadly ovate, obovate to obdeltoid, 5–7.5(–11) × (6–)8–10 mm, apex irregular undulate to crenate	oblong to ovate, 7–14 × 3–6 mm, apex bilobed to irregularly tridentate	oblong, obdeltoid to broadly ovate, 8–10 × 4–8 mm, apex bilobed, crenate to deeply irregular trilobed-undulate	oblong to ovate, (4–)8–14 × 3–6.6 mm, apex bilobed, usually with 2–3 small teeth between lobes
Filament	subsessile	subsessile	subsessile	(1.5–)2–3 mm long