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Chromosome number of some species of Hymenophyllaceae from Thailand

SIWAKORN CHOKRASSAMEEHIRUN[1,♥], EKAPHAN KRAICHAK[2,3], TASSANAI JARUWATTANAPHAN[1,3,♥♥]

[1]Department of Horticulture, Faculty of Agriculture, Kasetsart University. 50 Ngam Wong Wan Rd., Lat Yao, Chatuchak, Bangkok 10900, Thailand. Tel.: +66-2561-4891, ♥email: siwakorn.chok@ku.th, ♥♥tassanai.j@ku.th
[2]Department of Botany, Faculty of Science, Kasetsart University. 50 Ngam Wong Wan Rd., Lat Yao, Chatuchak, Bangkok 10900, Thailand
[3]Biodiversity Center Kasetsart University. 50 Ngam Wong Wan Rd., Lat Yao, Chatuchak, Bangkok 10900, Thailand

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Abstract. *Chokrassameehirun S, Kraichak E, Jaruwattanaphan T. 2024. Chromosome number of some species of Hymenophyllaceae from Thailand. Biodiversitas 25: 4389-4396.* Hymenophyllaceae, known by the common name filmy ferns family, a basal family of leptosporangiate ferns, comprises ca.450 species and is distinctive amongst the entire pteridophytes by mostly unistratrose lamina. Classification and evolution of the family have fascinated botanists for many decades. Chromosome numbers in this family provide insights into evolutionary trends and have been useful for genus delimitation in recent classification schemes. However, interest in studying chromosome counts has declined in recent years. This study aimed to provide additional chromosome counts from Thailand species. Mitotic chromosomes were observed by using the hematoxylin squash technique. Five species belonging to *Abrodictyum*, *Crepidomanes*, and *Hymenophyllum* were examined, including *H. acanthoides* (Bosch) Rosenst. (2*n*=2*x*=41) and *H. longifolium* Alderw. (2*n*=2*x*=72), which were recorded for the first time. All specimens are of the diploid cytotype based on the chromosome base number of the genus. The results support aneuploid reduced evolution in *Hymenophyllum*, especially in subg. *Hymenophyllum*, with *H. acanthoides* showing a unique number, is assumed to have been reduced from 2*n*=42, which is the most typical within the subgenus. Additionally, *A. obscurum* (Blume) Ebihara & K.Iwats. var. *obscurum* (2*n*=2*x*=66) and *H. exsertum* Wall. ex Hook. (2*n*=2*x*=42) show consistent numbers within the species, whereas *C. minutum* (Blume) K.Iwats. (2*n*=2*x*=72) displayed some variation within the species, suggesting multiple cytotypes and reticulate evolution. More sampling is still needed for all species to fill the gap in the evolution study for this fern family.

Keywords: Aneuploidy, cytotype, diploid, *Hymenophyllum*, *Trichomanes*

INTRODUCTION

Hymenophyllaceae Mart. or the filmy ferns family, are early leptosporangiate ferns in the order Hymenophyllales, consisting of an estimated 450 species (PPG I 2016). Unistratose laminae are distinct traits when compared to other fern families. The infrafamilial classification has been discussed for many decades (Copeland 1938, 1947; Morton 1968; Pichi Sermolli 1977; Iwatsuki 1984, 1985), resulting in various classification schemes. Genetic studies have revealed the deep evolutionary history and for long supported traditional bigeneric systems of the family (Dubuisson 1997; Pryer et al. 2001). Afterward, a classification of nine generic systems was proposed by Ebihara et al. (2006) and adopted to our current knowledge of the family (Lindsay et al. 2009; Ebihara et al. 2010; Del Rio et al. 2017; Saïd et al. 2017; Senterre et al. 2017; Hsu et al. 2019a, 2019b; TPG 2019; Chen et al. 2017, 2020; Iwatsuki et al. 2019; Vasques and Ebihara 2022; Dubuisson et al. 2023; Iwatsuki and Ebihara 2023). Data from phylogenetic, cytology, and morphology analyses support the nine generic systems. Therefore, it has been generally agreed for evolution and recent classifications aspects.

Classification of Hymenophyllaceae tends to be related to the basic chromosome number of each genus or even subgenus in *Hymenophyllum* Sm. (Ebihara et al. 2007; Hennequin et al. 2010; Abreu et al. 2024). In *Trichomanoidae sensu* (Ebihara et al. 2006), each genus represents a series of aneuploid reduced numbers from the base number (*x*) =36 (Ebihara et al. 2007). Consistently reported numbers are *x*=36 in *Callistopteris* Copel., *Crepidomanes* (C.Presl) C.Presl, *Polyphlebium* Copel., and *Vandenboschia* Copel.; *x*=34 in *Didymoglossum* Desv.; *x*=33 in *Abrodictyum* C.Presl; and lastly *x*=32 in *Cephalomanes* C.Presl and *Trichomanes* L. (Ebihara et al. 2007; Abreu et al. 2024). On the other hand, the genus *Hymenophyllum* displays a high diversity of chromosome numbers. Hennequin et al. (2010) counted meiotic chromosomes (*n*) of some taxa using the squash technique for accuracy and combined the results with several chromosome reports to estimate ancestral chromosome numbers of the genus. Suggested chromosome-reduced evolution was proposed in *Hymenophyllum*, especially in subg. *Hymenophyllum*, which had the lowest chromosome number and the greatest variation in chromosome numbers. Each subgenus was conserved or reduced in number. For certain subgenera that belong to Thai floras, *n*=36 was reported in subgenus *Globosa* (Prantl) Ebihara & K.Iwats., subg. *Pleuromanes* (C.Presl) Ebihara & K.Iwats. and subg. *Sphaerocionium* (C.Presl) C.Chr.; *n*=28 in subg. *Mecodium* Copel.; *n*=11 to 28 in subg. *Hymenophyllum*, where *n*=36 is accepted as the plesiomorphic character of the family (Ebihara et al. 2006,

2007; Hennequin et al. 2010; Iwatsuki and Ebihara 2023). Given that *Hymenophyllum* shows the greatest variation in chromosome number, the lack of cytotaxonomic data presents a crucial challenge for delimiting or classifying taxa within the subgenus in recent studies.

Furthermore, within *Trichomanes s.l.*, the *Crepidomanes minutum* (Blume) K.Iwats. species complex provides an intriguing case for using cytological data. By observing phylogenetic data, chromosome counts, and mode of reproduction, Nitta et al. (2011) investigated the origin of the complex and confirmed hybridization among the species, maintained by apogamy and polyploidy that independently evolved multiple times.

According to the reviewed cytotaxonomy of Hymenophyllaceae by Abreu et al. (2024), only 37% of the species in the family are backed by records for chromosome number data. Therefore, both mitotic and meiotic chromosome counts are still needed at present to elucidate the evolutionary background for the applicable taxonomic system of the family. Especially in Thailand, cytological studies in filmy ferns are scarce compared to those of other fern groups (Paitoonyakul 2018; Limpanasittichai and Jaruwattanaphan 2022), as data from only two diploid species were recorded from Paitoonyakul (2018), and there are no reports of chromosome number hitherto from Thailand. This study aims to record the chromosome number of some species of Hymenophyllaceae from Thailand using a reliable technique to provide some cytological data on the filmy fern family.

MATERIALS AND METHODS

Plant materials and cultivating

Living specimens were collected from natural habitats during a field expedition in 2023-2024. The fully fertile plants were collected as voucher specimens, and the rest of the specimens were used for cytology investigation. Specimen identification followed Flora of Thailand (Tagawa and Iwatsuki 1979), Flora of China (Jiaxi et al. 2013), Flora Malesiana (Iwatsuki and Ebihara 2023), and taxonomic publications on specific groups (Sangrit 2008; Chen et al. 2017; Hsu et al. 2019a; Iwatsuki et al. 2019), infrafamilial classification system (e.g., genus, subgenus, and section) followed Ebihara et al. (2006). The living specimens were maintained in closed transparent plastic containers under saturated humidity and low light conditions, supplied by a 16W fluorescent bulb for 12 hours daily. *Sphagnum* moss was used as the growing medium, and monthly spray with ¼ (a quarter) concentration of commercial liquid fertilizer (HYPONeX® 6-10-5) was provided. Voucher specimens were deposited at the Forest Herbarium (BKF), Department of National Parks, Wildlife and Plant Conservation, Thailand, and Plants Diversity Laboratory, Department of Horticulture, Faculty of Agriculture, Kasetsart University.

Chromosome observations and data analysis

Somatic chromosomes were counted using the hematoxylin squash method, reported in Jaruwattanaphan et al. (2013) and Limpanasittichai and Jaruwattanaphan (2022). New growth parts (e.g., young frond, rhizome, and roots) were cut and pre-treated immediately with 2 mM 8-hydroxyquinoline at 16-18°C for 8 hrs. or in cold water at 4-5°C for 24 hrs. (Takamiya 1993; Osalou et al. 2013). This was followed by fixation using acetic-alcohol (glacial acetic acid: absolute ethanol, 1:3) fixative solution at 5-6°C for 12-16 hrs. Observations were made using cell hydrolysis with 1N-hydrochloric acid (HCl) at 60°C for 10 min in *Hymenophyllum* or 15-20 min in *Trichomanes s.l.* due to the hardness of the cells (see results and discussion), then squashed with 1% hematoxylin for 15 min., observed under a light microscope (1000×), and photographed with a digital camera. Illustrations were made using Adobe Illustrator (Adobe Inc.). Chromosome numbers were compared with previous studies and reports in Thailand and other countries (Hennequin et al. 2010; Nitta et al. 2011; Paitoonyakul 2018; Abreu et al. 2024). Ploidy levels were estimated by comparing the chromosome base number of each genus from Ebihara et al. (2006, 2007) and Iwatsuki and Ebihara (2023).

Only clear mitotic cells were counted and illustrated. Due to the limited growth of specimens, only a limited number of specimens are recorded. Species, locality, and collector number are summarized in Table 1.

RESULTS AND DISCUSSION

Note on observation techniques

It has long been accepted that Hymenophyllaceae ferns are difficult to cultivate due to the required special growth conditions, which make them very slow-growing in *ex-situ* conditions, even with optimum humidity and fertilization.

Table 1. Specimens used in cytology study

Species	Collector number	Locality/floristic region[1]	Figure
Abrodictyum obscurum var. *obscurum*	*S. Chokrassameehirun 19-11*	Phatthalung/ PEN	1.A
Crepidomanes minutum	*S. Chokrassameehirun 19-02*	Phatthalung/ PEN	1.B
Hymenophyllum acanthoides	*S. Chokrassameehirun 22-74*	Phatthalung/ PEN	1.C
Hymenophyllum exsertum	*S. Chokrassameehirun 20-34*	Loei/ NE	
	S. Chokrassameehirun 20-80	Phitsanulok/ N	1.D
Hymenophyllum longifolium	*S. Chokrassameehirun 22-62*	Phatthalung/ PEN	1.E

Note: [1]floristic region of Thailand. N: Northern, NE: North-eastern, PEN: Peninsular (van Welzen et al. 2011)

Our cultivating protocols are usable for maintaining growth and development for some species, including *Abrodictyum obscurum, Cephalomanes javanicum* (Blume), C.Presl, *Crepidomanes minutum*, *Cr. christii* (Copel.) Copel., *Didymoglossum sublimbatum* (Bosch) Ebihara & K.Iwats., *Hymenophyllum acanthoides* (Bosch) Rosenst., *H. denticulatum* Sw., *H. exsertum* Wall., *H. polyanthos* (Sw.) Sw., and *Vandenboschia auriculata* (Blume) Copel., but the growing method showed some disadvantages in certain species with specific habitats, such as the epilithic-rheophytic species *V. maxima* (Blume) Copel., and the high-altitude species *H. pallidum* (Blume) Ebihara & K.Iwats.

All mitotic cell counts in this study were obtained from unfolding fronds. The rhizomes and roots from some species were also put through fixation, but appropriate mitotic cells were more likely found in the frond segments. This is possibly due to the limited number of initial cells in roots and rhizomes, while greater number in the developing frond segments. Thickness of the cell wall is one of the problematic issues. Hydrolysis with 1N-HCl for 10 min was decent for squashing the lamina cells of *Hymenophyllum*. However, *Trichomanes s.l.* presented a harder cell wall in all parts, even in lamina cells. Adjusting hydrolysis time, temperature, or concentration of HCl for the hydrolysis step was tested for some samples. High concentrations of HCl can cause direct damage to cells and chromosome structure, incurring chromosome fragmentation that can make an error in the counting step. Enzymatic hydrolyses were also used in our preliminary study, but the results are still unclear. The chromosomes from enzymatic hydrolyses have low clarity after staining of 1% hematoxylin or aceto-orcein, compared to the use of HCl hydrolyses. We suggest HCl hydrolyses at 60°C for 10 min. in *Hymenophyllum* and 15-20 min. in *Trichomanes s.l.* are appropriate for mitotic chromosome counts for Hymenophyllaceae.

Previous studies of chromosome numbers of Hymenophyllaceae are mainly records from meiotic cells, possibly due to the abovementioned problems. Fixation in the field is required for meiotic chromosome counts with the acetic-alcohol (1:3) fixative solution being used in many studies (Manton and Sledge 1954; Walker 1966; Braithwaite 1969; Tindale and Roy 2002; Ebihara et al. 2002; Hennequin et al. 2010). The advantage of meiotic chromosome counts is due to the continuous division of spores in sporangium, giving more possibility to observe during meiotic division. Furthermore, the hydrolysis step is not necessary for sporangium, making this approach more advantageous in chromosome observation. However, misinformation can occur through many processes, such as misidentification, miscount by overlapped chromosomes, or fragmentation of the chromosomes. The use of mitotic count compared to meiotic may be useful to confirm meiotic succession or aneuploidy events.

Chromosome number

Six specimens belonging to five species (Figure 1.A-E) were in the suitable phase for chromosome counts. Figure 2 shows images of clear and countable cells for each species, and Table 2 summarizes pre-treatment protocols, chromosome number, and estimated ploidy levels.

***Abrodictyum* C.Presl subg. *Pachychaetum* (C.Presl) K.Iwats.**

Chromosome base number (x)=33 (Ebihara et al. 2006; Iwatsuki and Ebihara 2023).

Abrodictyum obscurum (Blume) Ebihara & K.Iwats. var. *obscurum*

Specimens examined: *S. Chokrassameehirun 19-11* (Figure 1.A).

$2n=2x=66$ [diploid].

This species is widely distributed. Previous studies proposed $n=33$ from Ceylon (Sri Lanka) (Manton and Sledge 1954), India (Ghatak 1977; Manickam and Irudayaraj 1988; Bir and Verma 2010; Vijayakanth et al. 2018), Bukit Timah in Malaysia (Manton 1954), and the Solomon Islands (Braithwaite 1969). These mentioned records were solely obtained from meiotic observation. This study is the first record of mitotic counts from the margin of young frond segments. The result of mitotic counts (Figure 2.A) is consistent with meiotic counts, showing diploid cytotype and possibly sexual reproduction due to no irregular meiosis observed reports. The base number of the genus $x=33$ is unique among all genera in the family (Braithwaite 1969). Cytological data proved useful for genus delimitation, while morphological characters are complexes.

Table 2. Results of mitotic chromosome counts and pre-treatment protocols of some Hymenophyllaceae

Genus/subgenus	Chromosome base number[1]	Species	Collector no.	Pre-treatment	Chromosome number	Figure
Abrodictyum/*Pachychaetum*	x=33	*A. obscurum* var. *obscurum*	*19-11*	8-hydroxyquinoline	$2n=2x=66$	2A
Crepidomanes/*Crepidomanes* sect. *Gonocormus*	x=36	*C. minutum*	*19-02*	8-hydroxyquinoline	$2n=2x=72$	2B
Hymenophyllum/ *Hymenophyllum*	x=11 to 28	*H. acanthoides**	*22-74*	Cold water	$2n=2x=41$	2C
		H. exsertum	*20-34*	Cold water	$2n=2x=42$	-
			20-80	Cold water	$2n=2x=42$	2D
Hymenophyllum/*Globosa*	x=36	*H. longifolium**	*22-62*	8-hydroxyquinoline	$2n=2x=72$	2E

Notes: [1] chromosome base number for each genus/subgenus following Ebihara et al. (2006) and Iwatsuki and Ebihara (2023); [2] $2n$: somatic chromosome, $2x$: diploid cytotype; *First chromosome count record for species

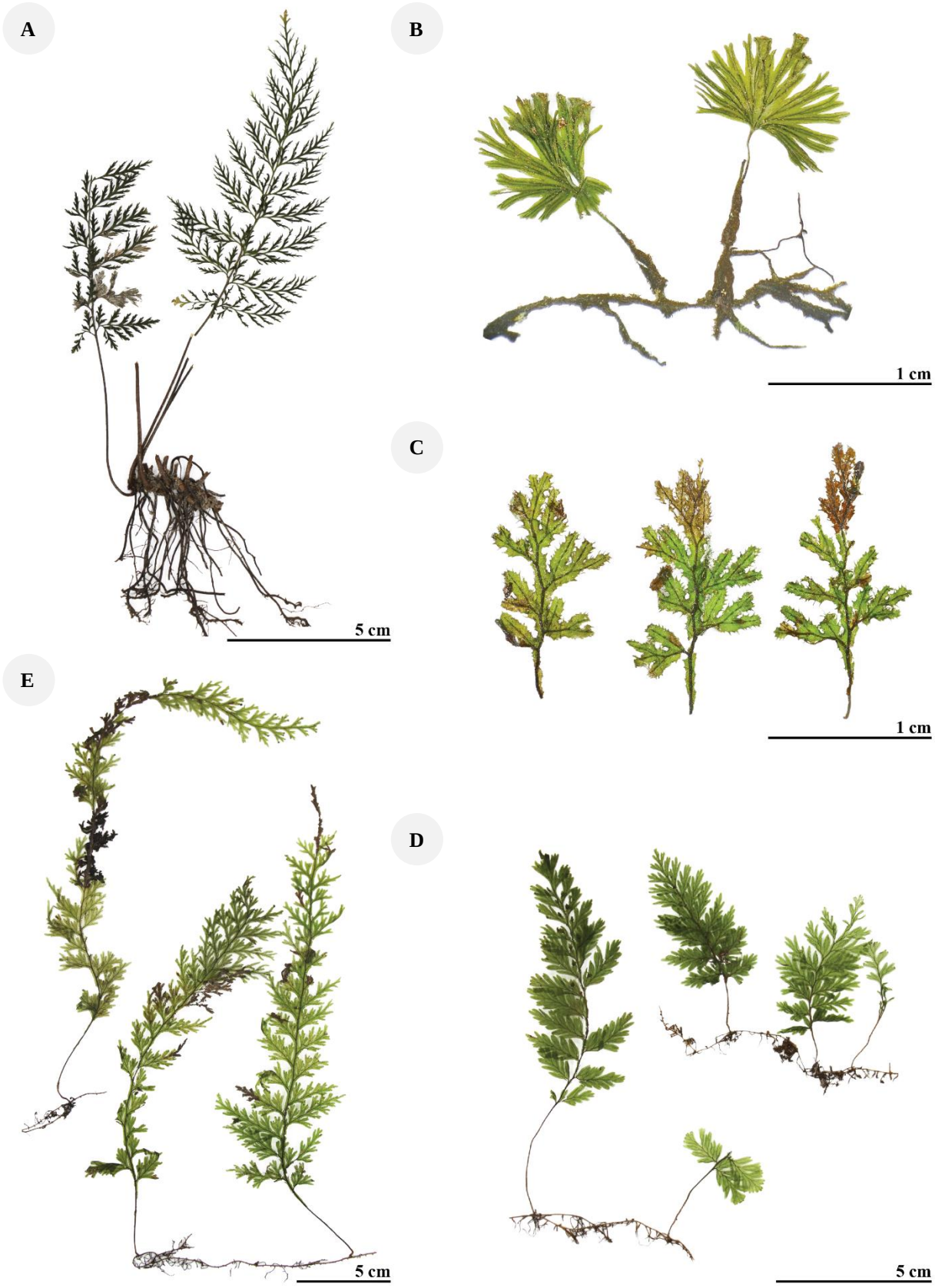

Figure 1. Voucher specimens. A. *Abrodictyum obscurum* var. *obscurum*; B. *Crepidomanes minutum*; C. *Hymenophyllum acanthoides*; D. *Hymenophyllum exsertum*; E. *Hymenophyllum longifolium*

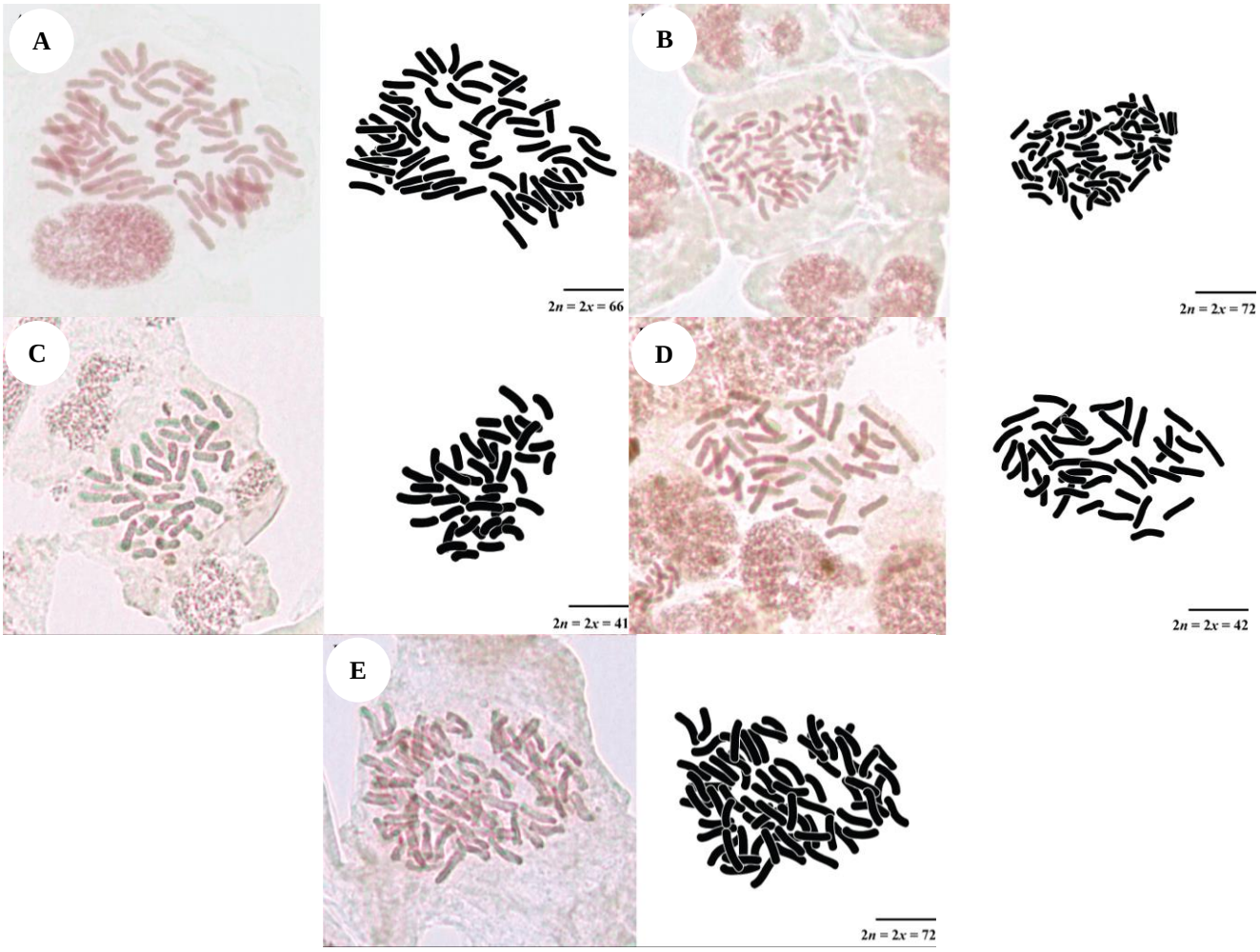

Figure 2. The mitotic chromosome of some ferns in the family Hymenophyllaceae. A. *Abrodictyum obscurum* var. *obscurum*; B. *Crepidomanes minutum*; C. *Hymenophyllum acanthoides*; D. *Hymenophyllum exsertum*; E. *Hymenophyllum longifolium*. Scale bar: 10 µm

Morphological variation of *A. obscurum* is recognized (Iwatsuki and Ebihara 2023), with two varieties presented (i) *A. obscurum* var. *obscurum* has a wide distribution in Thailand and the chromosome number of n=33, 2n=66; and (ii) *A. obscurum* var. *siamense* (Christ) K.Iwats. which is only known from two locations in Thailand (including type locality), and from adjacent areas recorded by Jiaxi et al. (2013) and Iwatsuki and Ebihara (2023) and there is still a lack of cytological data. Only a few morphological characters are used to discriminate between these two varieties, i.e., wings of rachis and dilated involucres are presented in *A. obscurum* var. *siamense*. On the other hand, wingless rachis and hardly dilated involucres are used to specify *A. obscurum* var. *obscurum*. The key from morphological characters is obscure, indicating the difficulty in using these characters alone for species distinction. Our phylogenetic results (Chokrassameehirun S 2024, unpublished data) agreed with the note on variety in Iwatsuki and Ebihara (2023), which revealed that *A. obscurum* var. *siamense* comprises an independent phylogenetic lineage. Thus, the addition of cytological data may be used to shed light on this topic. The uniqueness of the chromosome base number of *Abrodictyum* may help in genus discrimination, especially from *Cephalomanes*, which has similar characters but differs in chromosome base number.

***Crepidomanes* (C.Presl) C.Presl subg. *Crepidomanes* sect. *Gonocormus* (Bosch) K.Iwats.**

Chromosome base number (x)=36 (Ebihara et al. 2006; Iwatsuki and Ebihara 2023)

Crepidomanes minutum (Blume) K.Iwats.

Specimens examined: *S. Chokrassameehirun 19-02* (Figure 1.B).

2n=2x=72 [diploid].

One of the species with the greatest varieties of chromosome number, *Cr. minutum* is considered a species complex. Our study was based on the Iwatsuki and Ebihara (2023) species concept, although synonymous taxa of *Cr. minutum* are recorded as distinct species in Abreu et al. (2024). Chromosome numbers have been recorded ranging from n=36 from the Solomon Islands (Braithwaite 1969), Vanuatu, New Caledonia (Braithwaite 1975), Ryukyu Islands (Mitui 1976), India (Ghatak 1977), and Australia (Tindale and Roy 2002); n=72 from the Solomon Islands (Braithwaite 1969) and Australia (Tindale and Roy 2002); 2n=72 from Japan (Nitta et al. 2011); 2n=108 from Malaysia (Bell

1960) and Vanuatu, New Caledonia (Braithwaite 1975). Irregular meiosis was detected in India (Manickam and Irudayaraj 1988), and apogamous taxa were confirmed by Nitta et al. (2011). The counted specimen from peninsular Thailand (Figure 2B) is in accordance with the diploid cytotype record from Japan (2*n*=2*x*=72). Voucher specimens are non-proliferate, flabellate fronds, epilithic dominated near steam in ca. 400 msl.

Crepidomanes minutum is complicated in species concept, with the proliferation of the fronds recognized as a key character of this monotypic section of the genus *Crepidomanes* (Ebihara et al. 2006). Nitta et al. (2011) detected reticulate evolution using comprehensive morphology, sequences of nuclear and chloroplast DNA, cytology, and spore counts, covering wide distribution areas in the Old World tropics. The results showed three major clades nested within a single species: (i) the African clade; (ii) clade I from East Asia and Pacific distribution with uniform morphological characters and only a few hybrid events; and lastly (iii) clade II from Southeast Asia and South Pacific, which contains a large amount of variation resulting from hybrid events. The hybrid taxa are stabilized through apogamy and polyploidy mechanisms. Thailand samples are nested in Clade II. The frond morphology of counted specimens is similar to specimens from other areas, but no relations between frond morphology, chromosome number, and phylogenetic relations were detected. Further investigation of chromosome numbers from various populations may deepen our understanding of these mechanisms in this species complex.

Hymenophyllum* Sm. subg. *Hymenophyllum

Chromosome base number (*x*)=11-28 (Ebihara et al. 2006; Iwatsuki and Ebihara 2023)

Hymenophyllum acanthoides (Bosch) Rosenst.

Specimens examined: *S. Chokrassameehirun 22-74* (Figure 1.C).

2*n*=2*x*=41 [diploid].

This is the first chromosome number record for this species. The number 2*n*=41 (Figure 2.C) was confirmed by two mitotic fixed cells from a single voucher specimen, the newly observed and unique number from an entire species in subg. *Hymenophyllum*. According to the base number, it is suggested that this is a diploid cytotype species, possibly resulting from irregular meiosis. However, there is still a lack of meiotic chromosome behavior observed from our available materials.

Hymenophyllum acanthoides belongs to the largest subgenus in *Hymenophyllum*, subg. *Hymenophyllum*, consisting of ca. 100 species. Analysis of the phylogenetic relationship revealed the existence of *H. acanthoides* clade within the subgenus, supported by Hennequin et al. (2010) and Del Rio et al. (2017). The trend of chromosome number reduction from *n*=22 to *n*=21 is observed in the *H. acanthoides* clade *sensu* Hennequin et al. (2010). Our results present a transition event of reduction in chromosome number from *n*=21 to *n*=20 in *H. acanthoides* species. Aneuploid reduction seems to be a main mechanism in the evolution of subg. *Hymenophyllum*, especially in the *H. acanthoides* clade. On the other hand, the sister clade of the genus *Trichomanes s.l.* (*sensu* Ebihara et al. 2006) exhibited much less variation of chromosome numbers within each genus compared to *Hymenophyllum* (Hennequin et al. 2010; Abreu et al. 2024).

This species, *H. acanthoides* is recognized as closely related to *H. denticulatum* in morphological characters and is strongly supported to be a sister clade in phylogenetic relationships (Del Rio et al. 2017). Discriminate characters between these two species were illustrated by Iwatsuki and Ebihara (2023), mainly based on the continuity and degree of crispiness on the margin of segments. Sangrit (2008) studied rhizome anatomy, and the results revealed the similarity of these species, as no discrimination between the two taxa was detected from rhizome anatomy. Nevertheless, the cytological study of *H. denticulatum* is still unsolved, and more sampling of these two species should be made to clarify their relation. In addition, observation of meiotic and mitotic cells based on the same voucher specimens may be useful to determine aneuploid reduction events that resulted in high variations of chromosome number within the subgenus (Abreu et al. 2024).

Hymenophyllum exsertum Wall.

Specimens examined: *S. Chokrassameehirun 20-34, 23-80* (Figure 1.D).

2*n*=2*x*=42 [diploid].

As one of the most common species, it is recorded in all regions of Thailand. It has a wide distribution, ranging from India, Southern China, Myanmar, Thailand, Laos, Cambodia, Vietnam, and Peninsular Malaysia (Iwatsuki and Ebihara 2023). Our observation is in congruence with many meiotic chromosome studies, *n*=21 recorded from India and Sri Lanka (Manton and Sledge 1954; Mehra and Singh 1957; Ghatak 1964; Irudayaraj and Manickam 1987; Manickam and Irudayaraj 1988). Our result is the first mitotic count for *H. exsertum* (Figure 2.D), which confirmed the diploid cytotype of this species. The chromosome number is constant for this species. Our data also supports the phylogenetic position of this species belonging to subg. *Hymenophyllum* rather than *Mecodium*, which has the chromosome base number *x*=28 (Ebihara et al. 2006; Hennequin et al. 2006, 2010; Del Rio et al. 2017). Unique morphological characters are fronds being bipinnatifid, flat, entire at the margin, and densely covered with long brown hairs at the abaxial surfaces of axes. It is easily distinguished from other members of subg. *Hymenophyllum*, which are serrated or toothed at the margin of segments (Iwatsuki and Ebihara 2023).

***Hymenophyllum* Sm. subg. *Globosa* (Prantl) Ebihara & K.Iwats.**

Chromosome base number (*x*)=36 (Ebihara et al. 2006; Iwatsuki and Ebihara 2023)

Hymenophyllum longifolium Alderw.

Specimens examined: *S. Chokrassameehirun 22-62* (Figure 1.E).

2*n*=2*x*=72 [diploid].

The mitotic chromosome number 2*n*=72 is the first record for this species (Figure 2.E). According to the base

number x=36 of subg. *Globosa* (Hennequin et al. 2010), the diploid cytotype is proposed based on our results.

Chromosome counts for subg. *Globosa* is still needed; nine of ca. 25 species display the uniform number of n=36 (Iwatsuki and Ebihara 2023; Abreu et al. 2024) and 2n=72 (in *H. javanicum* Spreng., Manton and Sledge 1954). Phylogenetic data support the relationship of subg. *Globosa* as the sister lineage to subg. *Sphaerocionium*, which shows stability in the chromosome number of this clade (Del Rio et al. 2017).

The morphology of this species is closely related to *H. badium* Hook. & Grev. and especially *H. junghuhnii* Bosch as mentioned by Iwatsuki and Ebihara (2023), differing by flat laminar, uniform shorter stipe, and reniform involucres with fused clavate receptacles. Additional data for this species and closely related species is still limited. Phylogenetic analysis and more chromosome observations should be made to clarify the relationship within the subgenus.

In conclusion, mitotic chromosome numbers of some Hymenophyllaceae from Thailand are consistent with the recent classification and phylogenetic relationship. The chromosome numbers of five diploid species from three genera were accounted. First records of chromosome number for *H. longifolium* and *H. acanthoides* were observed, with the latter species revealing some interesting chromosome number reduction in evolution within subg. *Hymenophyllum*. Currently, a coverage sample is still needed to the extent of species, and more mitotic together with meiotic chromosome observation may shed light on the classification and reflect the deep evolutionary background of the family.

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