Reticulate evolution in the *Pteris fauriei* group (Pteridaceae)

Yi-Shan Chao1*, Atsushi Ebihara2, Wen-Liang Chiou3, Jer-Min Tsai4, Yu-Wen Huang1 & Tom A. Ranker5

The *Pteris fauriei* group (Pteridaceae) has a wide distribution in Eastern Asia and includes 18 species with similar but varied morphology. We collected more than 300 specimens of the *P. fauriei* group and determined ploidy by flow cytometry and inferred phylogenies by molecular analyses of chloroplast and nuclear DNA markers. Our results reveal a complicated reticulate evolution, consisting of seven parental taxa and 58 hybrids. The large number of hybrid taxa have added significant morphological complexity to the group leading to difficult taxonomic issues. The hybrids generally had broader ranges and more populations than their parental taxa. Genetic combination of different pairs of parental species created divergent phenotypes of hybrids, exhibited by both morphological characteristics and ecological fidelities. Niche novelty could facilitate hybrid speciation. Apogamy is common in this group and potentially contributes to the sustainability of the whole group. We propose that frequent hybridizations among members of the *P. fauriei* group generate and maintain genetic diversity, via novel genetic combinations, niche differentiation, and apogamy.

Hybridization instantly creates novel combinations of genes and genomes and, therefore, can lead to rapid species differentiation. Reticulate evolution of ferns was first reported in *Asplenium* species. Subsequently, a growing number of studies have documented reticulate evolution in ferns, such as in *Dryopteris*, *Pteris*, and *Vandenboschia*. However, the role of reticulate evolution in promoting diversification remains unclear. For example, with various phenotypes arising quickly, hybridization may promote adaptive radiation. In angiosperms, rapid diversification is driven by various morphological, physiological, and genetic characters, as well as the origin of physical barriers that may combine to promote different ecological fidelities between parental and hybrid species.

Hybrids may spread to new ecological habitats, differing from those of the parental species, thus reducing competition with parental species and potentially facilitating hybrid speciation. A long-term field experiment of *Helianthus* provided direct evidence of hybridization driving adaptive radiation. In other words, ecological divergence of hybrids is likely to be related to the success of hybrid speciation, but the role of niche differentiation in the evolution of hybrid species in ferns has been little studied. Ecological differentiation in hybrid zones was first proposed in the *Pteris quadriaurita* complex and then reported in *Polystichum imbricans* (D.C.Eaton) D.H.Wagner and *Polystichum munitum* (Kaulf.) C.Presl (Dryopteridaceae). In ferns, hybridization is often accompanied by apogamy. Apogamy is found in 3% to 10% of fern species and results from the deregulation of reproductive pathways. Apogamous hybrid ferns are reported in many cryptic complexes, especially in *Aspleniacae*, *Dryopteridaceae*, and *Pteridaceae*. Apogamous species often have limited genetic variation compared to close sexual relatives, and apogamy has been considered a dead end for fern evolution; however, it is common and could play a special role in *Pteris*, *Dryopteris varia*, and *Diplazium hachijoense* complexes.

Interestingly, apogamous *Pteris* species are most prevalent in East Asia and South Asia and mostly found in sect. *Campteria*. The *Pteris fauriei* group (Pteridaceae), belonging to sect. *Campteria*, includes more than 20 taxa (18 species) with bipinnatifid laminae. Apogamy of many taxa in this group is probably associated with hybridization. In addition, similar morphology among species in this group is thought to be due to...
were marked (Fig. 1). The cpDNA topology was similar to the previously published group in the cpDNA phylogenetic analyses. For each species, types. Phylogenetic statistics are shown in Table 2. There were 20 cpDNA haplotypes; some were specific to one P. fauriei group in this study (Supplementary Table 1) were clustered and named as haplotypes and allele.

P. fauriei occurs in open habitats51,58. Most taxa occur in shady environments, like P. latipinna, or semi-open habitats, like P. fauriei, although P. minor occurs in open habitats53,58.

We addressed the following questions of the P. fauriei group: (1) Are any species of the P. fauriei group of hybrid origin? Chloroplast and nuclear DNA data were analyzed phylogenetically and integrated with ploidy level data to infer a possible network of hybridization. (2) Is morphological diversity related to hybridization? Morphological characteristics and genetic composition of each taxon were compared. (3) Is niche diversity related to hybridization? General attributes of ecological niches were compared among taxa. (4) What is the role of apogamy in the evolution of the P. fauriei group? Contributions and/or disadvantages of apogamy in hybridization were also discussed.

Results

Morphological and habitat characters. Samples of the Pteris fauriei group were collected from Eastern Asia, including Japan, Taiwan, China, Vietnam, and Malaysia, and included 20 taxa (18 species; Supplementary Table 1). No material of P. ochrominis, P. biaurita, P. minor, or P. oshimensis var. oshimensis, and P. wulaiensis. These are all diploids except for P. arisanensis, which has some homoyzgous triploid individuals. Only three of the diploid taxa—P. boninensis, P. minor, and P. oshimensis var. oshimensis—had 64 spores per sporangium, which indicated that they reproduce sexually. The other plants had 32 spores per sporangium, which we inferred to be apogamous (Table 1; Supplementary Table 1). It has been reported that sexual Pteris species produce 64 spores per sporangium whereas apogamous species produce 32 spores per sporangium, especially in P. fauriei and P. minor. Each plant produced only one type of sporangium.

Ploidy levels and reproductive modes. There were diploids and triploids in the P. fauriei group based on flow cytometry data; some named taxa had both diploids and triploids. Previous studies on ploidy levels in the P. fauriei group are cited in Table 1. There were seven taxa with homozygous nDNA genotypes that could be potential parental taxa (inferred by nDNA described in the section below): P. arisanensis, P. biaurita, P. boninensis, P. latipinna, P. minor, P. oshimensis var. oshimensis, and P. wulaiensis. These are all diploids except for P. arisanensis, which has some homoyzgous triploid individuals. Only three of the diploid taxa—P. boninensis, P. minor, and P. oshimensis var. oshimensis—had 64 spores per sporangium, which indicated that they reproduce sexually. The other plants had 32 spores per sporangium, which we inferred to be apogamous (Table 1; Supplementary Table 1). It has been reported that sexual Pteris species produce 64 spores per sporangium whereas apogamous species produce 32 spores per sporangium, especially in P. fauriei and P. minor. Each plant produced only one type of sporangium.

The phylogeny of chloroplast DNA and nuclear DNA. The sequences of rbcL, matK, IBR3, and Knox3 of the P. fauriei group in this study (Supplementary Table 1) were clustered and named as haplotypes and allele types. Phylogenetic statistics are shown in Table 2. There were 20 cpDNA haplotypes; some were specific to one species and some were shared by several species. We included different taxa, different species and/or one species with different cpDNA haplotypes, of the P. fauriei group in the cpDNA phylogenetic analyses. For each species, samples with different cpDNA haplotypes were included, and the samples with morphology identical to type specimens were marked (Fig. 1). The cpDNA topology was similar to the previously published Pteris phylogeny60, which resolved the P. fauriei group as part of sect. Camptera, although the supporting value was not very high.

Taxa belonging to the P. fauriei group are coded based on their haplotypes, such as ca, cc, and cf. Most species clustered within the main fauriei clade, but P. confusa (cc), P. aff. confusa (cu), P. kiuschiuenis (ck), and P. setulosocostulata (ch), as well as some plants of P. arisanensis (cx, cxx) and P. biaurita (ci, cxx) had distinct phylogenetic positions outside of the main clade. Only P. austrotaiwanensis (ct), P. boninensis (cb), P. confusa (cc), P. kiuschiuenis (ck), P. natiensis (cn), P. pseudowulaiensis (cw), and P. setulosocostulata (ch) had their own unique cpDNA haplotypes (Fig. 1), most taxa had more than one cpDNA haplotype. Some taxa shared haplotypes, indicating shared maternal lineages, such as P. arisanensis and P. biaurita (cxx), P. arisanensis and P. latipinna (ca); P. fauriei, P. minor, P. laurisilvica, and P. oshimensis var. oshimensis (cf); P. oshimensis var. paraemeiensis and P. satsumana (cs); and P. wulaiensis and P. fauriei (cy). The two varieties of P. oshimensis have different cpDNA haplotypes. Based on this fact, together with their distinctly different morphologies, we suggest that P. oshimensis var. paraemeiensis is a new species awaiting taxonomic revision.

The phylogenetic analysis of the Knox3 alleles supported six clades, which we labeled B, C, D, E, F + G, and H (Fig. 2a.). Group A is related to P. minor, and groups F and G correspond to P. arisanensis and P. biaurita, respectively, and are interdigitated. The IBR3 topology (Fig. 2b) approximately corresponded to that based on Knox3. Together with ploidy data, the genotypes of IBR3 and Knox3 were determined (Supplementary Table 1). Most of the genotypes were found in two or more samples from each taxon, except P. kawabatae, in which each individual sampled had a unique genotype (Table 1). The IBR3 gene exhibited fewer alleles than the Knox3 gene (Table 1) and had fewer genotypes, labeled as S, T, V, W, X, and Y. For example, samples with Knox3 genotype A1A1 and A4A4 were both IBR3 genotype T1T1 (Table 1). In some taxa, however, IBR3 exhibited more variation
| Scientific name | Type | Hybrid formula | Taxon1 | Taxon2 | cpDNA haplotype | Ploidy | Reproductive mode | Maternal lineage | Distribution, elevation (1988, 1996, m) | Phyletic data of previous studies |
|-----------------|------|----------------|--------|--------|----------------|--------|-------------------|-----------------|----------------------------------------|--------------------------------|
| **P. arisanensis** | T | DT7F7F3 | EV3YF7 | κα | Φκ | 48 | 27 | V87 | Chao214 | Taiwan, Japan |
| **P. austrotaiwanensis** | T | DT7F7F5 | EY21V22 | κα | Φκ | 48 | 32 | W23 | Hsu s.n.2013016 | Taiwan, Japan |
| **P. boninensis** | T | - | D7F4F5 | ηα | Φη | 48 | 32 | W23 | Chao217 | Taiwan, Japan |
| **P. confusa** | T | - | F7G1S14 | κε | Φε | 48 | 32 | Y21 | Chao217 | Taiwan, Japan |
| **P. fauriei** | T | - | A1A6D7 | ηα | Φη | 48 | 32 | T1 | Chao217 | Taiwan, Japan |
| **P. kawabatae** | T | - | C3E2V41 | κε | Φε | 48 | 32 | Y86 | Chao217 | Taiwan, Japan |
| **P. kiuschiuensis** | T | - | C3D4H1 | κα | Φκ | 48 | 32 | Y11 | Chao217 | Taiwan, Japan |
| **P. latipinna** | T | - | D4D7W28 | κα | Φκ | 48 | 32 | W23 | Chao217 | Taiwan, Japan |
| **P. m. oshimensis var. oshimensis** | T | - | A4D7W5 | κα | Φκ | 48 | 32 | T1 | Chao217 | Taiwan, Japan |
| **P. natiensis** | T | - | C6T21 | κα | Φκ | 48 | 32 | W14 | Chao217 | Taiwan, Japan |
| **P. oshimensis** | T | - | C5YF7 | κα | Φκ | 48 | 32 | Y87 | Chao217 | Taiwan, Japan |
| **P. wulaiensis** | T | - | D4D7 | κα | Φκ | 48 | 32 | W23 | Chao217 | Taiwan, Japan |

Continued
Table 1. The ploidy levels, cpDNA haplotypes, nDNA genotype, inferred maternal lineage, paternal lineage, and geographic distribution of samples in the *Pteris fauriei* group. Plants having identical morphology with the type specimens are indicated as Y. – No data. = The same as the above field. * Unasserted alleles. †Parents in the inferred hybrid formula are based on the species (or taxa) with homozygous genotypes.

| Dataset                   | Number of sequences | Haplotypes/alleles numbers | Total characters | Parsimony-informative characters | Log-likelihood score for ML tree |
|---------------------------|---------------------|-----------------------------|------------------|----------------------------------|----------------------------------|
| *rbcL* + *matK*           | 57                  | 19                          | 2184             | 165                              | −6583.6080                       |
| Knox3                     | 46                  | 38                          | 493              | 109                              | −2096.8907                       |
| IBR3                      | 39                  | 34                          | 397              | 45                               | −1268.7633                       |

Table 2. The characters of DNA datasets of *Pteris fauriei* group.

Parent and hybrid taxa assignment. By comparing cpDNA haplotypes (Fig. 1) and the phasing of nDNA genotypes (Fig. 3), the maternal lineages of the nDNA genotypes were inferred (Table 1). For example, the cf, cf', and cb cpDNA haplotypes were always present along with the group A allele of nDNA, and ca of cpDNA was found with allele D7 of *Knox3* nDNA. A reticulogram based on the *Knox3* gene was constructed, onto which we mapped ploidy levels, habitats, and reproductive modes (Fig. 5); the taxa correspond to the taxa in Table 1. Sexual plants were few, so we assumed that the probabilities of backcrossing with parents and introgression were low, especially with the apogamous diploid. Furthermore, the two nDNA markers presented similar topologies.

Based on the *Knox3* gene, 72 genotypes were inferred, including 14 putative parental genotypes and 58 genotypes of hybrid origin (Table 1). All species were identified based on morphology. Plants with homozygous genotypes or some sexual diploid genotypes possessing alleles from the same groups, are proposed as possible parental taxa. Seven putative parental taxa were discovered. Strictly speaking, only samples morphologically identical with type specimens of *P. arisanensis* (F group, F7F7F7), *P. minor* (A group except A7, A4, A11A13, A16A6, A6A6), and *P. latipinna* (D7D7) were homozygous, which suggests that they were not hybrid taxa. Most taxa in the *P. fauriei* group, however, appeared to be of hybrid origin. Although the other three putative species had homozygous samples, such as *P. minores* belonging to group G (samples with G1G1), *P. oshimensis var. oshimensis* belonging to A4 (Kuo 3445, A4A4), and *P. wulaeae* belonging to D4 (Ebihara et al. 3234, D4D4), they also had heterozygous taxa. It was difficult to separate the homozygous samples and heterozygous samples based on their morphology.
Pteris latipinna (D7D7) was the putative parental lineage of 18 hybrid taxa (Fig. 4), far more than any other species. Pteris wulaiensis (D4D4) was the second most important parental lineage, contributing to eight taxa. Homozygous P. oshimensis var. oshimensis (AAA4) was inferred to be the maternal parent of hybrid P. oshimensis var. oshimensis (A4D1) and P. cf. fauriei (A4D7).

The Knox3 alleles of F and G groups, corresponding to P. arisanensis and P. biaurita, respectively, were clustered together within one clade (Fig. 2). The two species can be identified by morphology (especially the costal veins, described in the section below) and were phylogenetically close based on Knox3. Some taxa were inferred...
to arise from hybridization of the two species, with each species serving as either paternal or maternal parent, such as F16G1 (P. arisanensis ♂ × P. biaurita ♂) and F7G1 (P. biaurita ♀ × P. arisanensis ♂). However, the possible parental individuals (F7F7F7 in P. arisanensis; G1G1, G1G4, G1G3 in P. biaurita) were all apogamous. We have not found sexual diploid individuals of the two species.

Pteris latipinna was also involved in hybrid formation with each of those two species, such as D7F4F5 in P. arisanensis and D7G1 in P. biaurita.

Unique alleles only appeared in a few rare taxa. The C3 allele was found in P. kawabatae (C3D1, C3D7E2, C3E2), P. kiuschiuensis var. kiuschiuensis (C3H1, C3D4H1), P. natiensis (C3D7, C3D4D7), and P. satsumana (B1C3). Allele E2 was only found in P. kawabatae (C3D7E2, C3E2). Alleles H3, H5, and H10 were unique to Pteris setulosocostulata (D4H4H10, B2H3H5). Allele H1 was found in P. kawabatae (B4D5H1) and P. kiuschiuensis var. kiuschiuensis (C3H1, C3D4H1, D4D7H1). The relationships of P. setulosocostulata to P. kawabatae and P. kiuschiuensis var. kiuschiuensis were unclear because the sample sizes were small for the latter two taxa.

Genotypes resolving puzzles of morphology and habitats. The diverse morphology of the P. fauriei group appeared to be an outcome of a large number of hybridizations. By comparing morphological characters and molecular data (Table 1), the association of specific morphological character states with particular genotypes was explored. Some character states were only found in individuals with particular alleles (Table 3). We present apparent associations between morphological and ecological variation and genotypic markers as hypotheses that could be tested with more detailed analyses. We found that hybrid taxa exhibited the morphology associated with their parental taxa (Table 1).

Plants with homoygous genotypes provided more apparent evidence of character state/genotype associations, than did heterogeneous taxa (Tables 1 and 3). Number of pairs of basiscopic secondary pinnae were useful key characters in this group. In P. minor individuals with two or more pairs of basiscopic secondary pinnae or with tripartite laminae possessed alleles A1, A6, and A13. Pteris latipinna (D7D7) had the largest pinnae and the fewest lateral pinnae of all taxa; other taxa with wide pinnae had allele D7, including P. fauriei, P. kawabatae, P. laurisilvicola, and P. natiensis. It appears that the P. latipinna genome is associated with broad pinnae.

Figure 2. Nuclear DNA phylogeny of the Knox3 gene (a) and the IBR3 gene (b) of the Pteris fauriei group. ML bootstrap support values are indicated on each branch.
occurrence at high elevation, above 1000 m. Hybrids (with both F and G group alleles) had broad elevational.

\( \textit{P. minor} \) which might imply the decline of parental taxa. For example, \( \textit{fauriei} \) network in the \( \textit{P. fauriei} \)

\( \textit{P. arisanensis} \) We have clarified the hybridization patterns of \( \textit{Campteria} \) is a key mechanism facilitating the diversification of sect. \( \textit{P. fauriei} \) occurs across a broader area from Japan, Taiwan, and eastern China.

cases of apparently extinct diploid parental taxa are common in many fern complexes and in the \( \textit{P. fauriei} \) group as well. The distributions of extant parental taxa are much narrower than those of the hybrids, which might imply the decline of parental taxa. For example, \( \textit{latipinna} \) (apogamous; semi-shady), is an intermediate habitat between its parental taxa (sexual; open) and (apogamous; semi-shady), is an intermediate habitat between its parental taxa.

Cases of apparently extinct diploid parental taxa are common in many fern complexes and in the \( \textit{P. fauriei} \) group as well. The distributions of extant parental taxa are much narrower than those of the hybrids, which might imply the decline of parental taxa. For example, \( \textit{latipinna} \) (apogamous; semi-shady), is an intermediate habitat between its parental taxa (sexual; open) and (apogamous; semi-shady). By contrast, homoygous plants of \( \textit{P. arisanensis} \) and \( \textit{P. biaurita} \) were located at high and low elevation, respectively, and putative hybrids of the two species showed a wider elevational range than the parents, essentially representing the addition of ranges of both parental taxa.

Frequent hybridization with low introgression is found in hybrid zones of cacti, apparently due to postzygotic barriers. Our results also suggest that hybridization has enhanced range expansion of the entire group. We found that \( \textit{confusa} \) and \( \textit{praeternissa} \) have the same maternal lineage. However, because of limited materials, the hybridization involving \( \textit{confusa} \) is still unclear.

The assignment of homoeologs is complicated in taxa involved in hybridization and polyploidy (tetraploid, etc.). Recently, more applicable methods for phasing gene copies into polyploid subgenomes are now in development, focusing on several loci or target capture data. Even though we found no evidence of possible introgression in the \( \textit{P. fauriei} \) group, we revealed that multiple hybridizations contributed to offspring exhibiting diverse phenotypes. Frequent hybridization with low introgression is found in hybrid zones of cacti, apparently due to postzygotic isolation.

Diversification of the \( \textit{P. fauriei} \) group appears to be related to different environments. For example, light intensity of \( \textit{P. fauriei} \) (apogamous; semi-shady), is an intermediate habitat between its parental taxa \( \textit{P. minor} \) (sexual; open) and \( \textit{P. latipinna} \) (apogamous; semi-shady). By contrast, homoygous plants of \( \textit{P. arisanensis} \) and \( \textit{P. biaurita} \) were located at high and low elevation, respectively, and putative hybrids of the two species showed a wider elevational range than the parents, essentially representing the addition of ranges of both parental taxa.

Cases of apparently extinct diploid parental taxa are common in many fern complexes and in the \( \textit{P. fauriei} \) group as well. The distributions of extant parental taxa are much narrower than those of the hybrids, which might imply the decline of parental taxa. For example, \( \textit{latipinna} \) is an important parent, while the known populations are fewer than ten in China and Taiwan and \( \textit{P. minor} \) is limited to Iriomote Isl. (Japan) and Taiwan. Their hybrid \( \textit{P. fauriei} \) occurs a across a broader area from Japan, Taiwan, and eastern China. \( \textit{Pteris oshimensis} \) var. \( \textit{oshimensis} \) is distributed across Japan, but the paternal plants are only found in Amami Is, Japan. Thus, the ranges of hybrids often exceed those of their parents. The wide distribution of the \( \textit{P. fauriei} \) group also suggests that hybridization has enhanced range expansion of the entire group.

After long-distance dispersal, apogamy and gametophytic selfing could be adaptive because either is more likely to produce a new population than would sexual reproduction between gametophytes. Apomictic angiosperms (i.e., asexual reproducing via seeds) tend to predominate in environments unfavorable for sexual reproduction, such as at higher latitudes and elevations, and have a wider distribution than their sexual relatives. The apogamous triploid fern \( \textit{Myriopteris gracilis} \) has a wide distribution which might be related to its ability to reproduce asexually. Niche differentiation of apogamous and sexual fern taxa has also been documented previously, similar to what we have observed in the \( \textit{P. fauriei} \) group in the current study.
the primers for Miseq analyses. We tested the IBR3 group are the same as those listed in Table 1.

The two heatmap columns show the corresponding alleles of the two nDNA genes and are colored by the marginal posterior probability of the phase assignment. Another column presents the mean marginal probability across the two loci of the phasing assignment per tip. Taxa in red are putative parents. All the samples of the Pteris fauriei group are the same as those listed in Table 1.

those differences result from reproductive modes and/or higher fitness of certain genotypes needs to be explored further.

Within the P. fauriei complex, apogamy is the main reproductive mode with only three sexual taxa. In general, sexual taxa, and lineages with low genetic variation, are thought to be less “adaptable” than are sexual taxa84. Furthermore, without recombination, deleterious mutations could accumulate and decrease the fixation of beneficial mutations (Muller’s ratchet)86,87. However, new genetic combinations from hybridization could induce phenotypic differentiation of a hybrid from its progenitors88,89. In the P. fauriei group, the extant hybrid taxa, even being apogamous, are apparently the survivors of natural selection and have putatively retained adaptive genotypes that arose from hybridizations, even though some of their parental taxa may be extinct.

Furthermore, apogamous taxa have evolutionary advantages that might overcome the potential deleterious effects of low genetic variation. Epigenetic modification has been proposed to explain high fitness of apogamous taxa84. Moreover, Klekowski90 proposed that genetic segregation is possible via homoeologous chromosome pairing during sporogenesis leading to genetic variation in apogamous ferns as was found in the study of Cyrtomium fortunei (Dryopteridaceae)91,92.

Of the seven diploid putative parental taxa, five are apogamous. We hypothesize that some parental taxa with apogamy in the P. fauriei group were primarily sexual and then apogamy developed subsequent to the production of hybrids. Apogamous homozygous diploids are more likely derived from sexual diploids rather than from hybrid taxa. The exact mechanisms of sexual taxa giving rise to apogamous taxa could be related to environmental factors such as light, water, and sugar83–85. Some critical genes expressed during reproduction could control the process of apogamy86–88. Apogamous gametophytes could contribute functional male gametes, which could account for the origin of some triploid taxa in the P. fauriei group.

The putative reticulate evolution of the P. fauriei group demonstrates a pattern of hybridization in evolutionary radiation, with limited or no introgression. Parental taxa have close phylogenetic relationships and novel genetic combinations in hybrids led to a rapid increase of phenotypic variation, such as diversification of niche fidelities, compared to parental taxa. Apogamy leads to the genetic fixation of some successful hybrid lineages under natural selection. While some parental species could be extinct, some of their genetic diversity will be preserved within the hybrids. Hybridization could lead to abundant taxa, more phenotypic variation, and broader distributions (as discussed above). Our ongoing and future research will explore the relationship of niche differentiation to hybridization, especially by exploring ecophysiological characters and environmental conditions. We hope to know if hybridization plays a role to maintain genetic variation and increase adaptation of ferns.

Methods

Sampling. Voucher specimens of samples were deposited at the herbarium of the Taiwan Forestry Research Institute (TAIF; Supplementary Table 1). All sampled plants were identified based on the morphology of type specimens and original protologues; the plants that were morphologically the most similar to the types were marked as representatives of specific species. To identify the plants, morphological characteristics were examined following Chao, et al.51. Use of plant material in the study complied with relevant institutional, national, and international guidelines and legislation.

Ploidy analysis and reproductive systems. Ploidy levels of samples were determined by flow cytometry, if fresh leaves were available, following the methods Chao, et al.51. Chromosome numbers of some samples had been counted in previous studies46,52. Previously published accounts of ploidy levels and reproductive modes of the P. fauriei group were also used (Table 1).

The fact that the sexual Pteris plants produce 64 spores per sporangium and apogamous plants produce 32 or fewer spores per sporangium was used to infer the reproductive mode of each plant, corresponding to sexual or apogamous84,85,91. Five mature sporangia were picked randomly from each plant and spores were counted under a microscope.

DNA isolation, amplification. Materials for molecular analyses were preserved in silica gel. Total genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method102. Two plastid gene markers, rbcL and matK, were amplified by the primers employed in previous Pteris studies84. The two nuclear DNA (nDNA) putative single-copy genes, Knox3 and IBR3, were analyzed by single-strand conformation polymorphism (SSCP; see description below) and sequenced on the Illumina Miseq platform. Five primers for Knox3 were designed based on transcriptome sequencing data104. Knox3PF2 (ACA TTC AAG GAG CAG CCA CCG CAC C). All primers for Miseq were designed to target sequences shorter

Figure 3. The phasing inference of the Knox 3 gene and the IBR3 gene of the Pteris fauriei group by homologizer77. Bayesian posterior probabilities of branches are indicated, and the values < 0.5 are not shown.
than 500 bp. Both forward and reverse primers for Knox3 and IBR3 were synthesized with an 8-base barcode to produce amplicons; the barcodes were designed as recommended by Roche.

**Figure 4.** Phylogenetic network of the *Pteris fauriei* group, based on Knox3 gene. Color of line indicates lineage of Knox3 grouping. A group in red; B group in light purple; C group in orange; D group in yellow green; E group, in blue; F group in green; G group in limon. The dash black lines mean hybrid taxa. The taxa correspond to samples in Table 1.
Figure 5. A reticulogram of the *Pteris fauriei* group, based on the Knox3 sequences. The taxa correspond to the genotypes in Table 1. Maternal contributors of hybrids are shown as red arrows and paternal contributors as blue arrows. Ploidy levels are indicated by symbols: ellipse for diploids, triangle for triploids, and rectangle for unknown ploidy. Different kind of habitats are separated by colors inside symbols. Red or green symbol outlines indicate reproductive mode (sex and apogamy, respectively). Undiscovered taxa have dashed-lined outlines. Alleles of Knox3 and cpDNA haplotypes of each taxon are also presented.

**Single-strand conformation polymorphism.** Some Knox3 data were analyzed by SSCP. The SSCP analyses were conducted by isolating the nDNA loci from PCR products for each individual through separation on SSCP gels, following the methods of Ebihara, et al. Individual bands were isolated from gels and purified by the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd. Taipei, Taiwan) and amplified by primers Knox3PF2NE and Knox3PR3 for further Sanger sequencing.

**Library preparation, sequencing and quality assessment for NGS.** Each amplicon was extracted with the illustra GFX PCR DNA and Gel Band Purification Kit (GE, UK). The amplicon mixtures were pooled with equal quantities of DNA, then a total amount of 150 ng of amplicons was used as input material for the DNA library preparations. The sequencing library was generated using the Truseq Nano DNA HT Sample Prep Kit (Illumina, USA) following the manufacturer’s recommendations, and index codes were added to each sample. DNA fragments were ligated with the adapter for Illumina sequencing, followed by further PCR amplification. Then the PCR products were purified (SPRIselect reagent, Beckman) and DNA size spectra were determined using an Agilent 2100 Bioanalyzer and quantified with a Qubit fluorometer (Invitrogen, Carlsbad, California, USA). Finally, the DNA libraries were sequenced using the Illumina Miseq platform, and 300 bp paired-end reads were generated. Sequencing output was deposited in GenBank (Supplementary Table 1).

**Bioinformatic analysis for nDNA data.** Raw data were cleaned by FastQC and Trimmomatic to remove the adapter and low quality bases and reads. The forward and reverse reads were merged using PEAR. The cleaned data were demultiplexed and clustered (including chimeras removed), and chimeras were removed using PURC. We kept the clusters comprising the three (for diploid samples) or four highest number of reads (for triploid or ploidy unknown samples). The barcode sequences were identified and removed, that is, each read was assigned to its source accession, then primers were trimmed. The sequences obtained from SSCP and Sanger
sequencing were used as the reference sequences for the clustering of consensus sequences. The sequences representing clusters of less than 100 original reads were removed. The consensus sequences of each sample were the alleles of nDNA genes and were ready for downstream phylogenetic analyses. The sequences of the **Knox3** gene, both from SSCP (and Sanger sequencing) and NGS, were pooled together.

**Data analysis.** Sequences were automatically aligned using MUSCLE and then manually edited with BioEdit 7.1.1. Using DnaSP, the sequences of the cpDNA and two nDNA markers were clustered by haplotypes and type of alleles, respectively. To exclude errors in amplicon data, we only kept the alleles found in two or more samples. We hypothesized that the taxa that had only one allele type were homozygous and classified them as parental taxa (non-hybrid taxa), regardless of whether their reproduction modes were sexual or apogamous. Allele numbers of genotypes were supported by ploidy data. When the allele number was smaller than the ploidy level, it was difficult to infer the exact allelic composition; in those cases, “*” stands for unassorted alleles.

Maximum likelihood analyses were performed for cpDNA haplotypes and nDNA sequences using the program GARLI v.2.0.10. *Pteris* species in sect. *Campteris* were included, and species in other sections, including *P. bella* Tagawa, *P. formosana* Baker, *P. kidoi* Sa.Kurata, *P. longipes* D.Don, and *P. wallichiana* J.Agardh, were chosen as outgroups. For the three sequence datasets—cpDNA, *Knox3*, and *IBR3*—three phylogenetic analyses were conducted by ten independent runs, from different random sequence-order starting trees, based on automatic termination following 10,000 generations without a significant topological change. The ML bootstrap support for each clade was assessed by performing 1000 bootstrap replicates, each replicate with one single tree search with the same search parameters as above. A 50% majority rule consensus tree was then calculated using PAUP* v. 4.0b10. Gaps were treated as missing data. Then the nDNA genotypes, *Knox3* and *IBR3*, were compared visually to the cpDNA markers to infer possible hybridization patterns. A phylogenetic network was visualized with Dendroscope 3.

We also used homologizer to phase the alleles of the two nDNA genes (linking the tree of the two genes) and infer a phylogeny. The potential parental taxa with homozygous nDNA genotypes were fixed firstly to infer the pattern of the subgenome (or allele) evolution of this group. The two genes were modelled by an independent

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**Table 3.** The genetic lineage inferences of morphological and habitat characters, based on the *Knox3* gene marker (Table 1) of *Pteris fauriei* group. The taxa of *Pteris fauriei* group exhibit the morphology corresponded to their own genotypes. The alleles corresponding to the character state are difficult to infer.

| Characters | State 1 | Alleles | State 2 | Alleles | State 3 | Alleles |
|-----------|---------|---------|---------|---------|---------|---------|
| 1) Lamina ratio of length to width | 1.1–1.3 | A1, C3, D7 | 1.4–1.7 | Undetermined, except A1, C3, D7 | 1.8–2 | Undetermined, except A1, C3, D7 |
| 2) Stipe color | Stramineous or green | Except D4 | Red-brown | D4 |
| 3) Stipe base thick | 2.5–4 mm | Except A4, D5 | Stipes < 2.5 mm | A4, D5 |
| 4) Exaggerated basiscopic pinnacles | 1 pair | Undetermined | 2 or more pairs | A1, A4, A6, H1 | Almost triplicate | A1, A4, A6 |
| 5) Number of lateral pinnae | < 6 | C3, D4, D7 | 6–8 | – | ≥ 9 | A4, H1 |
| 6) Pinna angles against rachis | 60–70° | Undetermined, except C3 | 71–80° | Undetermined, except C3 | 81–90° | C3 |
| 7) Pinna straight or incurve | Straight | Except C3, H1 | Incurved | C3, H1 |
| 8) Pinnule width | > 3 mm | D4, D5 | ≤ 3 mm | Except D4, D5 |
| 9) Pinna stalks | Distinct | D4 | Sessile | Except C3, D4, H group | Basal segments connecting to midribs | C3, H group |
| 10) Basal pinnules of lateral pinnae | Not decurrent, falcate | – | Decurrent, triangular | A4, A7, D7, F |
| 11) Pinnae apices | Acute or caudate, short tails < 2.5 cm | – | Caudate, long tails > 3 cm | A7, C3, D7 |
| 12) Pinna width | > 3 cm | A4, D4, H1 | 3–4 cm | – | > 4 cm | D7 |
| 13) Length ratio of basiscopic pinnules with acrosopic ones | 1–1.4 | – | 1.5–2 | A5, C3 |
| 14) Pinna width variation | Equally wide | – | Narrowed at base | D4, D7 | Widest at base | A4 |
| 15) Pinnae apices of sterile fronds | Acute | – | Round | F1, H1 |
| 16) Angle of pinnules against costae | 60–70° | – | 71–80° | – | 81–85° | H1 |
| 17) Venation | Free | Except F & G groups | Costal veins triangular | F group | Costal veins areolate | G group |
| 18) Distribution elevation | ≤ 1000 m | Except F group | > 1000 m | F group | – |
| 18) Habitats | Full sun, seacoast | A1, A6 | Semi–shade, near seacoast | Under forest | – |
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Author contributions

Y.S.C conceived and designed the research. Y.S.C and Y.W.H. carried out the experiments and performed the analyses; J.M.T. managed the computer science; Y.S.C. and A.E. collected the samples and data. Y.S.C., A.E., W.L.C., and T.A.R. discussed the results and wrote the manuscript.

Competing interests

The authors declare no competing interests.
Additional information

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Correspondence and requests for materials should be addressed to Y.-S.C.

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