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Patterns of hybrid formation among cryptic species of bird-nest fern, *Asplenium nidus* complex (Aspleniaceae), in West Malesia

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In order to clarify patterns of hybrid formation in the *Asplenium nidus* complex, artificial crossing experiments were performed between individuals of genetically differentiated groups based on the sequence of the *rbcL* gene, including *A. australasicum* from New Caledonia, *A. setoi* from Japan and several cryptic species in the *A. nidus* complex. No hybrid plants were obtained in crosses between nine of the 16 pairs. Even for pairs that generated hybrids, the frequency of hybrid formation was lower than expected given random mating, or only one group was able to act as the maternal parent, when the genetic distance (Kimura's two parameter) between parental individuals was at least 0.006. Sterile hybrids were produced by three pairs that were distantly related but capable of forming hybrids. Considering the results of the crosses together with the genetic distance between the parental individuals, it seems that the frequency of hybrid formation decreases rapidly with increasing divergence. The frequency of hybrid formation has not been previously examined in homosporous ferns, but it seems that a low frequency of hybrid formation can function as an important mechanism of reproductive isolation between closely related pairs of species in the *A. nidus* complex in addition to hybrid sterility. *Botanical Journal of the Linnean Society*, 2009, **160**, 42–63.

ADDITIONAL KEYWORDS: artificial crossing – *rbcL*.

INTRODUCTION

Recent data from various animal taxa, including *Drosophila*, frogs, rotifers and cichlids, support the hypothesis that reproductive isolation increases with genetic distance, which can be expected to increase with time since divergence (Coyne & Orr, 1989; Fu, Hagiwara & Hirayama, 1993; Mckaye *et al.*, 1993; Gleason & Ritchie, 1998; Sasa, Chippindale & Johnson, 1998; Presgraves, 2002). Hybridization is considered a common event among plants but is relatively rare among animal taxa (Arnold, 1997). In various plant groups, not only closely related sister species but also distantly related species sometimes form hybrids (e.g. Alice *et al.*, 2001; Yokoyama, Fukuda, Yokoyama *et al.*, 2002; Scareli-Santos *et al.*, 2007). Although an uneven distribution of hybridization has been recognized among taxonomic groups (Stace, 1975; Ellstrand, Whitkus & Rieseberg, 1996) and even within a single genus (Cayouette & Catling, 1992), the frequency of hybrid formation would be expected to decrease with genetic divergence. A survey of patterns of hybrid formation in plants may provide a novel perspective on the role of post-mating isolating mechanisms in speciation. There are, however, only a few prior studies that have focused on the correlation between the frequency of hybrid formation and genetic distance (Scacchi, Angels & Lanzara, 1990; Kim & Jansen, 1998).

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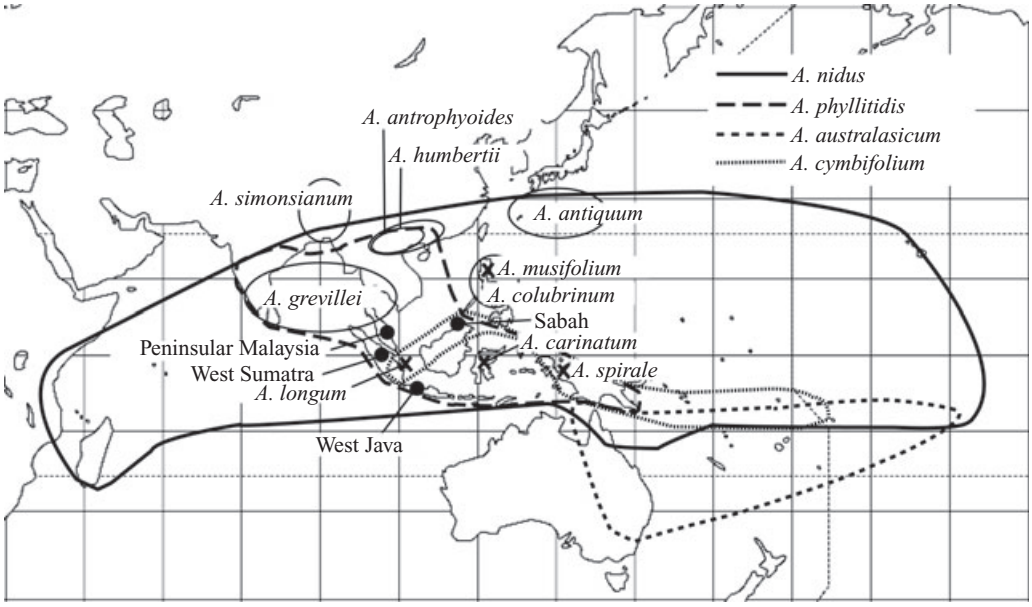


Figure 1. Distribution of *Asplenium* section *Thamnopteris*. Crosses indicate the type localities for the four species, the distribution of which have not been described in Holttum (1974). Solid circles indicate the investigated localities in west Malesia.

In ferns and fern allies, natural hybrids between various taxa have been reported (Knobloch, 1996) and a considerable volume of data on hybrid formation in natural populations is available. In particular, natural hybrids in Aspleniaceae became well known owing to the excellent work of Wagner (1954) on reticulate evolution of North American species. Murakami *et al.* (1999a) observed natural hybridization to occur only between closely related *Asplenium* spp. Similarly, Perrie & Brownsey (2005) reported that the *Asplenium* spp. participating in hybridization in New Zealand formed a closely related group in the phylogenetic tree based on *rbcL* sequence data, whereas non-hybridizing species had closer affinities to species from outside New Zealand.

Artificial crossing experiments to obtain hybrid ferns are also well established; these artificial crosses use gametophytes, which can be easily handled during crossing because of their small size and independence from sporophytes (Lovis, 1968). Most studies have focused on the degree of sterility in the obtained hybrids (e.g. Gibby & Walker, 1977; Fraser-Jenkins & Gibby, 1980; Gibby, 1982; Masuyama & Watano, 1994) and few data are available in the literature on the capacity to form hybrids or the frequency of hybrid formation (Boom, 1980; Schneller, 1981). The capacity to form hybrids has not been well assessed in ferns because no controls were included in the classic artificial crossing experiments. However, the inability to form hybrids can play an important role in reproductive isolation, in addition to hybrid sterility. In the present study, we performed artificial crossing experiments and examined the capacity of various pairs to form hybrids in order to study the correlation between the frequency of hybrid formation and genetic distance.

We used the *Asplenium nidus* L. complex as material for our artificial crossing experiments. *Asplenium nidus* is assigned to *Asplenium* L. section *Thamnopteris* Presl. Section *Thamnopteris* is a group of epiphytic ferns with sessile simple fronds attached to the caudex in an ascending spiral arrangement with submarginal veins connecting the lateral veins. It is distributed in the tropics and subtropics of the Old World from East Africa to Hawaii (Fig. 1; Holttum, 1974). Thirty-three species have been described (Ching, 1964; Holttum, 1974) and 15 species were monographed by Holttum (1974). *Asplenium nidus sensu* Holttum (1974) is morphologically variable in size and shape of fronds and length of sori and the other 14 species can be distinguished from *A. nidus* by distinctive characters including dilated or narrow bases of fronds or sharply keeled costa (Holttum, 1974). However, neither *Asplenium* section *Thamnopteris* nor plant materials identified as *A. nidus* was monophyletic based on molecular phylogenetic trees constructed on the basis of *rbcL* sequences (Murakami *et al.*, 1999a; Yatabe & Murakami, 2003; Perrie & Brownsey, 2005). Several other species of *Asplenium* are nested within *A. nidus* and the species boundaries of *A. nidus* and related species are not clear.

Cryptic species may be common in fern taxa, reflecting the lack of morphological divergence accompanying speciation events (Paris, Wagner & Wagner, 1989). Species should be delimited on the basis of genetic discontinuities and cryptic species represent distinct evolutionary lineages because they are reproductively isolated (Paris *et al.*, 1989). *Asplenium nidus* may include several cryptic species. In west Java, *A. nidus* was found from the lowlands to the highlands and exhibited five *rbcL* sequence types (Murakami *et al.*, 1999b). Individuals with these five *rbcL* sequence types are genetically differentiated by allozyme polymorphisms and are also ecologically differentiated by their habitat preference (the position on the tree trunk and the altitude at which they grow) (Murakami *et al.*, 1999b; Yatabe & Murakami, 2003). Yatabe *et al.*, (2001) performed crossing experiments using individuals of this species and obtained data indicating that some pairs of individuals with different *rbcL* sequences may be incapable of generating F1 hybrids. These results suggest that individuals with these *rbcL* sequence types are separate cryptic species. In order to examine the degree of reproductive isolation among closely related pairs of species, it is not enough to use only species that have been already described or are morphologically distinguishable; it is also necessary to examine pairs of cryptic species.

Furthermore, to study the correlation between capacity to form hybrids and divergence time, it is necessary to estimate the genetic distance, which is correlated with divergence time, between parental individuals in crossing experiments. In Aspleniaceae, however, as in many other plant taxa, various levels of polyploidy exist and reticulate events have occurred (Wagner, 1954). Chromosome numbers have been investigated for four species of the *Asplenium nidus* complex and only tetraploids with $2n = 144$ have been recorded to date [*A. australasicum* (J. Sm.) Hook.: Tindale & Roy, 2002; *A. antiquum* Makino: Kawakami, 1970; *A. nidus*: Bir, 1960; Abraham, Ninan & Mathew, 1962; Kawakami, 1970; Koul, 1970; Yatabe *et al.*, 2001; *A. setoi* N. Murak & Seriz.: Nakato, 1987]. Perrie & Brownsey (2005) suggested that the clade in which the *A. nidus* complex nests may be ancestrally tetraploid, as no diploid counts are known. Furthermore, the phylogenetic trees for the complex based on the maternally inherited *rbcL* (Gastony & Yatskievych, 1992) were highly concordant with dendrograms based on biparentally inherited allozymes, although the topologies were not entirely consistent because of a difference in the position of the individuals from Cat Ba Island in Vietnam (Murakami *et al.*, 1999c). Therefore, a phylogenetic tree based on *rbcL* may reflect evolutionary history sufficiently accurately and the genetic distance evaluated based on *rbcL* may be adequate as a measure of divergence to study the correlation between capacity to form hybrids and divergence time.

In the present study, we carried out artificial crosses between pairs of *A. nidus* complex individuals with various *rbcL* types and focused on the questions of how widespread hybrid formation between distantly related species is in this group and how the capacity to form hybrids and the frequency of hybrid formation change with divergence. Most of the materials used in this study were collected from the west Malesian region, including west Java, and using our data we are also able to estimate the number of cryptic species distributed in that region.

MATERIAL AND METHODS

Material

Two hundred and fifty-one individuals of the *A. nidus* complex were collected from west Malesia between 1997 and 2002. The *rbcL* gene was sequenced in order to determine how many *rbcL* sequence types were distributed in this region. The specific localities we investigated were west Java, peninsular Malaysia, west Sumatra and Sabah (Fig. 1). Voucher specimens have been deposited in the herbarium of the Graduate School of Science, Kyoto University (KYO). Details of the localities investigated are provided in Table 1. Voucher information for individuals collected from outside west Malesia that were used for phylogenetic analysis is listed in Table 2.

For artificial crossing experiments, fresh green leaves with mature sori were collected. At least four leaves were collected from each individual, one of which was used for DNA extraction and allozyme analyses, another leaf was kept in KYO as a voucher specimen and the remaining specimens were used for spore collection. The materials used for artificial crossing experiments are listed in Table 3.

rbcL sequencing

Total DNA was extracted using 2 × hexadecyl trimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA) in accordance with the method of Doyle & Doyle (1987).

Polymerase chain reaction (PCR) amplification of *rbcL* fragments was carried out using the method of Murakami *et al.* (1999a). The PCR products were purified using a Gene Clean III kit (BIO101, Vista, CA, USA) and then used as templates for direct sequencing. Sequencing reaction mixtures were prepared using a Big Dye terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Foster, CA, USA) and the reaction mixtures were analysed on an

Table 1. Investigated localities and the number of individuals of each *rbcL* sequence type found in west Malesia

Locality (abbreviation)	*rbcL* sequence type (number of individuals)
West Java	
Halimun National Park (HA)*	A(16), B(10), C(15), D(8), E(4)
Gunung Gede Pangrango National Park, West Java*	C(22), D(8)
Bogor Botanical Garden (BO)*	E(5)
Peninsular Malaysia	
Cameron Highland (CA)*	AII(1), E(17), F(27), G(9), H(5)
Fraser's Hill (FR)*	AII(3), E(12), EIII(1), EIV(1), F(5), G(2)
Mt Tahan*	E(1), F(1)
Kuala Lumpur*	G(2)
West Sumatra	
Within a radius of 50 km from Padang City (PA)*	AII(4), E(14), EII(1), F(12), FII(1), G(3), K(4)
Sabah	
Mt Kinabalu National Park	AII(10), E(8), I(2), I-II(4), H(5), J(1), G(7)

*Localities investigated in previous studies.

Applied Biosystems Model 377 automated sequencer (Perkin Elmer Applied Biosystems). Sequences were aligned using Sequence Navigator software (Perkin Elmer Applied Biosystems).

Phylogenetic analyses

The *rbcL* sequence data matrix contained data from 62 accessions, including *Hymenasplenium hondoense* (N. Murak. & Hatan.) Nakaike as an outgroup, deemed appropriate from a recent phylogenetic study (Schuettpelz & Pryer, 2007). Seventeen species of Aspleniaceae in addition to those in *Asplenium* section *Thamnopteris* were also included because the monophyly of section *Thamnopteris* has not been supported (Murakami *et al.*, 1999a; Perrie & Brownsey, 2005) and these 17 species formed clades with species of section *Thamnopteris* (Murakami *et al.*, 1999a; Schneider *et al.*, 2004; Perrie & Brownsey, 2005; Schneider *et al.*, 2005). The relevant database accession numbers are listed in Table 2. Phylogenetic analysis was performed with the maximum parsimony method using PAUP version 4.0b8 (Swofford, 2000) with the following options: heuristic search mode with 10 000 random-addition-sequence replicates, tree bisection–reconnection (TBR) branch swapping, MULTrees option on. Character state changes were treated as equally weighted. Branch support was estimated by bootstrap analyses (Felsenstein, 1985) with full heuristic searches, 1000 bootstrap replicate, 10 random-addition-sequence replicates per bootstrap replicate, TBR branch swapping and MULTrees option on, and saving all trees.

Reciprocal crossing experiments

In order to determine whether cross-fertilization is possible between individuals with various *rbcL* sequence types or individuals with the same *rbcL* sequence, we performed artificial crossing experiments and control crosses (Yatabe *et al.*, 2001). The number of nucleotides differing in the *rbcL* sequence and the genetic distance based on the *rbcL* sequence (Kimura, 1980) between the pairs used for the artificial crossing experiments varied from 1 to 25 and from 0.001 to 0.021, respectively.

Spores of each sporophyte used as a parent were sown on inorganic nutrient medium in Petri dishes 9 cm in diameter and were cultivated in a chamber, following the methods of Watano & Masuyama (1991). Information on the individuals used as spore sources is given in Table 3. Forty days after spores were sown, we obtained gametophytes with archegonia, which functioned as females, although gametophytes were not always found with antheridia. If necessary, we re-sowed the spores around the mature gametophytes. The spores were induced to germinate and to produce antheridia, probably owing to the presence of antheridogen (Näf, 1968) secreted by the mature female gametophytes. Forty days after re-sowing, we obtained gametophytes with antheridia, which functioned as males.

The method used to carry out the artificial crosses between hypothetical types X and Y is illustrated in Figure 2. Twenty gametophytes with archegonia of type X were transferred into the dish flooded with water in which many gametophytes with antheridia of type Y were growing (Fig. 2II). The same number of gametophytes of type X were also transferred into a dish of type X gametophytes as a control (Fig. 2I). These procedures were carried out reciprocally (Fig. 2III, IV) and the dishes were left overnight. We placed these 80 gametophytes on new medium and dried them for 1 h in a germ-free chamber (Clean Bench; Sanyo, Osaka, Japan) in order to prevent subsequent fertilization.

After 1 month, we began counting the number of juvenile sporophytes growing on the gametophytes. After 3 months, we analysed the allozymes of all the sporophytes obtained. Because the gametophytes of homosporous ferns are hermaphroditic and not entirely dichogamous, the gametophytes functioning

Table 2. Voucher information for phylogenetic analysis based on *rbcL* sequences

Species	Voucher	Locality	DDBJ no.
Asplenium section Thamnopteris			
A. antiquum Makino			
JP-Yakushima	JY 5151 (TI)	Suzunoko River, Yaku Is., Kagoshima Pref., Japan	AB013237
A. aff. antiquum			
VN-Sapa	KI *et al.* 94-V259 (TI)	Sapa, Hoang Lien Son Prov., Vietnam	AB013244
A. antrophyoides Christ			
CH-Menglun	KI *et al.* 100413 (KYO)	Menlun, Xishuanbanna, Yunnan Prov., China	AB097592*
A. australasicum (J. Sm.) Hook.			
AU-Brisbane	NS s.n. Aug.7, 1997 (KYO)	Brisbane, Australia	AB013249
New Caledonia	NM 97-N014 (KYO)	Mt Mou, Is. Grande Terre, New Caledonia	AB013250
A. cymbifolium Christ			
MY-Tawau	YY *et al.* 00-MY06 (KYO)	Tawau, Sabah Prov., Malaysia (cultivated in UKM)	AB097593*
A. nidus L.			
Type A	YY 98-ID14 (KYO)	Mt Halimun National Park, West Java, Indonesia	AB023500
Type AII	YY & AT 00-ID43 (KYO)	Lembah-harau, West Sumatra, Indonesia	AB023503*
Type B	KI 97-ID03 (KYO)	Mt Halimun National Park, West Java, Indonesia	AB023501
Type C	KI 97-ID23 (KYO)	Mt Halimun National Park, West Java, Indonesia	AB013245
Type D	YY 98-ID151 (KYO)	Mt Gede, West Java, Indonesia	AB023502
Type E	NM 97-ID35 (KYO)	Bogor Botanical Garden, West Java, Indonesia	AB023508
Type EII	YY & AT 00-ID01	Lembah-anai, West Sumatra, Indonesia	AB097595*
Type EIII	YY *et al.* 00-MY66 (KYO)	Fraser's Hill Pahang Prov., Malaysia	AB097596*
Type EIV	YY *et al.* 00-MY71 (KYO)	Fraser's Hill Pahang Prov., Malaysia	AB097597*
Type F	NM *et al.* 98-MY09 (KYO)	Selangor Prov., Malaysia (cultivated in National Univ. of Malaysia)	AB042147
Type FII	YY & AT 00-ID02 (KYO)	Airsirah, West Sumatra, Indonesia	AB097598*
Type G	NM *et al.* 98-MY01 (KYO)	Kampang Batu Tiga-tapah, Pahang Prov., Malaysia	AB042150
Type H	NM *et al.* 98-MY03 (KYO)	Mt Tahan, Pahang Prov., Malaysia	AB042144
Type I	NM *et al.* 98-MY31 (KYO)	Mesilau, Mt Kinabalu, Sabah Prov., Malaysia	AB097599*
Type I–II	YY *et al.* 00-MY01 (KYO)	Mesilau, Mt Kinabalu, Sabah Prov., Malaysia	AB097600*
Type J	YY *et al.* 00-MY03 (KYO)	Near Mt Kinabalu National Park Headquarter, Sabah Prov., Malaysia	AB097601*
Type K	YY & AT 00-ID11 (KYO)	Airsirah, West Sumatra, Indonesia	AB097602*
Bhutan	M. Okubo 99-B01 (KYO)	Mo Valley, Bhutan	AB042146
CH-Hekou1	NM & XC 95-2851 (TI)	Hekou, Yunnan Prov., China	AB023503
CH-Hekou2*	YY *et al.* 01-C01 (KYO)	Hekou, Yunnan Prov., China	AB097603*
JP-Amami	NM 94-J022 (TI)	Mt Yuwan, Amami Is., Kagoshima Pref., Japan (cultivated at Univ. of Tokyo)	AB013239
TH-Suthep	NF *et al.* 94-T382 (TI)	Mt Suthep, Chiang Mai Prov., Thailand	AB013247
TH-SK	NM *et al.* 99-T04 (KYO)	Phru to Daeng/Phru Sirindhorn, Sungai Kolok Distr., Thailand	AB042152
TH-WWA1	NM *et al.* 99-T17 (KYO)	Wildlife Watching Area, behind Headquarters of the Bala-Hala Wildlife Sanctuary, Waeng Distr., Thailand	AB042153

Table 2. *Continued*

Species	Voucher	Locality	DDBJ no.
TH-WWA2	NM *et al.* 99-T18 (KYO)	Wildlife Watching Area, behind Headquarters of the Bala-Hala Wildlife Sanctuary, Waeng Distr., Thailand	AB042149
TH-Sirindhorn1	NM *et al.* 99-T06 (KYO)	Sirindhorn Fall, Bala-Hala Wildlife Sanctuary, Waeng Distr., Thailand	AB042145
TH-Sirindhorn2	NM *et al.* 99-T11 (KYO)	Sirindhorn Fall, Bala-Hala Wildlife Sanctuary,Waeng Distr., Thailand	AB042151
VN-CatBa	KI *et al.* 94-V374 (TI)	Cat Ba Island, Hai Phong Prov., Vietnam	AB013246
VN-Concuong1	NF *et al.* 95-V2416 (TI)	Concuong, Vinh Prov., Vietnam	AB023504
VN-Concuong2	NF *et al.* 95-V2440 (TI)	Concuong, Vinh Prov., Vietnam	AB023505
VN-Concuong3	NF *et al.* 95-V2443 (TI)	Concuong, Vinh Prov., Vietnam	AB023506
VN-Dalat	KI *et al.* 98-V518 (KYO)	Dalat, Dalat Prov., Vietnam	AB023507
VN-TamDao	KI *et al.* 94-V339 (TI)	Tam Dao, Vinh Phu Prov., Vietnam	AB013248
VN-Bavi	KI *et al.* 94-V320 (TI)	Mt Bavi, Ha Noi Prov., Vietnam	AB013251
A. phyllitidis Don			
CH-Mengla	KI *et al.* 100462 (KYO)	Menla, Xishuanbanna, Yunnan Prov., China	AB097594*
A. setoi N. Murak. & Seriz.			
JP-Okinawa	SS 71596 (AICH)	Urazoe, Okinawa Is., Okinawa Pref., Japan	AB013243
JP-Daitoh	NM 96-J101 (TI)	Kita-Daitoh Is., Daitoh Is, Okinawa Pref., Japan	AB013241
JP-Iriomote	NM 98-J001 (KYO)	Iriomote Is., Okinawa Pref., Japan	AB013234
Other species of Aspleniaceae			
A. angustum Sw.			AY300106
A. anisophyllum Kunze			AY300107
A. bulbiferum G. Forst.			AY283226
A. feei Kunze & Fee			AF525267
A. gemmiferum Schrad.			AY300117
A. griffithianum Hook.	NM J93-001 (TI)	Yaku Is., Kagoshima Pref., Japan	AB013252
A. hookerianum Colenso			AY283229
A. lamprophyllum Carse			AY283230
A. oblongifolium Colenso			AY283231
A. obtusatum G. Forst.			AY300130
A. prolongatum Hook.	SN & KO 25 (KYO)	Funada, Kiho, Mie Pref., Japan	AB014691
A. richardii (Hook. f.) Hook. f.			AY300138
A. sandersonii Hook.			AF525274
A. serratum L.			AY300141
A. shuttleworthianum Kunze			AY283235
A. simplicifrons F. Muell.			AY300142
A. theciferum (Kunth) Mett.			AF336099
Outgroup			
Hymenasplenium hondoense (N. Murak. & Hatanaka) Nakaike	NM 596920 (KYO)	Hayama, Kouchi Pref., Japan	AB014705

*Sequence data corrected for the first time in this study.
Abbreviations in parentheses indicate harbaria where the vouchers are kept.
Country names: AU, Australia; CH, China; JP, Japan; MY, Malaysia; TH, Thailand; VN, Vietnam.
Collectors' names: AT, A. Takano; JY, J. Yokoyama; KI, K. Iwatsuki; KO, K. Oohora; MH, Mitsuyasu Hasebe; NF, N. Fukuoka; NM, N. Murakami; NS, N. Sahashi; SN, S. Nogami; RI, R. Ito; SS, S. Serizawa; UH, U. Hapid; XC, X. Cheng; YH, Y. Higuchi; YT, Y. Takahashi; YY, Y. Yatabe.

Table 3. Plant materials for artificial crossing experiments and their allelomorph

Pair of *rbcL* sequence type Type X × Type Y	Parent 1 Type X	Parent 2 Type Y	Locus	Genotype	
				Parent 1	Parent 2
Type A × Type B	HA-98068	HA-98074*	lap	ii	cc
	HA-98068	HA-98080*	lap	ii	ch
	HA-98084	HA-98075*	lap	dj	cc
Type A × Type C	HA-98068	HA-98140*	lap	ii	bg
	HA-98084	HA-98101*	lap	dj	gg
	HA-98084	HA-98134*	lap	dj	gg
Type B × Type C	HA-00020	HA-00025	lap	cc	gg
	HA-00021	HA-00025	lap	cc	gg
Type B × Type G	HA-00020	PA-00051	tpi-3	bb	cc
	HA-00009	CA-00050	tpi-3	bb	cc
Type B × *A. australasicum*	HA-00020	NC-99002	lap	cc	kk
			pgm-1	aa	bb
			skd	ab	cc
	HA-00021	NC-99002	lap	cc	kk
			pgm-1	aa	bb
			skd	ab	cc
Type C × Type E	HA-00020	CA-00060	pgm-2	aa	ef
	HA-00020	PA-00020	pgm-2	aa	df
	HA-00025	BO-00001	pgm-2	aa	ff
	HA-00025	PA-00020	pgm-2	aa	df
Type C × Type F	HA-00020	CA-00028	tpi-1	ee	dd
	HA-00025	PA-00017	tpi-1	ee	dd
Type E × Type E	CA-00060	BO-00001	tpi-2	aa	cc
			g6p	bb	cc
	CA-00060	PA-00020	tpi-2	aa	cc
Type E × *A. setoi*	BO-00001	IR-98001	lap	ff	ci
Type E × Type EII	BO-00001	PA-00001	tpi-2	cc	aa
	PA-00020	PA-00001	tpi-2	cc	aa
Type E × Type F	FR-00064	PA-00017	tpi-3	bb	aa
			pgm-2	dd	ab
	PA-00020	PA-00017	tpi-3	bb	aa
			pgm-2	df	ab
	PA-00020	CA-00028†	tpi-3	bb	aa
			pgm-2	df	aa
	BO-00001	PA-00017†	tpi-3	bb	aa
			pgm-2	ff	ab
Type E × Type G	PA-00020	CA-00050	lap	fh	eg
			tpi-3	bb	cc
	PA-00020	PA-00051	lap	fh	gg
			tpi-3	bb	cc
	CA-00060	CA-00050	lap	ff	eg
			tpi-3	bb	cc
Type E × *A. australasicum*	BO-00001	NC-99002	lap	ff	kk
			skd	aa	cc
	PA-00020	NC-99002	lap	fh	kk
			skd	aa	cc
Type F × Type G	CA-00028	CA-00050	tpi-3	aa	cc
			6pg	bb	cc
	PA-00017	CA-00050	tpi-3	aa	cc
			6pg	bb	cc
	PA-00017	PA-00051	tpi-3	aa	cc
			6pg	bb	cc

Table 3. *Continued*

Pair of *rbcL* sequence type Type X × Type Y	Parent 1 Type X	Parent 2 Type Y	Locus	Genotype	
				Parent 1	Parent 2
Type G × *A. australasicum*	CA-00050	NC-99001	lap	eg	kk
	PA-00051	NC-99002	lap	gg	kk
A. setoi × *A. australasicum*	IR-98001	NC-99001	lap	ci	kk
			skd	ab	cc
			pgm-2	aa	ff
	IR-98001	NC-99002	lap	ci	kk
			skd	ab	cc
			pgm-2	aa	ef
	IR-98001	NC-99001†	lap	ci	kk
			skd	ab	cc
			pgm-2	aa	ff
	IR-98002	NC-99009†	lap	ci	kk
			skd	ab	cc
			pgm-2	aa	ef

Notes: *rbcL* types correspond to those of Table 5 and Figure 4. Two capital letters in names of plant materials indicate the localities where they were collected.
*Examined in Yatabe *et al.* (2001).
†For evaluation of the frequency of hybrid formation.
BO, Bogor Botanical Garden, West Java; CA, Cameron Highland, Malaysia; FR, Fraser's Hill, Malaysia; HA, Halimun National Park, West Java, Indonesia; IR, Iriomote Island, Japan; NC, New Caledonia; PA, within a radius of 50 km from Padang city, West Sumatra, Indonesia.

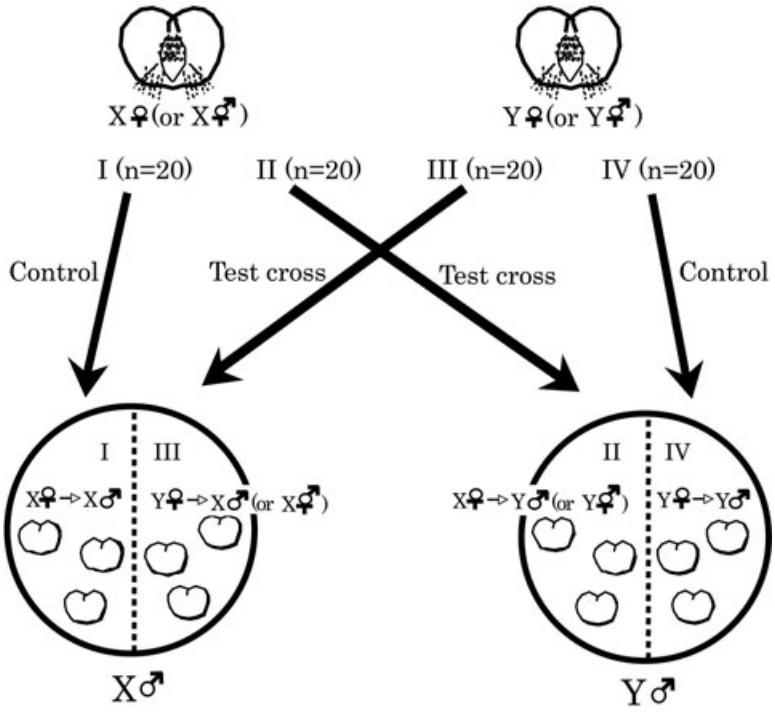


Figure 2. Method of artificial experiments. Details are provided in MATERIAL AND METHODS.

as females sometimes bear not only archegonia but also antheridia. Therefore, intragametophytic mating *sensu* Klekowski (1968), the selfing of hermaphrodites, may occur in the crossing tests. Allozyme polymorphisms were used to identify hybrid plants based on the heterozygous patterns that combined the patterns of their parents, inferred genotypes of which are shown in Table 3. There are three possible kinds of matings in the crossing tests, intergametophytic mating between type X and Y, intergametophytic mating between the gametophytes of the same type and intragametophytic mating. Non-hybrid sporophytes in the crossing tests are considered to be the result of intragametophytic mating or intergametophytic mating between gametophytes of the same type. This method is appropriate to assess the capacity for forming hybrids because the number of gametophytes with antheridia can be maximized by observing their development and the density of sperm of one parental type in the dishes flooded with antheridia can be raised substantially.

Evaluation of the frequency of hybrid formation

The above-mentioned reciprocal crossing experiment is inadequate for evaluating the frequency of hybrid formation, so another artificial crossing experiment was performed for pairs of individuals with different *rbcL* sequence types. In this experiment, deviation from the Hardy–Weinberg equilibrium was evaluated when gametophytes of two *rbcL* types were grown together. The main difference between this approach for evaluation of the frequency of hybrid formation and the reciprocal crossing experiments described in the previous section is the proportion of sperm of the two parental types. In this approach, an extreme bias of sperm density should be avoided by the following procedure.

Spores of the two sporophytes used as parents were sown together on inorganic nutrient medium in Petri dishes 9 cm in diameter and were cultivated in a chamber. Forty days after the spores were sown, we obtained gametophytes with archegonia. We re-sowed the spores of both parental sporophytes around the mature gametophytes to obtain gametophytes with antheridia. For each pair of parental sporophytes, five treated Petri dishes were prepared. Forty days after spores were re-sown, the treated Petri dishes were flooded with water.

After 2 months, 20 juvenile sporophytes growing on the gametophytes were collected from each Petri dish. We determined the *rbcL* sequence type of these juvenile sporophytes using single-strand conformation polymorphism (SSCP) analysis (Yap & McGee, 1994). Because *rbcL* is encoded by the plastid genome and is maternally inherited (Gastony & Yatskievych, 1992), it is possible to estimate how frequently an individual of a given *rbcL* sequence type has become the maternal parent of the juvenile sporophytes obtained in each crossing experiment. Out of the five dishes prepared, we chose the dish in which this ratio deviated least from 1 : 1 in order to evaluate the frequency of hybrid formation efficiently.

From the chosen dish, 96 juvenile sporophytes were collected and planted on new medium in Petri dishes. Two or 3 weeks after collection, these sporophytes had grown enough for allozyme analysis. For all these individuals, allozyme polymorphisms were assessed in order to identify the hybrid plants, based on the heterozygous patterns compared with those of their parents, the genotypes of which are shown in Table 3. For the individuals identified as hybrid plants, *rbcL* sequence type was identified using SSCP analysis in order to identify the maternal parent. Based on the number of sporophytes generated by each pair of parents, deviation from the number expected under random crossing was assessed using the χ^2-test.

This method could not be used for closely related pairs because the maternal parents of the obtained sporophytes could not be identified using SSCP analysis; therefore, we crossed two *rbcL* types of *A. nidus* (types E and F), and *A. australasicum* and *A. setoi*, using four parental individuals for each pair.

In order to test for deviation from the expected number of sporophytes under random crossing, it is necessary to assume random mating of gametophytes. We also tested for random mating of gametophytes originating from the same sporophyte. For four of eight sporophytes used in the crossing experiments, more than one heterozygous locus was found and random mating of their gametophytes was tested. The heterozygous loci are listed in Table 4. Spores of one sporophyte were sown on inorganic nutrient medium in Petri dishes and, 40 days later, the spores were re-sown around the mature gametophytes. Forty days after the spores were re-sown, the treated Petri dishes were flooded with water. After 2 months, 20 juvenile sporophytes growing on the gametophytes were collected from each Petri dish. For all of these individuals, allozyme polymorphisms were examined for heterozygous loci to determine their genotypes and the null hypothesis that the expected numbers of the three genotypes (two kinds of homozygotes and heterozygotes) are equal to the observed numbers was tested using a χ^2-test.

Allozyme analyses

Fresh leaves were ground in 1.0 mL of cold extraction buffer containing 0.1 mM Tris-HCl, 1 mM EDTA (4NA), 10 mM KCl, 10 mM $MgCl_2$, 0.4% 2-mercaptoethanol

Table 4. Numbers of sporophytes obtained from mating between gametophytes originating from the same sporophyte and tests of random mating in those gametophytes

Plant materials	Locus	Number of homozygotes (pp)	Number of heterozygotes (pq)	Number of homozygotes (qq)	χ^2-test probability
PA-00020 (Type E)	pgm-2	3	12	5	0.55
	lap	3	11	6	0.57
CA-00028 (Type F)	skd	6	10	4	0.82
IR-98001 (*A. setoi*)	skd	4	10	6	0.82
	lap	6	9	5	0.86
NC-99001 (*A. australasicum*)	hk	5	11	4	0.86

Notes: Two capital letters in names of plant materials indicate the localities where they were collected.
CA, Cameron Highland, Malaysia; IR, Iriomote Island, Japan; NC, New Caledonia; PA, within a radius of 50 km from Padang City, West Sumatra, Indonesia.

and 10% polyvinylpyrrolidone with the pH adjusted to 7.5. Enzymes were resolved on 7.5% polyacrylamide gels following the procedures of Shiraishi (1988). Leucine aminopeptidase (LAP), 6-phosphogluconate dehydrogenase (6PGD), glucose-6-phosphate dehydrogenase (G6PD), triosephosphate isomerase (TPI), phosphoglucomutase (PGM), hexokinase (HK) and shikimate dehydrogenase (SKD) were examined, also following the method of Shiraishi (1988).

Loci were numbered, with the most anodal form designated '1' and so on, when more than one isozyme existed for an enzyme. Allelomorphs were designated similarly at each locus, with the most anodal form designated 'a' and progressively slower forms 'b', 'c' and so on.

SSCP analysis

The primers SSCP-2F (5′-CACGGTATGCATTTTCGT GT-3′) and SSCP-2R (5′-TAGATACCCAATCTTGAG TG-3′) were designed and used for PCR amplification of a 193 bp *rbcL* fragment. The PCR products were separated on a GenePhor unit (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 5 °C. After electrophoresis, the gels were stained using a DNA silver staining method on a Hoefer apparatus (Amersham Pharmacia Biotech). The electrophoretic patterns are shown in Figure 3.

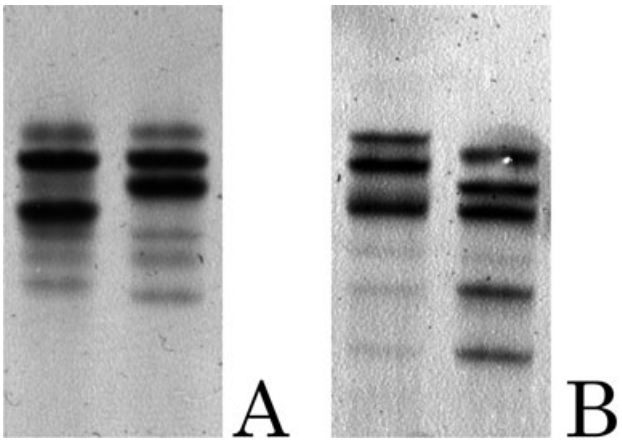


Figure 3. Band patterns of single-strand conformation polymorphisms (SSCP) in partial sequences of *rbcL*. A, Type E (left) and Type F (right). B, *A. setoi* (left) and *A. australasicum* (right).

Determination of spore germination in hybrids

Sporophytes identified as hybrids based on the allozyme composition were cultivated in a greenhouse of Kyoto Botanical Garden or Tsukuba Botanical Garden for at least 5 years. Spores were collected from the cultivated sporophytes and cultured on inorganic nutrient medium in the same way as described earlier. One month after sowing, the germination rate of the cultures was checked for about 200 spores using a dissecting microscope (MZ8; Leica Microsystems, Wetler, Germany). If the first rhizoidal cell had emerged, spores were judged to have germinated.

Morphological observation of spores in hybrids

To assess the morphological features of hybrid spores, we used the techniques described by Takamiya (1993). Young fertile pinnae were fixed in a 3 : 1 solution of ethanol : acetic acid for 3 h. Sporangia were then squashed in 2% aceto-orcein. Spores were observed using a light microscope (BX-51; Olympus,

Tokyo, Japan) and photographs were taken with a digital camera (DP-70; Olympus).

RESULTS

Observed *rbcL* sequence types in west Malesia and phylogenetic analysis

We sequenced a 1191-bp nucleotide region of *rbcL* for 88, 87, 39 and 37 individuals collected from west Java, peninsular Malaysia, west Sumatra and Kinabalu, respectively, including the individuals used for artificial crossing experiments. Seventeen *rbcL* sequence types were found, including the five types previously found from west Java (types A–E; Murakami *et al.*, 1999b; Yatabe *et al.* 2001). We named the other types AII, EII, EIII, EIV, F, FII, G, H, I, I-II, J and K. Only one nucleotide differed between the pairs E and EII, E and EIII, E and EIV, F and FII, and I and I-II; these pairs were found in the same localities (Table 1). At least five nucleotides differed between types designated using different letters. The accession numbers of these sequences in the DNA Database of Japan are shown in Table 2. The number of individuals of each *rbcL* sequence type found from each locality is shown in Table 1.

Maximum parsimony analyses of *rbcL* sequences produced 21 equally most-parsimonious trees. The strict consensus tree showing bootstrap percentages is shown in Figure 4. The monophyly of *Asplenium* section *Thamnopteris* was not supported because nine species of *Asplenium* from other sections of *Asplenium* were nested within the clade. In section *Thamnopteris*, two major clades exist, clades Z and W. Clade W was supported by a high bootstrap value (99%) and includes *A. australasicum* from New Caledonia and Australia, *A. setoi* from Japan and *A. nidus* from various localities in Asia (Fig. 4). The sequence of *A. setoi* from Iriomote Island, Japan, is the same as that of type E. Within clade W, two further clades exist, clades W-1 and W-2, which were supported by 100 and 84% bootstrap, respectively. Clade W-1 includes types A, AII, C, E, EII, EIII, EIV, F, FII, H and K. Clade W-2 includes types B, G, I and I-II. Clade Z includes *A. antiquum* Makino from Japan, *A. cymbifolium* Christ from west Malesia, *A. phyllitidis* Don, *A. antrophyoides* Christ from China and *A. nidus* from some parts of Asia, including west Malesia (types D and J), Bhutan and Vietnam (Fig. 4).

Reciprocal crossing experiments

After repeating the reciprocal crossing experiments 80 times between randomly chosen pairs, data for 42 crosses were obtained, which are shown in Table 5. In these crosses, sporophytes were obtained for both of the controls (Fig. 2I, IV), but no sporophytes were obtained for any one of the controls in the remaining failed tests. The successful tests included the three crosses between parental individuals with identical *rbcL* sequences (crosses between different individuals of type E and between type E and *A. setoi* from Japan) and crosses between individuals with different *rbcL* sequence types. The number of sporophytes obtained and the number identified as hybrids between parental individuals in these 42 crosses are also shown in Table 5. No sporophytes obtained in control crosses were identified as hybrids.

In 26 of the 42 crosses, no sporophytes were identified as hybrids based on allozyme analyses, although some sporophytes were obtained even in the test crosses. These 26 crosses were between types A and B, types A and C, types B and C, types B and G, type B and *Asplenium australasicum*, types C and E, types E and G, types F and G and type G and *A. australasicum*. The frequency with which juvenile sporophytes grew on gametophytes in the controls ranged from 20 to 95%. Most of these frequencies in the controls were significantly higher than in test crosses (as assessed by Fisher's exact probability test; Table 5).

In the other 16 crosses, sporophytes identified as hybrids were obtained (Table 5, Fig. 5). In crosses between types C and F and type E and *A. australasicum*, hybrids were obtained in one of the two test crosses and only individuals of type F and *A. australasicum* were maternal parents of the hybrids obtained. In crosses between individuals of type E, type E and *A. setoi*, types E and EII, types E and F, and *A. setoi* and *A. australasicum*, hybrids were obtained in both test crosses (Fig. 2II, III). In crosses between individuals of type E, type E and *A. setoi* and types E and EII, the number of hybrids obtained ranged from 16 to 35. In crosses between types E and F and *A. setoi* and *A. australasicum* the numbers of hybrids obtained were relatively small, ranging from five to 11.

The relationship between genetic distance [as calculated using Kimura's two-parameter (K2P) method based on *rbcL* sequence] and the number of hybrids obtained in the reciprocal cross experiments is shown in Figure 6.

Frequency of hybrid formation between types E and F and *Asplenium setoi* and *A. australasicum*

The numbers of sporophytes obtained from mating between gametophytes originating from the same sporophyte did not deviate from Hardy–Weinberg expectations for any of the six cases of four sporophytes (Table 4). Therefore, it can be assumed that gametophytes that originate from the same sporophyte mate randomly. To evaluate the frequency of

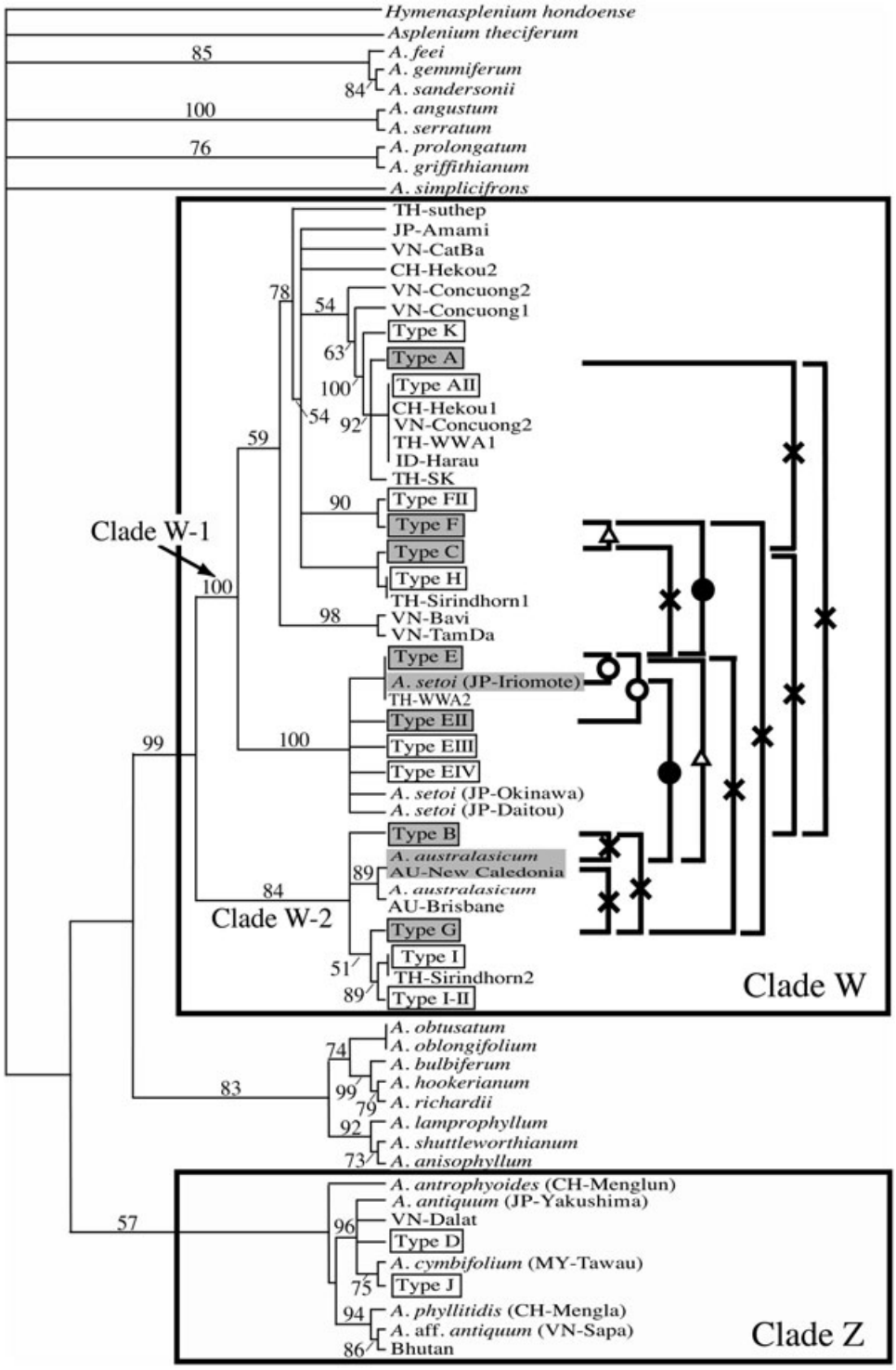


Figure 4. The strict consensus tree of the 21 most-parsimonious trees based on *rbcL* sequence data for Aspleniaceae, including the *A. nidus* complex. Types A, AII, B, C, D, E, EII, EIII, EIV, F, FII, G, H, I, I-II, J and K in boxes correspond to the *rbcL* sequence types of *A. nidus* in the text. The numbers above the branches are bootstrap percentages (> 50%). Materials used for artificial crossing experiments are shaded. Circles, triangles and crosses indicate the pairs giving rise to bidirectional hybrids, asymmetrical hybrids and no hybrids in reciprocal crossing experiments, respectively. Solid circles indicate the pairs where the frequencies of hybrid formation were lower than the Hardy–Weinberg expectations under random crossing (see Fig. 7).

Table 5. The frequencies of occurrence of juvenile sporophytes and hybrids in reciprocal crossing experiments among *rbcL* types

Pair of *rbcL* sequence type Type X × Type Y	Parent 1 Type X	Parent 2 Type Y	I. $X^m X^p$	II. $X^m Y^p$ ($X^m \cdot ^p$)	III. $Y^m X^p$ ($Y^m \cdot ^p$)	IV. $Y^m Y^p$
Type A × Type B	HA-98068	HA-98074†	11**	0 (2)	0 (0)	10**
NN = 25	HA-98068	HA-98080†	13**	0 (5)	0 (0)	16**
K2P = 0.021	HA-98084	HA-98075†	15**	0 (0)	0 (1)	15**
	HA-98084	HA-98075†	11**	0 (0)	0 (2)	16**
	HA-98084	HA-98075†	12**	0 (1)	0 (9)	19**
Type B × Type C	HA-00020	HA-00025	7**	0 (0)	0 (0)	7**
NN = 18	HA-00021	HA-00025	15**	0 (0)	0 (0)	5*
K2P = 0.015						
Type F × Type G	CA-00028	CA-00050	8**	0 (0)	0 (0)	7**
NN = 18	PA-00017	CA-00050	11**	0 (2)	0 (0)	6*
K2P = 0.015	PA-00017	PA-00051	7*	0 (1)	0 (2)	13**
Type A × Type C	HA-98068	HA-98140†	4^{NS}	0 (1)	0 (2)	7^{NS}
NN = 17	HA-98084	HA-98101†	16*	0 (9)	0 (0)	15**
K2P = 0.014	HA-98084	HA-98134†	12^{NS}	0 (6)	0 (1)	8*
Type E × Type G	PA-00020	CA-00050	9**	0 (1)	0 (1)	15**
NN = 15	PA-00020	PA-00051	12*	0 (5)	0 (2)	14**
K2P = 0.013	CA-00060	CA-00050	5^{NS}	0 (1)	0 (0)	16**
Type C × Type E	HA-00020	CA-00060	5*	0 (0)	0 (0)	7**
NN = 14	HA-00020	PA-00020	4^{NS}	0 (0)	0 (0)	6*
K2P = 0.012	HA-00025	BO-00001	11**	0 (2)	0 (0)	16**
	HA-00025	PA-00020	11**	0 (0)	0 (2)	15**
Type E × *A. australasicum*	BO-00001	NC-99002	16**	0 (0)	14 (16)	9
NN = 14	PA-00020	NC-99002	12**	0 (1)	10 (10)	12
K2P = 0.012						
A. setoi × A. australasicum	IR-98001	NC-99001	13	4 (4)	7 (7)	18
NN = 14	IR-98001	NC-99002	14	7 (8)	4 (6)	17
K2P = 0.012						
Type E × Type F	FR-00064	PA-00017	14	3 (4)	2 (2)	7
NN = 13	PA-00020	PA-00017	6	2 (2)	3 (7)	12
K2P = 0.011						
Type C × Type F	HA-00020	CA-00028	9**	0 (0)	15 (19)	10
NN = 7	HA-00025	PA-00017	8**	0 (1)	6 (6)	5
K2P = 0.006	HA-00025	PA-00017	10**	0 (0)	11 (11)	9
Type B × Type G	HA-00020	PA-00051	7**	0 (0)	0 (0)	11**
NN = 6	HA-00009	CA-00050	11**	0 (0)	0 (1)	13**
K2P = 0.005						
Type B × *A. australasicum*	HA-00020	NC-99002	7*	0 (1)	0 (3)	17**
NN = 5	HA-00021	NC-99002	12*	0 (1)	0 (5)	18**
K2P = 0.004						
Type G × *A. australasicum*	CA-00050	NC-99001	6*	0 (0)	0 (8)	17*
NN = 5	PA-00051	NC-99002	17**	0 (5)	0 (0)	8**
K2P = 0.004						
Type E × Type EII	BO-00001	PA-00001	9	17 (18)	18 (18)	8
NN = 1	BO-00001	PA-00001	14	19 (19)	6 (6)	13
K2P = 0.001	BO-00001	PA-00001	9	8 (8)	17 (17)	7
	PA-00020	PA-00001	10	8 (8)	8 (8)	9
Type E × Type E	CA-00060	BO-00001	8	15 (17)	17 (18)	6
NN = 0	CA-00060	PA-00020	6	10 (10)	9 (9)	5
K2P = 0.000						
Type E × *A. setoi*	BO-00001	IR-98001	13	11 (12)	9 (12)	15
NN = 0						
K2P = 0.000						

Notes: I, II, III and IV correspond to those of Figure 2.

* and ** indicate that the number of the sporophytes obtained in the control are significantly higher than that in the treatment (*$P < 0.05$, **$P < 0.01$).

†Examined in Yatabe *et al.* (2001).

^{m} and ^{p} indicate the maternal and paternal parents, respectively (e.g. $X^m Y^p$ indicates the number of sporophytes obtained by the mating between maternal parent with Type X *rbcL* sequence and paternal parent with Type Y *rbcL* sequence). Numbers in parentheses indicate number of sporophytes obtained in each crossing test.

NN, number of nucleotide substitutions; K2P, genetic distance calculated by Kimura's two parameter method (Kimura, 1980); NS, non-significant. Two capital letters in names of plant materials indicate the localities where they were collected. BO, Bogor Botanical Garden, West Java; CA, Cameron Highland, Malaysia; FR, Fraser's Hill, Malaysia; HA, Halimun National Park, West Java, Indonesia; IR, Iriomote Island, Japan; NC, New Caledonia; PA, within a radius of 50 km from Padang City, West Sumatra, Indonesia.

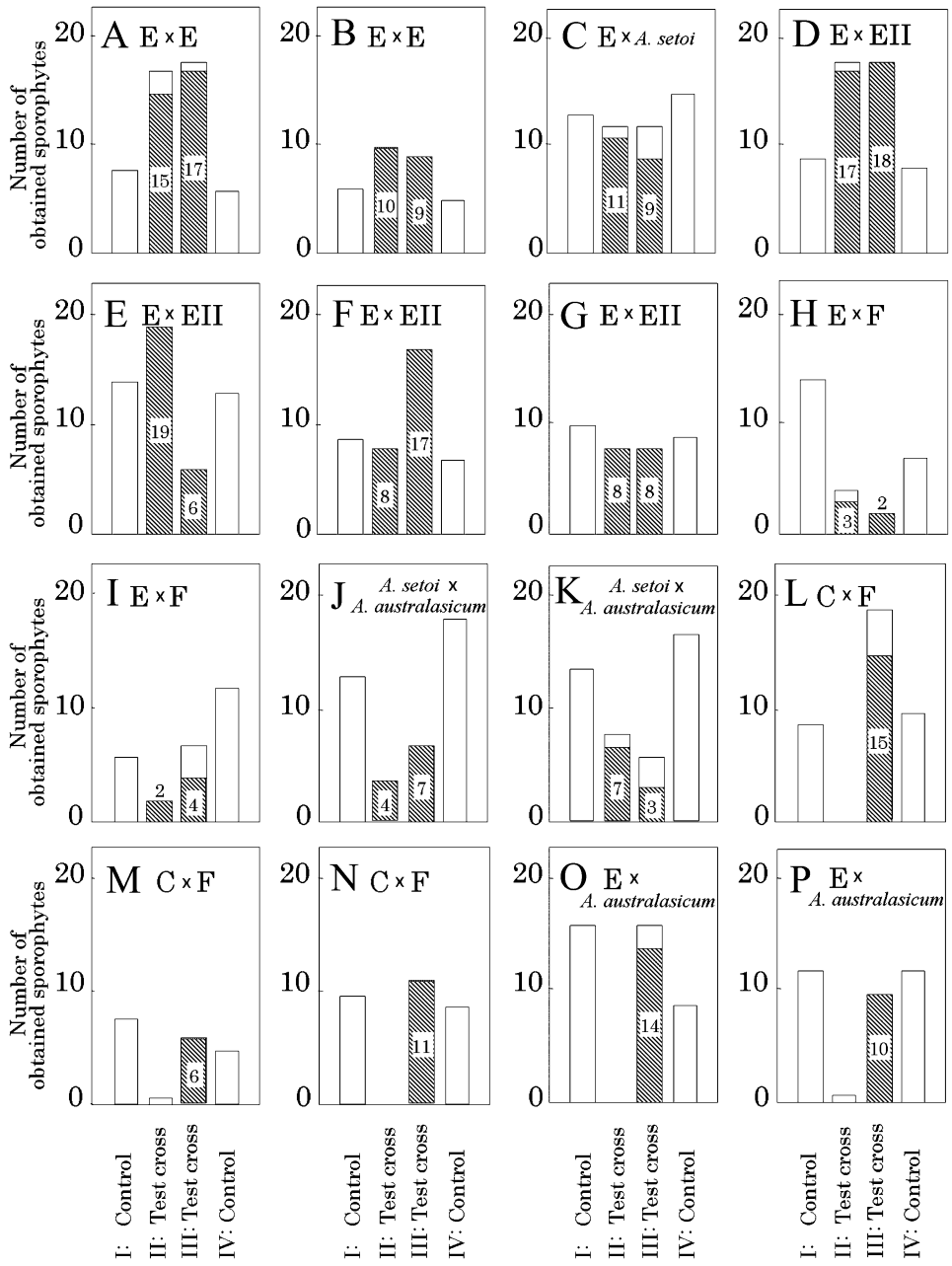


Figure 5. The number of sporophytes and hybrids obtained in reciprocal crossing experiments. Each figure corresponds to the results listed in Table 5. Bars indicate the numbers of sporophytes obtained and shaded parts indicate the numbers of sporophytes identified as hybrids based on allozyme patterns.

hybrid formation, the number of hybrids obtained was compared with the number of hybrids expected under random crossing (Table 6, Fig. 7). The frequencies of hybrid formation were significantly smaller than expected for both pairs as assessed using χ^2-tests. The frequencies of hybrid formation for crosses between type E and type F were at most 15.8% of the number expected under random crossing. For *A. setoi* and *A. australasicum*, the frequencies of hybrid formation were always less than 10% of the number expected under random crossing.

HYBRID STERILITY

Although survivorship of the hybrids was not high, after 5 years 36 hybrids still survived in the greenhouse, 18 of which produced fertile leaves (Table 7). The growth rates of hybrids and their spore germi-

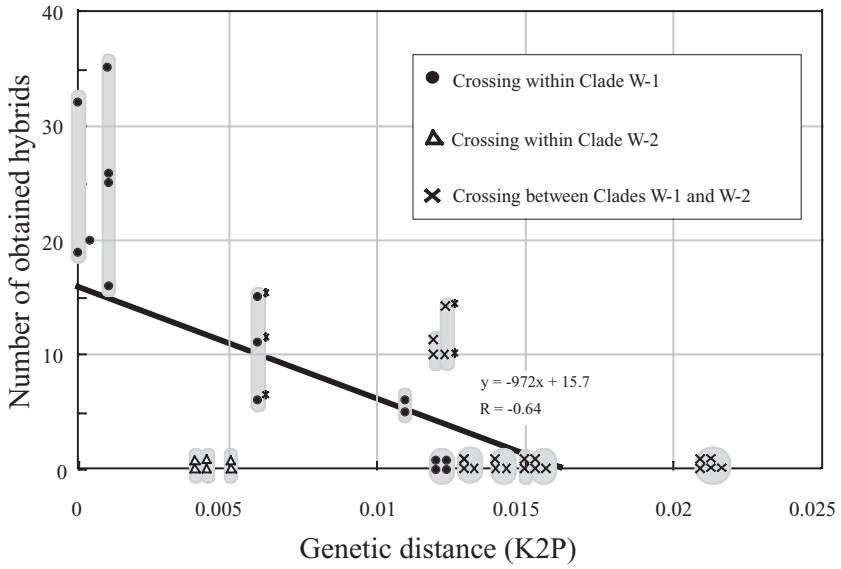


Figure 6. Relationship between genetic distance calculated by Kimura's two parameters method based on *rbcL* sequences and the number of obtained hybrids in reciprocal crossing experiments. Each plot indicates the total number of hybrids obtained in each crossing experiment listed in Table 5. Plots connected with shaded parts indicate the results obtained in the crossing between the same pairs. Asterisks indicate that only one of two parents used in reciprocal crossing experiments were maternal parents of the hybrids obtained.

nation rates varied with the parental pairs. All but one of the hybrids between individuals of type E from different localities and between types E and EII matured, with sporangia producing 64 normal-shaped spores (Fig. 8) and the germination rates of the spores ranging from 66 to 86%. All eight hybrids between *A. setoi* and *A. australasicum* and the hybrid between *A. australasicum* and type E produced fertile leaves. However, their sporangia usually contained 16 large, abnormally shaped spores (Fig. 9) and less than 3% of the spores germinated. Only two of eight hybrids between types E and F matured. These two hybrids, of which the maternal parent was type F, had low fertility. Their spores were variable in size (Fig. 10) and the germination rates were at most 1%. The only hybrid between types E and F for which the maternal parent was type E was immature and so there are no data available on its fertility. None of the hybrids between types C and F or between type E and *A. setoi* produced fertile leaves after 5 years in cultivation.

DISCUSSION

Capacity to form hybrids

In our crossing experiments, *rbcL* sequence types nesting within a monophyletic group (Fig. 4, clade W) were used as parental individuals. In 26 reciprocal crosses, no hybrids were detected, although some sporophytes were obtained in the test crosses as well as in the control crosses (Table 5). In most of these crosses, however, the numbers of sporophytes obtained in the control crosses were significantly higher than those in the test crosses (Table 5), suggesting that an abundance of gametophytes with antheridia, which functioned as males, existed in the dishes flooded with water, and that the gametophytes transferred into the dishes, which functioned as females, bore mature archegonia. The absence of hybrids suggests that reproductive isolation between parental individuals prevents hybrids forming. Pre-mating isolation barriers are defined as isolating barriers that impede gene flow before the transfer of sperm or pollen to members of other species (Coyne & Orr, 2004). In ferns, spores are dispersed by wind and fertilization is mediated by water. Therefore, it is unlikely that any external causes aside from geographical, habitat or temporal isolation function to effect pre-mating isolation. In artificial crossing experiments, given the high activity of fern spermatozoa (Igura, 1949) and the small size of Petri dishes, the above-mentioned pre-mating isolation factors cannot act. Therefore, the reproductive isolation observed in our crosses involves prezygotic isolation or zygotic sterility. Prezygotic isolation has been reported between two species of *Athyrium* Roth and *Dryopteris filix-mas* (L.) Schott (Schneller, 1981). It is not clear whether prezygotic isolation or zygotic sterility is the causative factor in the *A. nidus* complex.

Data from the 26 crosses suggest that the following nine pairs do not form hybrids: types A and B, types

Table 6. Evaluation of frequency of hybrid formation between Type E and Type F and between *Asplenium setoi* and *A. australasicum*

	Inbreed $X^m X^p$	Hybrid $X^m Y^p$	Hybrid $Y^m X^p$	Inbreed $Y^m Y^p$	
PA-00020 (Type E) × CA-00028 (Type F)					
Observed number	70	3	1	22	
(Observed frequency)	(0.729)	(0.031)	(0.010)	(0.229)	
Number expected under random crossing	54	19	17	6	
(Expected frequency)	(0.562)	(0.198)	(0.177)	(0.062)	
Ho/He		0.158	0.059		
χ^2-test probability					2.11E-16
BO-00001 (Type E) × PA-00017 (Type F)					
Observed number	64	1	0	31	
(Observed frequency)	(0.667)	(0.010)	(0.000)	(0.323)	
Number expected under random crossing	43	22	21	10	
(Expected frequency)	(0.451)	(0.226)	(0.215)	(0.108)	
Ho/He		0.046	0		
χ^2-test probability					1.01E-19
IR-98001 (*A. setoi*) × NC-99001 (*A. australasicum*)					
Observed number	33	1	2	60	
(Observed frequency)	(0.344)	(0.010)	(0.021)	(0.625)	
Number expected under random crossing	12	22	23	39	
(Expected frequency)	(0.129)	(0.225)	(0.235)	(0.410)	
Ho/He		0.046	0.088		
χ^2-test probability					5.57E-18
IR-98002 (*A. setoi*) × NC-99009 (*A. australasicum*)					
Observed number	26	0	1	69	
(Observed frequency)	(0.271)	(0.000)	(0.010)	(0.719)	
Number expected under random crossing	7	19	20	50	
(Expected frequency)	(0.076)	(0.195)	(0.205)	(0.524)	
Ho/He		0	0.051		
χ^2-test probability					1.26E-19

Ho, observed frequency of hybrids; He, frequency of hybrids expected under random crossing.
Two capital letters in names of plant materials indicate the localities where they were collected. BO, Bogor Botanical Garden, West Java; CA, Cameron Highland, Malaysia; NC, New Caledonia; IR, Iriomote Island, Japan; PA, within a radius of 50 km from Padang City, West Sumatra, Indonesia.
^{m} and ^{p} indicate the maternal and paternal parents, respectively (e.g. $X^m Y^p$ indicates the number of sporophytes obtained by the mating between maternal parent with Type X *rbcL* sequence and paternal parent with Type Y *rbcL* sequence).

A and C, types B and C, types B and G, type B and *A. australasicum*, types C and E, types E and G, types F and G and type G and *A. australasicum*. For these pairs, two to four different pairs of individuals were used for the repeated reciprocal crosses (Table 3), but no hybrids were detected (Table 5), supporting the conclusion that these pairs are unable to form hybrids. The genetic distances (K2P) between the individuals of these pairs range widely, from 0.004 to 0.021.

Hybrid sporophytes were obtained in 16 of the 42 crosses (Table 5, Fig. 5). In crosses between different individuals of type E, type E and *A. setoi*, types E and EII, types E and F and *A. setoi* and *A. australasicum*, hybrids were obtained for both of the test crosses (Fig. 2II, III). Therefore, these pairs are able to form hybrids bidirectionally. In crosses between closely related pairs (pairs of different individuals of type E, type E and *A. setoi* and types E and EII), for which K2P ranged from 0.000 to 0.001, large numbers of hybrids, between 16 and 36, were obtained. In fact, for closely related pairs (different individuals of type E and types E and EII), the numbers of hybrid plants in some test crosses were even larger than the numbers of sporophytes obtained in control crosses. In contrast, in crosses between relatively distantly related pairs, for which K2P ranged from 0.006 to 0.012 (types E and F, *A. setoi* and *A. australasicum*,

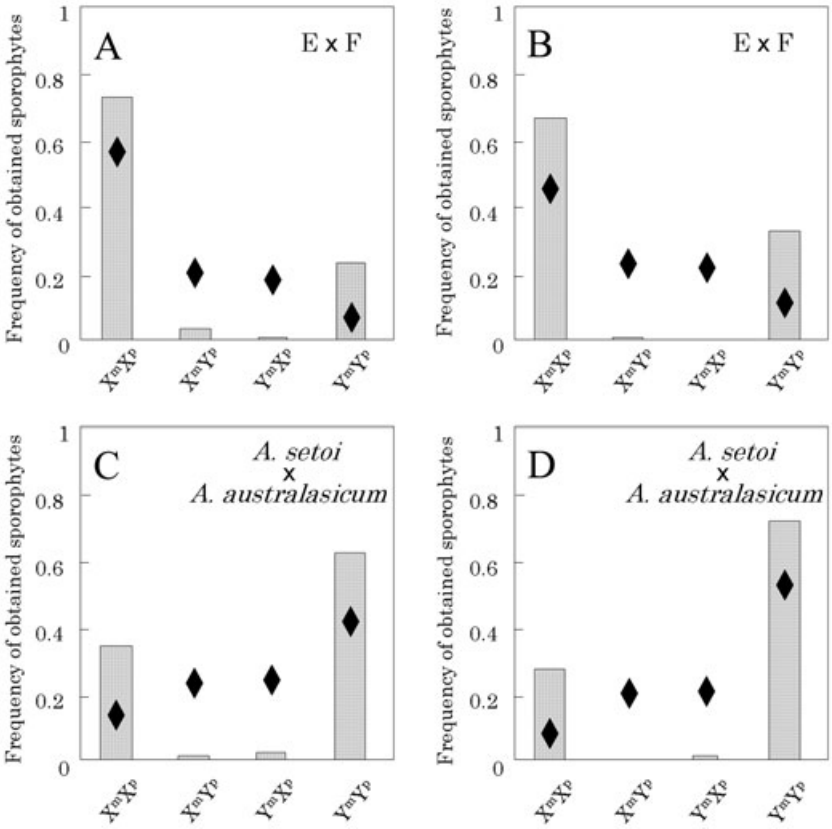


Figure 7. Frequencies of hybrid formation estimated between Type E and F and between *Asplenium setoi* and *A. australasicum*. Each figure corresponds to the results listed in Table 6. Solid diamonds indicate the frequencies expected under random crossing. A–B, X represents Type E and Y represents Type F. C–D, X represents *A. setoi* and Y represents *A. australasicum*.

types C and F and type E and *A. australasicum*), relatively small numbers of hybrids, between five and 15, were obtained, indicating low frequencies of hybrid formation or asymmetrical hybrid formation.

Correlation between capacity to form hybrids and genetic distance

Based on data from all the crossing experiments, the number of hybrids obtained decreased with genetic distance as assessed using *rbcL* sequences (correlation coefficient = –0.64, number of samples = 16; probability = 0.006, Fig. 6). There may, however, be statistical difficulty, because data points are not independent because the same parental individuals were used repeatedly and phylogenetic relationships exist among the pairs.

The data from this study suggest that hybrids do not often form between distantly related pairs because all five pairs in which K2P was higher than 0.013 were incapable of forming hybrids (Table 5, Fig. 6). The rapid loss of the capacity to form hybrids in the *A. nidus* complex seems to contrast strikingly with the abundant reports of experimentally produced hybrids (e.g. Lovis, 1968; Brownsey, 1976) and natural sterile hybrids (e.g. Knobloch, Gibby & Fraser-Jenkins, 1984; Iwatsuki, 1995) in Aspleniaceae. Although no putative natural hybrids have been reported within the *A. nidus* complex, sterile hybrids of *A. antiquum*, which has been assigned to *Asplenium* section *Thamnopteris*, and *A. prolongatum* Hook. were found in Japan, for which K2P is 0.031 (Murakami *et al.*, 1999a). Putative natural hybrids have been reported in the genus *Asplenium* even between *A. pseudo-wilfordii* Tagawa and *A. yoshinagae* Makino, *A. wrightii* Mett. & Kuhn and *A. ritoense* Hayata, *A. sarelii* Hook. and *A. ruprechtii* Kurata and *A. incisum* Thumb. and *A. ruprechtii*, for which K2P is at least 0.027 (Murakami *et al.*, 1999a). The frequencies with which natural hybrids occur have not, however, been examined, even in the localities where the parental species grow together. It is possible that the frequencies with which hybrids form may not always be high in natural populations, even between

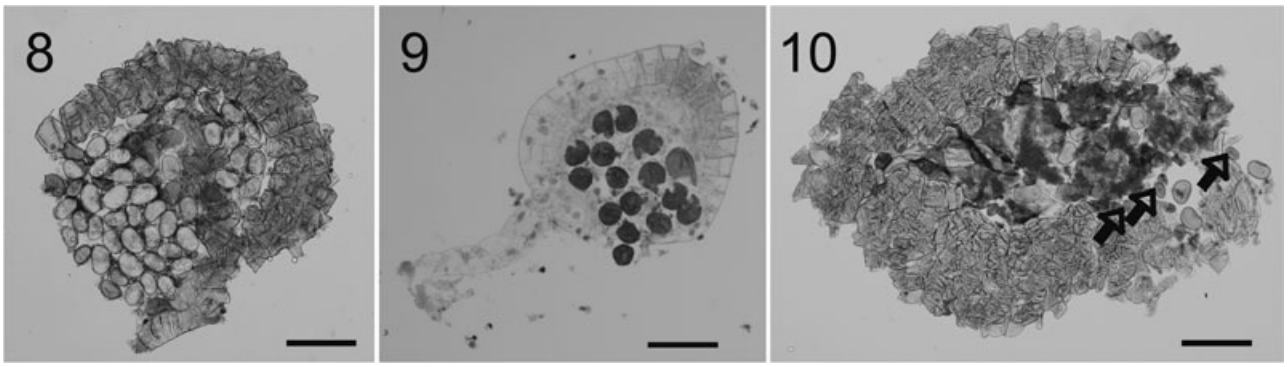
Table 7. The number of mature hybrids and the germination rates of their spores

Pair of *rbcL* sequence type	Maternal parent	Paternal parent	Number of hybrids cultivated for 5 years	Number of mature hybrids	Germination rates of spores in each hybrid (%)	
Type E × *Asplenium australasicum*	NC-99002 (aus)	PA-00020 (E)	1	1	< 1	
NN = 14						
K2P = 0.012						
A. setoi × A. australasicum	NC-99001 (aus)	IR-98001 (set)	2	2	0	< 1
NN = 14	NC-99002 (aus)	IR-98001 (set)	2	2	< 1	< 1
K2P = 0.012	IR-98001 (set)	NC-99001 (aus)	2	2	3	0
	IR-98001 (set)	NC-99002 (aus)	2	2	0	< 1
Type E × Type F	FR-00064 (E)	PA-00017 (F)	1	0		
NN = 13	PA-00017 (F)	FR-00064 (E)	3	0		
K2P = 0.011	PA-00017 (F)	PA-00020 (E)	5	2	0	< 1
Type C × Type F	CA-00028 (F)	HA-00020 (C)	3	0		
NN = 7						
K2P = 0.006						
Type E × Type EII	PA-00020 (E)	PA-00001 (EII)	3	2	84	86
NN = 1	PA-00001 (EII)	PA-00020 (E)	2	2	66	76
K2P = 0.001						
Type E × *A. setoi*	BO-00001 (E)	IR-98001 (set)	1	0		
NN = 0	IR-98001 (set)	BO-00001 (E)	6	0		
K2P = 0.000						
Type E × Type E	CA-00060 (E)	BO-00001 (E)	2	2	66	75
NN = 0	BO-00001 (E)	CA-00060 (E)	1	1	80	
K2P = 0.000						

Notes: K2P, genetic distance calculated by Kimura's two parameter method (Kimura 1980); NN, number of nucleotide substitutions.

Two capital letters in names of plant materials indicate the localities where they were collected. BO, Bogor Botanical Garden, West Java; CA, Cameron Highland, Malaysia; FR, Fraser's Hill, Malaysia; HA, Halimun National Park, West Java, Indonesia; IR, Iriomote Island, Japan; NC, New Caledonia; PA, within a radius of 50 km from Padang City, West Sumatra, Indonesia.

The letter(s) in parentheses indicate the *rbcL* sequence type of *A. nidus* or species name: aus, *A. australasicum*; set, *A. setoi.*



Figures 8–10. Fig. 8. Sixty-four normally shaped spores per sporangium from the hybrid between Type E and Type E2 (see Table 7). Fig. 9. Sixteen large and abnormally shaped spores per sporangium from the hybrid between *Asplenium australasicum* and Type E (Table 7). Fig. 10. Irregular shaped spores variable in size from the hybrid between Type F and Type E (Table 7) Arrows indicate small spores. Bars, 100 μm.

pairs that are capable of forming hybrids. We evaluated the frequency of hybrid formation for types E and F and *A. setoi* and *A. australasicum*, i.e. pairs that are distantly related but capable of forming hybrids bidirectionally in reciprocal crosses. The frequencies of hybrid formation were significantly lower than expected under random crossing for both pairs and were at most 15.8 % of the expected number (Table 6, Fig. 7). Therefore, even pairs that are capable of hybrid formation do not form hybrids with high frequency.

In Figure 6, we discriminate between crosses within one of the two major clades (clades W-1 and W-2) and crosses between these clades. In crosses between parental individuals nested within clade W-2, none of the three pairs (types B and G, type B and *A. australasicum* and type G and *A. australasicum*) could form hybrids, although the genetic distance among each of the pairs was at most 0.005. In contrast, hybrids were obtained in crosses between types C and F and types E and F, for which the genetic distances are 0.006 and 0.011, respectively. It is possible that the rate of decrease in hybrid formation differs among lineages. Cayouette & Catling (1992) discovered an unequal distribution of natural hybridization among the three subgenera of *Carex* L. (Cyperaceae). Unequal taxonomic distributions of hybridization are also demonstrated in fish (Hubbs, 1955) and birds (Grant & Grant, 1992). Therefore, it is suggested that divergence level and hybridization ability can evolve independently in plants as well as in animals.

Asymmetrical hybrid formation

Our results also suggest that asymmetric hybrid formation exists, because hybrids were obtained in only one of the two crosses between types C and F and type E and *A. australasicum* (Table 5, Fig. 5) and only individuals of type F and *A. australasicum* became maternal parents of hybrids resulting from these crosses. Barriers that cause asymmetric reproductive isolation in angiosperms are well documented by Tiffin, Olson & Moyle (2001). However, only a few studies have focused on asymmetrical reproductive isolation in homosporous ferns. Vogel *et al.* (1998) and Xiang *et al.* (2000) reported gender-biased hybridization in natural populations of *A. trichomanes* L. and *A. septentrionale* (L.) Hoffm. and *Dryopteris intermedia* (Muhl.) A. Gray and *D. carthusiana* (Vill.) H. P. Fuchs, respectively. Differences in breeding system may cause unidirectional hybrid formation because archegonia of outbreeding species tend to receive sperm from other gametophytes and become maternal parents. Inbreeding species, however, in which archegonia and antheridia reach maturity simultaneously, are unlikely to be the maternal parent in any hybridization event.

Although types C and E can be considered outbreeding species based on their low fixation indices ($F = 0.006$ and 0.054, respectively), when allozyme polymorphisms were analysed for populations from west Java (Yatabe, Darnaedi & Murakami, 2002), these types were not the maternal parent. With regard to type E, the data suggested that gametophytes mate randomly in Petri dishes (Table 4) and can act as the maternal parent of hybrids combining type E and *A. australasicum*. Therefore, differences in breeding system are unlikely to cause unidirectional hybrid formation in the *A. nidus* complex. Further investigation is required to identify the mechanisms of interspecific isolation that cause asymmetrical hybrid formation.

Hybrid sterility

The mature hybrids formed from phylogenetically distantly related pairs were all sterile (Table 7, Figs 9, 10). Out of three pairs that produced sterile hybrids, sporogenesis producing 16 large, abnormally shaped spores was observed in hybrids between *A. australasicum* and *A. setoi* and between *A. australasicum* and type E (Fig. 9). This type of sporogenesis has been reported in sterile fern hybrids and apomictic species (Morzenti, 1962, 1967; Evans, 1969; Vida & Reichstein, 1975; Pinter, 1995). The hybrids between types E and F produced sporangia containing spores variable in size, which is a characteristic of sterile fern hybrids (Fig. 10). Therefore, it is suggested that the distantly related pairs may be strongly reproductively isolated even if they produce hybrids.

Conclusion

Assuming that the *Asplenium nidus* complex is not a reticulate complex, each *rbcL* sequence type or each clade in the phylogenetic tree should represent separate evolutionary lineages. The spores of hybrids of individuals of type E from different localities (peninsular Malaysia and west Sumatra) germinated at high rates (Table 7), supporting the hypothesis that individuals with the same *rbcL* sequence are not reproductively isolated. Type E individuals were found in all four localities investigated in this study (Table 3) and are thought to be widely distributed in west Malesia. Three other *rbcL* sequence types, types EII, EIII and EIV, are also distributed in west Malesia. Type E individuals differ from each of these three types by only one nucleotide in *rbcL*. The high germination rates of spores of hybrids of type E and type EII suggest that these may be intraspecific variations because they are not reproductively isolated.

Whenever at least five nucleotides differed between *rbcL* sequence types, those *rbcL* types were always reproductively isolated in some way, whether by loss of the capacity to form hybrids, asymmetrical hybrid formation or hybrid sterility. Considering these results, it is likely that at least six cryptic species may exist in addition to the five cryptic species found from west Java (Yatabe *et al.*, 2002, types A, B, C, D and E). Haufler, Hooper & Therrien (2000) emphasized the role of ecological niche differentiation in speciation of the epiphytic fern genus *Pleopeltis* Humb. & Bonp. (Polypodiaceae) distributed in the Neotropics. It is also possible that these cryptic species in the *A. nidus* complex may occupy separate ecological niches because they differed in the elevation at which they occurred and the position on tree trunks on which they grew (Murakami *et al.*, 1999b; Yatabe & Murakami, 2003).

In addition to hybrid sterility, a low frequency of hybrid formation can affect reproductive isolation, even between closely related species, and hybrid formation between distantly related pairs is not widespread because the capacity to form hybrids and the frequency of hybrid formation seem to decrease rapidly with divergence. A task for the future is to determine whether prezygotic isolation or zygotic sterility is the mechanism responsible for reducing hybrid formation in the *A. nidus* complex.

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