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Ploidy level of the invasive weed *Rubus alceifolius* (Rosaceae) in its native range and in areas of introduction

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Abstract. A change in ploidy level could increase invasiveness of introduced plants in insular plant communities. To examine this question for *R. alceifolius*, we compared its ploidy level in its Asian native range and in the Indian Ocean islands where it has been introduced. We first counted chromosomes on root tips from a Vietnamese individual, which proved to be tetraploid ($2n = 4x = 28$). The nuclear DNA content of other individuals from the native range and areas of introduction was estimated using the flow cytometry method. The Vietnamese individual on which chromosomes were counted was added to the sample, to enable deduction of the ploidy level of all individuals from their nuclear DNA content. All individuals were found to be tetraploid, except 10 individuals from a single clone collected in a Vietnamese population, estimated to be triploid, and morphologically different of other individuals of this study. We showed that while polyploidy of the source population may have predisposed this plant to become a successful invader, its introduction into Indian Ocean islands was not associated with any change in ploidy level.

Key words: *Rubus alceifolius*, *Malachobatus*, Indian Ocean islands, invasive species, weeds, ploidy level, chromosome counts, flow cytometry.

According to Barrett and Richardson (1986), one of the most frequently recurring characters of invading plants is a high ploidy level. Examples are *Chondrilla juncea*, a weed in Australia (Bergman 1952, Cuthbertson 1974), the water hyacinth, *Eichhornia crassipes* invading Asian rivers (Barrett 1982), and *Spartina anglica* (Thompson 1991). Polyploidy allows the plant to diversify its genome, conferring adaptive plasticity (Roose and Gottlieb 1976, Bretagnolle et al. 1998), to hide deleterious alleles through an increase in frequencies of heterozygous loci (Bretagnolle et al. 1998), and to reduce the cost of inbreeding.

Rubus alceifolius Poir. (Rosaceae, subgenus *Malachobatus* Focke), is a simple-leafed southeast Asian bramble that has been a serious weed on La Réunion island since the early 20th century (Lavergne 1978, Quere 1990, Soulères 1990, Sigala and Lavergne 1996). Although the precise time of its introduction in this island is still unknown, records suggest an introduction sometime in the mid-19th century (in Lavergne 1978). *Rubus alceifolius* invades native vegetation and road edges from sea level to about 1600 meters elevation. Introduced populations of this bramble also occur on other Indian Ocean islands (Madagascar, Mayotte, Mauritius) and in Australia (Queensland). A previous study based on AFLP markers showed

that this weed was likely to have been first introduced in Madagascar from an unknown Asian locality, before invading the other Indian Ocean islands from Madagascar sources (Amsellem et al. 2000).

Ploidy level is quite variable in the genus *Rubus*, primarily due to a high potential for hybridization between more or less closely related taxa. These hybridizations often give rise to fertile hybrids with an intermediate ploidy level compared to their parents (Gustafsson 1943, Nybom 1986, Weber 1996). All species of the subgenus *Malachobatus* are suspected to be polyploid (from $2n = 4x = 28$, to $2n = 14x = 98$) (Nybom 1986, Thompson 1997). The objective of this study was to compare ploidy level of *R. alceifolius* in its native range and in areas of introduction, to examine whether its aggressiveness in insular plant communities might be associated with a change in ploidy level.

Ploidy determinations have traditionally been done only by chromosome counts, but this method is laborious, particularly if many individuals need to be evaluated. More recently, flow cytometry has become a commonly used procedure for determining the DNA content of plant nuclei, and deducing ploidy level. For assessment of ploidy of a single plant or plant populations, it is much more rapid and convenient than root tip chromosome counts (de Laat et al. 1987, Bennett and Leitch 1995, Dolezel 1998, Brummer et al. 1999).

In this study, we determined ploidy level of a reference individual, by chromosome counts on root tips, and then used flow cytometry to compare ploidy level of this individual with that of *R. alceifolius* in different localities in its native range and its area of introduction.

Materials and methods

Vocabulary. In this study, we will use the term "locality" to design an entire region in the distribution of *R. alceifolius*, such as an island or a country. "Population" will be used to designate groups of individuals geographically close enough to exchange genes. "Individual" will represent a ramet, well delimited in space. "Genet" will be used to regroup several ramets originating from a single genetic individual, where this could be determined by molecular markers. "Clone" will represent the individuals issued from the same ramet and vegetatively reproduced.

Plant material. We studied healthy individuals of *R. alceifolius* grown in a greenhouse in Montpellier, France, and originating from various geographic localities in the plant's native range and areas of introduction. Individuals from Madagascar (a total of 11 individuals from three populations), La Réunion (13 individuals from three populations), and Sumatra (13 individuals from four populations) were obtained from germinated seeds. Individuals from four populations in Thailand (12 individuals from populations sampled in the National Park of Kaho Yaï), and three populations in Vietnam (15 individuals) were harvested in their natural populations and transplanted into the greenhouse in Montpellier. The 15 Vietnamese individuals were sampled in different populations as follows: 10 individuals in Cuc Phuong, one individual in Lang Son, and four individuals in Tam Dao. The 10 Vietnamese samples from Cuc Phuong represent in fact one individual cloned through suckering and cuttings. The four samples considered in the population of Tam Dao represent also two genets, each having been cloned to give two ramets. In these two populations, individuals were well identified and constituted controls to test reliability and repeatability of the flow cytometry method. Voucher specimens were deposited at the herbarium of the CIRAD, Department of Breeding and Veterinary Medicine (EMVT), Montpellier, France.

Chromosome counts. Chromosome numbers were established from counts in root tips of a healthy Vietnamese individual (locality of Lang Son). We used the protocol modified by D'Hont et al. (2000). Actively growing roots (0.5 cm) were treated with 0.04% 8-hydroxyquinoline in the dark, at room temperature (RT) during four hours, and four hours at +4 °C. They were fixed in 3:1 ethanol:glacial acetic acid (v/v) at RT during 48 hours. To obtain protoplasts, root tips were rinsed in demineralized water two times for 10 minutes, hydrolyzed in 0.25 N HCl during 10 minutes rinsed again in water during 10 minutes, and bathed in 0.1 M citrate buffer ($pH = 4.5$) for 10 minutes. Meristem tips (1–1.5 mm) were cut and digested in an enzyme mixture (5% cellulase

Onozuka R-10 [Yakult Honsha Co. Ltd., Japan], 1% pectolyase Y-23 [Seishin Pharmaceutical Ltd., Japan]) during 45 to 60 minutes at 37 °C. Reaction was stopped by rinsing root tips twice in water during 10 minutes at RT. Meristem tips were then spread with a forceps in a drop of 3:1 fixative. Slides were then dried at RT for 20 minutes. Chromosomes were stained in a 2 μg/ml DAPI solution in a MacIlvaine buffer (citric acid 9 mM, disodium hydrogeno-phosphate 80 mM, magnesium Chloride 2.5 mM).

Chromosomes were observed with a microscope, under a wavelength of 349 nm. Chromosome counts were established based on observations of 10 cells in metaphase.

Flow cytometric analysis. Samples consisted of about 2 to 3 cm^2 of fresh foliar tissue, without any sign of necrosis, pathogen, or wound. To prevent such an artifact in our sample, we limited the chance of having a large proportion of nuclei in the G_2 stage of the cell cycle, by choosing tissues from well defined adult leaves. Tissues were finely chopped up with a razor blade in a Petri dish, in 900 μl of ice-cold buffer (pH = 9, 15 mM Tris-HCl, 2 mM EDTA, 80 mM KCl, 20 mM NaCl, 20% Triton X-100, and 0.1% mercaptoethanol). After fine chopping, 900 μl of buffer was added and homogenized. The suspension of isolated nuclei was filtered through a 50 μm nylon mesh, and about 900 μl of nuclei suspension was stained by adding 140 μl of propidium iodide (1 mg/ml). The solution was gently mixed, and left at RT for 1 to 3 minutes before being analyzed with the flow cytometer.

The flow cytometer was a FACScan, working with the software Cellquest (Becton Dickinson, Mountain View, CA). From laser excitation of propidium iodide at 488 nm, we measured reflection at 585 nm to read 2C nuclei DNA contents of 5000 nuclei per sample.

We used *Petunia hybrida* as a reference standard (2C = 3.35; Bennett and Smith 1976), because preliminary trials showed for this species 2C peaks close to those of *R. alceifolius*, and without any overlap, as recommended by Johnston et al. (1999). The use of an internal reference standard gave poor reading of results in peak qualities, probably resulting from interference between the staining solution and the genomes of the two species. For this reason we preferred to use the reference standard as external, and controlled every five samples to check the calibration of the flow cytometer.

The gain of the instrument was adjusted so that the G_0/G_1 peak of *P. hybrida* was on channel 800. The nuclear DNA content (in pg) of *Rubus* samples was estimated according to the equation: 2C nuclear DNA content = $(3.35 \times G_0/G_1$ peak mean of *Rubus*$)/(G_0/G_1$ peak mean of *Petunia*).

To correlate the 2C nuclei DNA content of samples with their ploidy level, we estimated with flow cytometry the 2C nuclei DNA content of the Vietnamese individual from which chromosome counts were made, through eight measures randomly conducted during the manipulation (these eight measures represent the sample size of Lang Son in this study). Assuming that ploidy level of individuals is proportional to their 2C nuclear DNA content, we deduced ploidy level of each sample according to the mean DNA content and the ploidy level of the Vietnamese individual from Lang Son.

Statistical analysis. Statistical analyses were performed using SAS (SAS Institute 1996, version 8, PROC GLM). Nested ANOVA and a Bonferroni multi-comparison test were used to analyze the variation in 2C nuclear DNA content among localities and among populations within localities.

Concerning repeated measures from Vietnamese clonal individuals (Cuc Phuong and Tam Dao) or the same individual (Lang Son), means of 2C nuclear DNA content were calculated for each population, and analyzed as a single value.

Results

Chromosome counts. Ten cells of the Vietnamese individual from Lang Son were counted, and all exhibited 28 chromosomes (Fig. 1). The basic chromosome number in the genus *Rubus* is x = 7 (Thompson 1997). Presence of 28 chromosomes thus corresponds to tetraploidy (2n = 4x = 28).

Flow cytometric analysis. Flow cytometric analysis of isolated nuclei resulted in histograms of their DNA content compared to that of the standard reference, and represented one peak corresponding to the G_0/G_1 nuclei of *R. alceifolius*. This peak represents the number of nuclei as a function of level of fluorescence. Only the samples with clear high peaks were retained for this study (Fig. 2). Details of

sampling, results of flow cytometry, and genome sizes (in Mbp), are given in Table 1.

The 2C nuclear DNA content among all localities ranged from 1.62 to 1.82 pg, except for the sample of Cuc Phuong (Vietnam) which displayed a content of 1.29 ± 0.08 pg. This last individual can be assumed to be triploid (Table 1).

Nested ANOVA indicated significant differences in 2C nuclear DNA content among localities and among populations within localities, if measures of the individual from Cuc

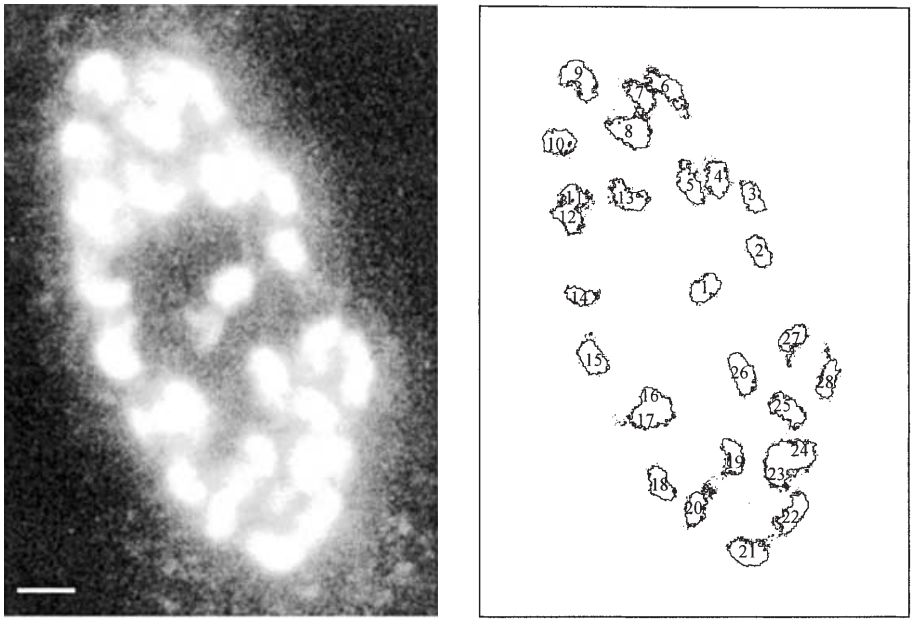

Fig. 1. Nuclear chromosomes of a Vietnamese individual of *R. alceifolius*, from the population of Lang Son. Bar scale represents 0.075 μm

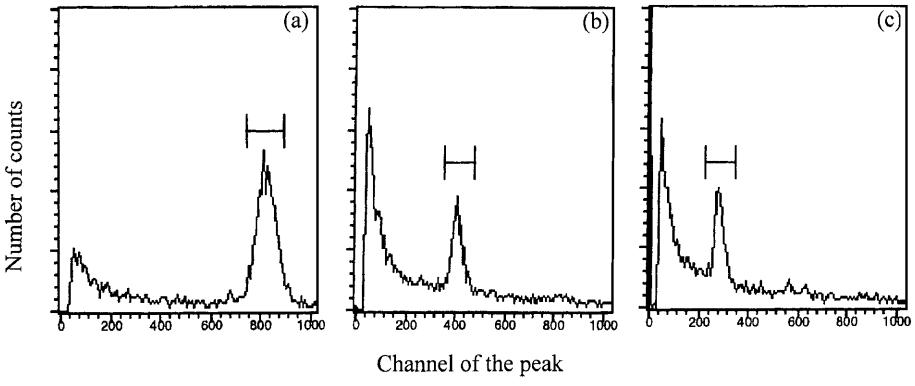

Fig. 2. Histogram of relative nuclear DNA content obtained during the analysis of *R. alceifolius* individuals. The gain of the instrument was adjusted so that the G_0/G_1 peak of *P. hybrida* was on channel 800 (**a**). Peaks of *R. alceifolius* from all populations from the native range and the area of introduction were centered on channel 400 (**b**), except those of samples from Cuc Phuong which were centered on channel 300 (**c**)

Table 1. Geographical origins of the studied individuals of *R. alceifolius*. Mean ± standard deviation of 2C nuclear DNA content (pg), ploidy level, Mean genome size (= MGS), and Bonferroni's groups (= BG; P = 0.05) are given for each population

Locality	Population	Coordinate	Sample size	Peak	2C nuclear DNA content	Ploidy level[a]	MGS (Mbp/1C)[b]	BG
Madagascar	Ampasimandinika	18°58′S; 49°13′E	5	407.38 ± 22.62	1.71 ± 0.09	4 ± 0	823 ± 46	A
	Beforona	18°44′S; 48°24′E	1	410.64	1.72	4	830	A
	Ranomafana	18°56′S; 48°25′E	5	432.81 ± 24.56	1.81 ± 0.10	4 ± 0	874 ± 50	A
	Whole locality		11	419.23 ± 24.81	1.76 ± 0.10	4 ± 0	847 ± 50	
La Réunion	Chemin de Malécage	21°04′N; 55°40′E	5	416.83 ± 8.27	1.75 ± 0.03	4 ± 0	842 ± 17	A
	Grand – Etang	21°04′N; 55°40′E	5	418.43 ± 24.16	1.75 ± 0.10	4 ± 0	845 ± 49	A
	Route des Ravines	21°04′N; 55°40′E	3	433.44 ± 26.18	1.82 ± 0.11	4 ± 0	876 ± 53	A
	Whole locality		13	421.28 ± 19.50	1.76 ± 0.08	4 ± 0	851 ± 39	
Sumatra	Kampung Simarmata	2°44′N; 98°42′E	3	426.42 ± 19.39	1.79 ±0.08	4 ± 0	862 ± 39	A
	Procecidochares	2°44′N; 98°45′E	3	423.39 ± 16.18	1.77 ± 0.07	4 ± 0	855 ± 33	A
	Simararunjung (Simpang)	2°42′N; 98°55′E	4	426.90 ± 19.01	1.79 ± 0.08	4 ± 0	863 ± 38	A
	Tuk-Tuk/Tomok	2°40′N; 98°51′E	3	417.44 ± 16.75	1.75 ± 0.07	4 ± 0	843 ± 34	A
	Whole locality		13	423.79 ± 16.08	1.77 ± 0.07	4 ± 0	856 ± 32	
Thailand	Kaho Yaï – population 1	14°18′N; 101°21′E	3	412.42 ± 28.16	1.73 ± 0.12	4 ± 0	833 ± 57	A
	Kaho Yaï – population 2	14°23′N; 101°22′E	3	409.66 ± 7.74	1.72 ± 0.03	4 ± 0	828 ± 16	A
	Kaho Yaï – population 3	14°21′N; 101°21′E	3	397.85 ± 10.04	1.67 ± 0.04	4 ± 0	804 ± 20	A
	Kaho Yaï – population 4	14°18′N; 101°21′E	3	412.49 ± 7.37	1.73 ± 0.03	4 ± 0	833 ± 15	A
	Whole locality		12	408.11 ± 14.93	1.71 ± 0.06	4 ± 0	825 ± 30	
Vietnam	Cuc Phuong	20°18′N; 105°35′E	1; 10 measures	307.22 ± 19.65	1.29 ± 0.08	3 ± 0	621 ± 40	B
	Lang Son[c]	21°75′N; 106°40′E	1; 8 measures	410.75 ± 3.83	**1.72** ± 0.02	4 ± 0	830 ± 8	A
	Tam Dao	21°27′N; 105°38′E	2; 4 measures	433.30 ± 28.32	1.81 ± 0.12	4 ± 0	875 ± 57	A
	Whole locality[d]		3; 12 measures	418.26 ± 18.75	1.75 ± 0.08	4 ± 0	845 ± 38	A
	All localities[d]			418.26 ± 19.11	1.75 ± 0.08	4 ± 0	845 ± 39	

[a] Ploidy level assuming it is proportional to DNA amount: pl = 4×2C nuclear DNA content/1.72.
[b] One copy of nuclear genome. 1 pg = 965 Mbp (Bennett and Smith 1976).
[c] Individual on which chromosomes were counted. Eight measures with flow cytometry were done to obtain reference mean 2C nuclear DNA content.
[d] Only tetraploid individuals are consisdered.

Table 2. Analysis of data on nuclear DNA content by a nested ANOVA. Analysis was made including Cuc Phuong samples and excluding these samples

Source of variation	df	Mean squares	F-value	P
Cuc Phuong individuals included				
Among localities	4	0.023	3.648	0.013
Among populations within localities	9	0.022	3.442	0.003
Cuc Phuong individuals excluded				
Among localities	4	0.009	1.372	0.262
Among populations within localities	8	0.006	0.917	0.514

Phuong are taken into account (Table 2). If the Cuc Phuong individual is omitted from the analysis, no difference among localities or among populations within localities is found (Table 2).

The Bonferroni multi-comparison test characterized the variation in 2C nuclear DNA content among all the populations taken into account (Table 1). The individual from Cuc Phuong is confirmed to have a consistently different 2C nuclear DNA content than other individuals from the native range and areas of introduction.

Discussion

Because root tips and chromosomes of *R. alceifolius* are small, it was difficult and problematic to obtain root cells in clear metaphase, and to count chromosomes on many individuals from a number of populations. We thus added the individual from Lang Son on which chromosomes were counted to the sample of individuals investigated through flow cytometry, to use it as a reference for examining ploidy level of *R. alceifolius*. This method offers a more rapid and reliable means to determine ploidy level than chromosome counting of root tips.

The eight replicated measures done on the individual from Lang Son gave the narrowest standard deviations obtained in this study for the estimation of 2C nuclear DNA content. The repeatability of measures of this sample validate the reliability of the technique, and the small variations detected are certainly due to residual imprecision. The measurements made on several ramets of the same clone (individuals of Cuc Phuong) allowed quantification of DNA variation from one clone to another. In this case, differences observed may be due to the accumulation either of methodological errors or to differences in the general health conditions of the different ramets grown from the individual. Moreover, a limited extent of intraspecific variation observed in the genome size of *R. alceifolius* could also reflect plasticity in the nuclear genome of this species, such as variations in the number of telomeric repeats (Shippen and McKnight 1998). Large intraspecific variations in genome size have been detected in *Musa* species (Dolezel et al. 1994, 1997), *Pisum sativum* (Cavallini et al. 1993), and *Glycine max* (Graham et al. 1994). For these two last species, more recent studies have not confirmed these variations (Baranyi and Greilhuber 1996, Greilhuber and Obermayer 1997, respectively). A third explanation for these observed variations could come from the importance of the proportion of nuclei in the G_2 stage of the cell cycle, with double DNA content compared to nuclei in the G_0 and G_1 stage. Even if this proportion is insufficient to present any ambiguity in interpreting the true ploidy level of individuals with flow cytometry, it could be sufficient to create artefactual intraspecific variation in measures of 2C nuclear DNA contents (de Laat et al. 1987, Blondon et al. 1994). All the variations observed in this study are insufficient to create any significant artefactual differences between the studied populations, and our results con-

firm previous findings that flow cytometry may be used for the precise and reproducible estimation of nuclear DNA content (Dolezel et al. 1994, 1997; Bennett and Leitch 1995; Johnston et al. 1999; Lysak et al. 1999).

Although *R. alceifolius* was not represented in any previous studies determining the polyploidy level of species from the subgenus *Malachobatus*, our study confirms that *R. alceifolius*, like those species studied previously, is also polyploid. Moreover, it was found to be tetraploid, as is *R. moluccanus* (Nybom 1986), considered to be its sister species. The 2C nuclear DNA contents of tetraploid and triploid *R. alceifolius* found in this study (2C = 1.75 pg and 1.29 pg, respectively) are consistent with the results obtained on *Rubus odoratus*, a diploid species with 2C = 0.8 pg (Bennett and Leitch 1995). Previous molecular genetic studies of *R. alceifolius* had already indicated that *R. alceifolius* was tetraploid, both in its native range and its areas of introduction. Microsatellite profiles from Asian and introduced populations exhibit individuals bearing three or four alleles for some amplified loci (Amsellem et al. 2001). However, the strong suspicion of null alleles for some loci prevented conclusions about the ploidy level of *R. alceifolius* based on these molecular markers. Studies with ALFP markers had also suggested the possibility that ploidy level was the same in the native range and areas of introduction. Those populations showed a number of band levels of the same range (Amsellem et al. 2000). The present work confirms the tetraploidy of *R. alceifolius*, both in Asian populations and those from Indian Ocean islands, showing that no change has occurred in ploidy level during the introduction of this weed. Thus, a change in ploidy level is not the cause of the remarkable capacity of *R. alceifolius* to invade plant communities in the Indian Ocean islands. Other investigations show that a switch occurred in the mating system of *R. alceifolius* between native and introduced populations (Amsellem et al. in press). High reproductive rate of this weed, associated both with the clonal propagation of genotypes well adapted for invading these insular environments, and the known susceptibility to invasion of insular communities by continental species (de Vos et al. 1956, Carlquist 1966, Kramer 1971, Pizzey 1980, van Riper and van Riper 1982, Barrett 1996), could be the major causes for the rapid spread of *R. alceifolius* through these insular communities. The polyploidy of the source population may have contributed to the invasive potential of this plant in its areas of introduction.

Observation of one triploid Vietnamese individual of *R. alceifolius* in the population from Cuc Phuong, while individuals from other Vietnamese populations have been shown to be tetraploid, is puzzling. The observation of triploidy of a single individual does not allow us to conclude about the existence or frequency of other triploid individuals in this population. If we assume that a population constitued of triploid individuals exists in Cuc Phuong, it is hard to envisage how such an islet of triploid individuals could endure among a sea of tetraploids in Vietnam and the rest of the species' Asian range. However, small morphological differences can be observed between Cuc Phuong individuals and those from other Vietmanese populations (T. Le Bourgeois, CIRAD, pers. comm.), suggesting that individuals from this population could belong to a taxon other than *R. alceifolius*. Nybom (1986) showed that hybridizations could occur between closely related species of the subgenus *Malachobatus*. Cuc Phuong individuals could be hybrids between *R. alceifolius* and a closely related species. The persistence of such hybrid triploid individuals could be ensured by asexual reproduction (vegetative propagation, but also apomixis), well documented in the genus *Rubus* but described only once in the subgenus *Malachobatus* (Nybom 1986, Amsellem et al. in press), although studies show that sexuality seems to be largely predominant in Asian species of the subgenus *Malachobatus* (Nybom 1986, Busemeyer et al. 1997, Amsellem et al. 2000, 2001, and in press). The hypothesis of an

hybrid asexual population of *R. alceifolius* in Cuc Phuong could be tested by screening a large number of individuals in wild populations, by comparison of morphological traits with those of other Vietnamese *R. alceifolius* proved to be tetraploid, by quantification of 2C nuclear DNA content of plant in this population with flow cytometry, and by experiments on reproductive biology.

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