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博 士 論 文

Reproductive ecology in a heterostylous aquatic plant

Nymphoides indica

異型花柱性水生植物ガガブタにおける繁殖生態学的研究

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神戸大学大学院自然科学研究科

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Doctoral Dissertation

**Reproductive ecology in a heterostylous
aquatic plant *Nymphoides indica***

(異型花柱性水生植物ガガブタにおける繁殖生態学的研究)

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Summary

The aim of this study is to elucidate the ecological driving force underlying the variation of floral morph composition in a heterostylous aquatic plant, *N.indica* and propose a strategy of conservation based on the ecological traits of *N.indica*.

In the first chapter, the quantitative evaluation was performed to know whether the trait of heterostyly was maintained or not in *N.indica*. Long-styled morph and short-styled morph were clearly discriminated by the different length of pistil and stamen. The pollen size of short-styled morph was larger compared to that of long-styled morph. The difference of pollen size between long-styled and short-styled morph was about $8\ \mu\text{m}$. Moreover, I conducted the following three treatments to determine compatibility between the two morphs: 1) intermorph pollination; 2) intramorph pollination; and 3) self-pollination. Fruit and seed set were observed only in intermorph pollination. These results showed that heterostyly of *N.indica* in Japan was rigidly maintained.

In the second chapter, the floral morph composition and seed set rate in the populations of *N.indica* were investigated. Four of 25 populations in Hyogo and Okayama Prefectures showed equal floral morph ratio. Ten populations were composed of biased floral morph ratio and the remaining 11 populations were monomorphic. The parameter $[|L-S| / (L+S)]$ representing the bias of floral morph composition in the populations was negatively correlated with the seed set. Legitimate pollination rate in a population with equal floral morph

ratio was higher than the populations with biased floral morph ratio and monomorphic ones. In the populations with biased floral morph ratio, the pollens on the stigma of the common morphs were dominated by pollen of the same morph, resulting in reduced seed set. Addition of the pollens of the opposite morph on the stigmas of the common morph brought about the increase of fruit and seed set rates. Intermorph pollination rate and the fruit and seed set rates of *N. indica* were highest when two floral morphs were located in the near distance in the populations. Various kinds of insects including the species of Lepidoptera, Diptera and Hymenoptera visited the flowers. However, geitonogamous pollination seemed to be common because of short flying distance of the pollinators.

These results showed that seed set of *N. indica* is limited by the shortage of compatible pollens and the success of intermorph pollination is influenced by the spatial distribution of the two floral morphs. As *N. indica* populations are endangered, conservation of the populations with high fecundity was proposed.

In the third chapter, genetic structure within and among populations was examined by allozyme analysis to determine whether the different reproductive modes in each local population reflect to the genetic structure and whether the balance of floral morph composition is maintained by gene flow. PGM, SkDH and PGI were polymorphic out of nine enzymes studied. Three genotypes of each enzyme were confirmed and the number of multilocus genotype (MLG) based on the combination of genotype of three polymorphic loci was eleven. Seed set rate was positively correlated with the number of MLG. Nine of eleven

MLGs occurred in two populations with equal floral morph and Hardy-Weinberg equilibrium showed that random mating has occurred in those populations. On the other hand, monomorphic populations were composed of a single genotype except one population. There are two hypotheses. One possibility is that some propagules from other populations have clonally propagated after immigration. Another possibility is that several genotypes including different floral morph types occurred but demographic stochasticity eventually eliminated all but one from the population. G_{st} that shows genetic differentiation was 0.5, which showed that gene flow was rare event.

The results showed that the establishment of seedlings contributed to the increase of genetic diversity in populations with equal floral morph composition.

In the fourth chapter, field survey from seed dispersal to the establishment of seedlings coupled with laboratory-based experiments was performed to know the environment conditions required for seed germination and the establishment of seedlings in *N.indica*. Seed germination occurred only in populations with equal floral morph ratio. The buried seeds following seed dispersal spatially distributed from the shore to offshore. However, seed germination was definitely limited to the water margin where amphibious and uliginous species grew or bare space after occasional water drawdown. The bank construction and absence of remarkable water fluctuation by the abandonment of water management prevented seeds from germinating in most of the populations. Seeds buried under the deep water level remained as seed

bank. The environment required for the establishment of seedlings was a period of unsubmerged condition. In the laboratory-based experiments, the cumulative germination rate in the incubator was high with light (14 h light / 10h Dark) and alternative temperature (25°C/12°C), which corresponded to the environmental condition of the water margin or bare space after occasional water drawdown in the field. This result showed that seed germination occurred not under the deep water but on the exposed ground. Cultivated experiment showed that inundation suppressed the growth when the developing stage of seedlings was younger, whereas the establishment of seedlings succeeded in spite of inundation when the developing stage was later.

In the fifth chapter, stochastic computer simulation combined with the field survey was performed to know whether the different reproductive modes give an influence to the floral morph composition or not. In the irrigation pond with small water fluctuation, the establishment of seedlings succeeded along the water margin when rootstocks or turions survived in the deep water level. Computer simulation supported that equal floral morph ratio will be brought about if the establishment of seedlings succeeds even in a low frequency.

Moreover, in the irrigation pond with remarkable water fluctuation, seedlings could establish in case of water drawdown although rootstocks or turions died. The computer simulation showed that equal floral morph ratio will be brought about if the sexual reproduction occurs more frequently than vegetative reproduction in case of water drawdown.

On the other hand, in the populations dominated by vegetative reproduction, the bias of floral morph composition has become more notable

for six years, which was also supported by computer simulation.

Thus, the theoretical and empirical analyses showed that the establishment of seedlings in the ecotone or in case of occasional water drawdown served to lead to the equal floral morph composition. However, the bias of floral morph ratio in the populations with only a vegetative reproduction became more notable in the future, which brings about decreasing seed production. The increase of populations with severely skewed floral morph composition will threaten to the long-term persistence of *N.indica*.

Balanced floral morph composition and genetic diversity in *N. indica* was brought about if the ecotone of water bodies and occasional water fluctuation occurred. However, the bank construction and abandonment of water management observed in most of the present populations will lead to the collapse of the balanced floral morph composition and inferiority of genetic diversity. To conserve *N.indica* populations, the presence of ecotone and the maintenance of water level fluctuation are needed.

Preface

Sexual dimorphism such as dioecy, gynodioecy, and heterostyly gives a strong influence to the mating pattern and population dynamics because the crossing patterns were severely limited. In particular, the influence of spatial patterns of compatible partners and plant density to reproductive success had been proved (Barrett & Thomson 1982; Feinsinger et al. 1991; House 1992, 1993; Wyatt & Hellwig 1979, Nishihiro & Washitani 1998).

Moreover, the factors determining population structure including the balance of sex composition had been investigated in dioecious species (review in Lloyd & Webb 1977; Cox 1981; Bierzycudek & Eckhart 1988; Eppley et al. 1998). Males and females are known to be spatially segregated by the mechanism of environmental sex determination and heritable variation for offspring sex composition in concert with limited dispersal of seeds (Bierzycudeka & Eckhart 1988; Houssard et al. 1994; Taylor 1996).

Compared to the dioecious species, the factors determining population structure in heterostylous species remains to be solved (Barrett et al. 1983; Morgan & Barrett 1988; Barrett et al. 1989p; Eckert & Barrett 1992 ; Eckert & Barrett 1995). For example, as Eckert & Barrett (1992) pointed out, there occur natural populations with different floral morph compositions in heterostylous plants, reflecting the history and ecology of the species. The clonality (Morgan & Barrett 1988; Eckert & Barrett 1992; Eckert & Barrett 1995), low outcrossing level (Barrett et al. 1989) and mating asymmetries among floral morphs

(Barrett et al. 1983) had been thought to result in the deviation from equal floral morph. The floral morph is genetically determined in heterostylous plants (Ganders 1979; Lewis & Jones 1992) and reproductive success from disassortative pollination to the establishment of seedlings leads to an equal floral morph composition.

In the most of heterostylous species, sexual reproduction occurs with vegetative reproduction (Ganders 1979). It is likely that the balance of reproductive modes within a species changes according to the environmental condition (i.e. the traits of habitat, the extent of disturbance and the rate of habitat succession) (Sebens & Thorne 1985; Philbrick & Les 1996; Damman & Cain 1998; Mandujano et al. 1998; Barsoum 2002 ; Winkler & Fisher 2002), which will bring about the further variation of floral morph composition within a heterostylous species. The environmental factor determining reproductive mode representing floral morph composition in case of heterostylous species has been not documented except *Decodon verticillatus* (Eckert et al. 1999; Dorken & Eckert 2001).

In case that heterostylous plant is endangered species, the balance of floral morph composition may give a critical influence to long-term persistence. In *Primula sieboldii*, the absence of pollinator, *Bombus diversus tersatus* queen, prevent outcrossed seeds through disassortative pollination from being produced because the habitat of the pollinators has been limited by the change of surrounding environment into urban areas and golf course (Washitani et al. 1994). According to the computer simulation based on the supergene model for heterostyly of *Primula* (Dorwick 1956), severe limitation of pollinator

availability is suggested to lose the occurrence of short-styled morph and bring about the increase of recombinant of homostyle floral morphs which can produce seeds by self pollination, which shows that genetic deterioration may be caused in the future (Washitani 1996).

Nymphoides indica O. Kuntze (Menyanthaceae) has a long-styled morph with the pistil longer than stamens and short-styled morph with stamens longer than pistil (Ornduff 1966). Reddy and Bahadur (1976) and Barrett (1980) investigated some traits of heterostyly in populations of *N. indica* in India and Brazil, respectively, and showed that intermorphic pollination is usually required for the fruit and seed sets.

N. indica reproduces vegetatively as well as sexually (Hamashima 1979, 1985). Floral morph composition of the populations has been shown to be diverse in Japan (Hamashima 1979). Some traits of heterostyly in *N. indica* in Japan had been qualitatively investigated. The observation of Hamashima (1979) supported the result by Reddy and Bahadur (1976) and Barrett (1980). Moreover, *N. indica* is an endangered plant (Environmental Agency of Japan 2000) and recently many populations have been declined due to the eutrophication of water and reclamation of habitats. Thus, isolation and fragmentation of habitats are more and more in progress. To know the environmental conditions underlying the variation of floral morph composition in *N. indica* may provide me a direction for conservation.

The aim of the study is to investigate the ecological driving force bringing about the variation of floral morph composition in *N. indica* and propose a strategy of conservation based on the ecological traits of *N. indica*.

To achieve the aim, chapters 1 and 2 focused on confirming whether the trait and function of heterostyly in the field condition is maintained or not. The quantitative re-evaluation was performed to determine whether the trait of heterostyly in *N.indica* was maintained (Chapter 1). Spatial distribution of pistil and stamen in long-styled and short-styled morph, the difference of the pollen size between both of the floral morphs and the compatibility traits were measured. To know the present status of floral morph composition and reproductive success including pollination success in the field condition, the floral morph composition, fruit and seed set rates and legitimate pollination rate within and among populations were measured in 25 populations in Higashi-Harima of Hyogo Prefecture and Okayama Prefecture. Disassortative pollination is likely to be promoted and seeds will be normally produced in populations with equal floral morph composition if the pollinators are available. On the other hand, in case of populations with biased floral morph composition or monomorphic, the shortage of compatible pollens may result in reduced seed set.

Chapters 3 and 4 focused on dissolving the means for regeneration in each population. To determine whether the different reproductive mode among populations reflects to the genetic structure or not and whether the balance of floral morph composition is maintained by gene flow or not, allozyme analysis was performed (Chapter 3). Field survey from seed dispersal to the establishment of seedlings combined with laboratory-based experiments was tried to investigate the environmental conditions required for the establishment of seedlings bringing about equal floral morph composition

(Chapter 4).

Finally, to elucidate the effect of different reproductive mode on the floral morph composition, stochastic computer simulation combined with the environmental situation for the establishment of seedlings and the field survey including the change of floral morph composition in the populations dominated by vegetative reproduction were tried (Chapter 5).

CHAPTER 1

Heterostyly in *Nymphoides indica* O.Kuntze (Menyanthaceae) in Japan

1.1. Introduction

Nymphoides (Menyanthaceae) is a cosmopolitan genus of 20 species of floating-leaved aquatic plants (Cook 1990). The various species show different reproductive systems, including dioecy, heterostyly, partial homostyly or a breakdown in heterostyly, and autogamy (Ornduff 1966; Barrett 1980; van der Velde & van der Heijden 1981; Marui & Washitani 1993).

Nymphoides indica O. Kuntze (Menyanthaceae) is a distylous, perennial plant (Ornduff 1966, 1970; Sculthorpe 1967). Reddy and Bahadur (1976) and Barrett (1980) investigated some traits of heterostyly in populations of *N. indica* in India and Brazil, respectively, and showed that intermorphic pollination is usually required for fruit and seed set. In Japan, *N. indica* grows in meso- to eutrophic lakes and ponds, mainly in the southwest (Kadono 1994). Hamashima (1979) observed fruit and seed set in *N. indica* in the Tokai Region, central Japan, and showed that floral morph composition influenced seed set.

The purpose of our study was to re-evaluate features of heterostyly in *N. indica* in Japan with special emphasis on quantitative aspects.

1. 2. Methods

1. 2. 1. The spatial distribution of the pistil and stamens

To confirm dimorphism in the flowers of *N. indica*, I measured the length of the anthers and stigmas in two floral morphs - long-styled and short-styled (Fig. 1-1). The flowers of both morphs were sampled in seven populations, as shown in Table 1-1. The floral morph composition in populations HB, HC, HE and HO (see Table 1-1) was so biased that I sampled only the dominant floral morphs. Twenty flowers (one per inflorescence) were collected from each population and fixed in FAA (formalin, acetic acid and ethanol) in the field. The length of the stamens and pistils were measured to 0.1 mm with an ocular micrometer. The five anthers in each flower were nearly equal in length, so I measured one stamen per flower. The length of the stamen and pistil was measured from the base of the ovary to the top of them. The difference between these two lengths is referred to as the staminate-carpellate distance (SCD) (the "MFD" of Marui & Washitani 1993).

1. 2. 2. Comparison of the pollen size between long-styled and short-styled morph

To compare the pollen size between long-styled and short-styled morphs, I collected flowers of each morph from cultivated plants that originated from the study populations labeled HC and HD in Table 1- 1. Twenty flowers were sampled from HD for long-styled morph and from HC for short-styled morph, respectively. The pollen grains were gathered from the flowers at anthesis on a

slide glass with fine forceps and soaked in 70% alcohol for preparation. The diameters of 400 grains from long-styled morphs and 600 grains from short-styled morphs were measured on photomicrographs.

1. 2. 3. Compatibility status

To confirm the compatibility status of *N. indica*, I conducted pollination experiments using cultivated plants from 15 to 30 August 1998. I compared the following three treatments: 1) intermorph pollination, receiving pollen grains from the opposite morph; 2) intramorph pollination, receiving grains from the same morph; and 3) self-pollination. The flower buds for cross-pollination were emasculated with fine forceps on the day before opening. Both long-styled and short-styled morphs were used as maternal and paternal parents reciprocally in the three treatments. The number of treated flowers was 17 to 22 for each treatment. Crossing was conducted from 8:00 a.m. until 12:00 at noon. After pollination, the whole plants were covered with semi-transparent cloth to exclude pollinators. I assumed that photosynthesis was not significantly reduced, as the cloth reduced irradiation by only 10%.

The fruits were collected after three weeks when they had matured. Fruit set rate (number of fertile fruits / number of pollinated flowers) and seed set rate (number of mature seeds / total number of ovules) were investigated under a stereomicroscope.

1. 3. Results

1. 3. 1. The spatial distribution of the pistil and stamens

The staminate-carpellate distance (SCD) of both morphs in seven populations is shown in Fig. 1-2. The differences between the lengths of stigma and anther were clear in both morphs, and the values show a discrete bimodal distribution, thus verifying the distinct differentiation of the two floral morphs.

1. 3. 2. Comparison of pollen size between long-styled and short-styled morph

Figure 1-3 and 1-4 shows the frequency distribution of pollen size of both morphs. The grain diameter was $31.7 \pm 1.5 \mu\text{m}$ (mean $\pm$ SD) (range: 27.5 – 35.8 μm ; N = 400) in long-styled flowers and $39.6 \pm 2.0 \mu\text{m}$ (mean $\pm$ SD) (range: 35.5 – 48.2 μm ; N = 600) in short-styled flowers. The difference in pollen size between the two morphs was about 8 μm .

1. 3. 3. Compatibility status

The results of the pollination experiments are shown in Table 1-2. In cross-pollination between long-styled and short-styled morphs the average seed set rate was about 74 % in long-styled and 76 % in short-styled morphs. No seeds were produced in intramorph pollination or in self-pollination in either morph.

1. 4. Discussion

According to the present study, the SCD of the flowers of *N. indica* showed a bimodal distribution corresponding to long-styled and short-styled morphs. This means that spatial separation of stigma and anther in one morph is maintained in *N. indica*; in other words, there is no “homostyle” morph. The

pollen size of the two floral morphs was also confirmed to be different, as has been reported by Ornduff (1966), Reddy and Bahadur (1976), and Hamashima (1979). The difference of 8 μ m was roughly in accordance with the results of Ornduff (1966) and Hamashima (1979), but was different from those of Reddy and Bahadur (1976), who reported that the difference of the pollen size was about 3.7 μ m in Indian populations. The disagreement may be due to differences in the measuring method, but geographical variations in pollen size can occur.

In pollination experiments, fruits and seeds were produced only by intermorph pollination. Although Barrett (1980) reported that self-incompatibility of *N. indica* was somewhat incomplete, our result indicates that self-incompatibility of the populations in Japan is strong as was reported for populations in India (Reddy & Bahadur 1976).

I confirmed that self-incompatible heterostyly is maintained in the populations of *N. indica* in Japan. There was no evidence suggesting breakdown of heterostyly in *N. indica*, as has been reported in closely-related *N. peltata*, which can produce some seeds by self and illegitimate pollination (Ornduff 1966; van der Velde & van der Heijden 1981; Marui & Washitani 1993).

CHAPTER 2

Floral morph composition and pollen limitation in seed set of the populations in a heterostylous aquatic plant *Nymphoides indica* O. Kuntze (Menyanthaceae)

2. 1. Introduction

In heterostylous or dioecious plants, the number and spatial pattern of compatible individuals and pollinator behavior have an influence to reproductive success of the populations because crossing patterns leading to successful seed set are limited.

In dioecious plants, Barrett & Thomson (1982) discussed the relationship between the spatial pattern of sexual morphs and fecundity in a population of *Aralia nudicaulis*. It was also shown that male density influenced to the fecundity of the female in three dioecious species, *Neolitsea dealbata*, *Litsea leefeana* and *Diospyros pentamera*, composed of different floral morph ratio (House 1992). Pollination success in female trees of *Neolitsea dealbata* was determined by the distance between male and female trees within a population.

Wyatt & Hellwig (1979) discussed the difference in fruit set among subpopulations composed of different floral morph ratio in some populations

of *Houstonia caerulea* in heterostylous plant. Feisinger et al. (1991) demonstrated the effect of floral neighborhood for successful seed set through the field experiments using self-compatible *Besleria triflora* and heterostylous *Palicourea lasiorrachis*. Spatial effects for reproductive success including pollination was compared between single and conjugated clones within a population in *Persicaria japonica* (Nishihiro & Washitani, 1998).

These studies suggest the availability of compatible pollens as a limiting factor of successful seed set in dioecious and heterostylous plants. Other published studies have found reproductive success from the process of the germination of seeds to establishment of the seedlings of these plants will determine population structure including sex ratio and floral morph composition and also genetic structure (Cox 1981; Bierzychudek & Eckhart 1988; Eppley et al. 1998). However, the mechanisms and processes of the maintenance of such population structures are still not well documented in heterostylous plants (Barrett et al. 1983; Morgan & Barrett 1988; Barrett et al. 1989; Eckert & Barrett 1992; Eckert & Barrett 1995). For example, as Eckert & Barrett (1992) stated, there occur natural populations with different floral morph compositions in heterostylous plants, in particular, monomorphic floral morph composition, floral morph composition dominated by one morph alone and floral morph composition of equal floral morph ratio, which reflect the history and ecology of the populations. Floral morph is genetically determined in heterostylous plants (Ganders 1979; Lewis & Jones 1992) and reproductive success through disassortative pollination leads to an equal floral morph ratio. Thus, comparative study of the populations in which different modes of

reproduction dominate will help me to understand the maintenance of population structure in these populations.

Nymphoides indica (Menyanthaceae), a perennial aquatic macrophyte, is a typical heterostylous plant displaying herkogamy and self-incompatibility (Ornduff 1966; Reddy & Bahadur 1976; Barrett 1980).

Floral morph composition of the populations has been shown to be diverse in Japan (Hamashima 1979). I undertook a comparative study of the reproductive success with special reference to the relationship between floral morph composition and seed set of the populations of the species.

N. indica is an endangered plant (Environmental Agency of Japan 2000) and recently many populations have been declined due to the eutrophication of water and reclamation of habitats. Thus, the isolation and fragmentation of habitats are becoming more common. To conserve the existent populations or to restore lost populations, scientific strategies are urgently needed. To plan the strategy of conservation, it is basically important to elucidate habitat requirements, life history traits, demography of the populations, and metapopulation structure of the species, if any. As the number of populations becoming extinct because of human interference is increasing (Gilpin & Soulé 1986), I needed to find the most effective strategy of conservation considering long-term persistence of the populations.

Taking into consideration ecological reproduction and conservation studies, I started a project to elucidate the ecological traits of *N. indica* including population processes and to propose the most effective strategy of the conservation of the populations. I began by comparing the reproductive success

in the populations with different floral morph composition. I then analyzed the factors influencing the reproductive success especially at pollination stage. In this chapter I will report the results of the studies and discuss their ecological and conservational implications.

2. 2. Materials and methods

2. 2. 1. Study species

N. indica reproduces vegetatively as well as sexually (Fig. 2-1; Hamashima, 1979, 1985). Swollen roots that are formed at the basal node of petiole from summer to autumn falls apart from the plants and plays the role of a turion for vegetative reproduction and overwintering. However, the plants produce many flowers from July to September. One to three flowers open in each inflorescence per day. Flower longevity is within one day and flowers begin to fade in late afternoon. According to our preliminary observation the activity of pollinators was almost exclusively confined to morning hours.

2. 2. 2. Floral morph composition of the populations and fruit and seed set

I studied floral morph composition extensively in 23 populations in the Higashi-harima area of the Hyogo Prefecture and two populations in the Okayama Prefecture, south-western Japan, from July to September 1997 and 1998 (Table 2-1). All populations grew in irrigation ponds.

To determine the ratio of the two floral morphs in each population, I collected between 30 and 118 flowering inflorescences according to population size from a wide area of each population at intervals of more than 1 m to avoid

collecting from the same plants. The morph type (long-styled or short-styled) for each inflorescence was determined in the field and recorded. As an indicator of the bias of composition of the two floral morphs, the parameter $|L - S| / (L + S)$ was calculated, where L and S are the numbers of long-styled and short-styled flowers (Nishihiro et al. 1998). The statistical significance of the bias was tested by the G-test. The fruit and seed set rates were investigated from late August to September 1997 and then during the same period 1998 in 20 of the 25 populations. Between 24 and 46 inflorescences were collected from each population. The fruit set rate was checked for all inflorescences collected at appropriate intervals and seed set rate was calculated as the average of six fruits per inflorescence. Only undamaged inflorescences were selected in populations where the seed predator *Bagous* sp. (Curculionidae) fed on the ovules and seeds.

2. 2. 3. Seed set and effective pollination

To determine how seed set was influenced by pollination with different kinds of pollens, I compared the pollen load and intermorph pollination rate of flowers among and within populations, using the difference in pollen sizes of two floral morphs. The diameters of pollen grains are $31.7 \pm 1.5 \mu\text{m}$ (mean $\pm$ SD) in long-styled morph and $39.6 \pm 2.0 \mu\text{m}$ (mean $\pm$ SD) in short-styled morph and can be clearly discriminated (Shibayama & Kadono 2003 a). The intermorph pollination rate is defined as the number of pollens of opposite floral morph / total number of pollens on the stigma.

2.2.3.1. Effective pollination among the populations with different floral morph ratios

In the present study, intermorph pollination rate was investigated in four populations (HA, HC, HD and HT) with different floral morph compositions as shown in Table 2- 1. The stigmas were collected from 40, 103, 93 and 45 flowers at appropriate interval in the four populations, respectively.

The collection of stigmas was done during 13:00 - 15:00 when active visitation of pollinators had ceased. The collected stigmas were immediately fixed with 70% alcohol in Eppendorf tubes (Eppendorf Co., Ltd., Japan). The deposited pollens hardly fell off the stigma because germination commenced immediately in most of the pollen irrespective of morph type. The fixed stigmas were stained with aceto-carmin on the slide glass and flattened by cover glass for observation. The number of pollens of each of the two floral morphs was counted under a microscope. The count was repeated three times for each stigma and the intermorph pollination rate was expressed as the average of the three counts. The data were analyzed with one-way ANOVA after arcsin transformed in long-styled flowers and Mann-Whitney *U*-test in short styled ones.

2.2.3.2. Spatial difference of the seed set and effective pollination of the common morph within the populations

To verify how spatial patterns of the two morphs affects the seed set, I investigated the pollen load and intermorph pollination rate of the common morph. In the common morph, the seed set is significantly limited in

populations because of the bias of floral morph ratio. An intensive study was conducted in two populations (HC and HD in Table 2-1). In HC short-styled morph was dominant and long-styled morph was dominant in HD.

In both populations, three or four quadrats of 1m×1m (meshed to 10 cm×10 cm) were set, covering the area with different floral morph ratio. The distribution of the plants in flower were patchy in HC. Only one patch included both of the two morphs. In HD flowering plants spread continuously but the floral morph ratio changed gradually along the line where three quadrats were set.

The stigmas of all the open flowers within the quadrats were sampled with their positional record on the map in early September 1998. The samplings were performed on fine days when pollinator behavior was active. At the same time, between 19 to 21 inflorescences were randomly collected to assess the state of fruit and seed set in each quadrat.

Fruit and seed set rates were determined in the same way. The differences of intermorph pollination rate and fruit and seed set rates in each quadrat were analyzed for dominant floral morph with one-way ANOVA after arcsin transformed.

2. 2. 4. Artificial pollen addition

To confirm the pollen limitation for seed production, artificial pollen addition was performed in two populations (HC and HD). The four treatments were conducted as follows: 1) intermorph pollination, 2) intramorph pollination, 3) self-pollination and 4) spontaneous pollination (control). The

short-styled and long-styled flowers were used as maternal plants in HC and HD, respectively.

The flowers for artificial pollination were selected from those of the inflorescences that seemed to fail in seed set in natural conditions to prove pollen limitation clearly. Such inflorescences could be easily selected because the fruits after anthesis remained undeveloped. The pollens for intermorph pollination were supplied from the plants cultivated at Kobe University which was collected in advance from HC and HD. They were derived from one genet, respectively. Intramorph pollens were derived from the other plants of the same morph of the study site and self-pollination was conducted in the same flower.

Each pollination treatment was performed with a fine forceps in the early morning when pollinators were not yet active on the fine day. Following the treatments, flowers were covered with semi-transparent bag to exclude pollinators except spontaneous pollination. Subsequently the bags were removed at about 3:00 p.m. when activity of pollinators ceased. Each treatment was conducted in flowers of 18 - 21 inflorescences. The pollinated flowers were marked with colored vinyl tape. The flowers were collected after 2 - 3 weeks and fruit and seed set rates were determined in the same way as above. The results were analyzed with one-way ANOVA .

2. 2. 5. Pollinators

Pollinators visiting the flowers of *N.indica* were investigated in five populations during the flowering period in 1998. Intensive observation of

pollinators was performed in HC and HD. The time for observation was 13 and 9.5 hours in HC and in HD during flowering time, respectively. The species of pollinators, frequency of pollinator's visit and flying distance from flower to flower were recorded in a quadrat of 2m×2m. The time for observation is from a.m. 8:30 to p.m.13: 00. Some visiting insects were sampled for identification.

The flying distance of the pollinators was estimated based on the meshes of 10 cm×10 cm as a measure.

2. 3. Results

2. 3. 1. Floral morph composition of populations and fruit and seed set

The frequencies of the two floral morphs in the 25 populations are shown in Table 2-1. Fourteen populations were composed of both long-styled and short-styled floral morphs. The ratio was 1:1 in four populations and in the remaining 10 populations, it deviated significantly from 1:1 (*G*-test, *P* < 0.05). Eleven populations were composed of monomorphic flowers, four long-styled and seven short-styled alone.

Fruit and seed set rates in 20 populations are shown in Table 2-2. Seeds and fruits were produced in the populations composed of both floral morphs. Both the fruit and seed set rates were higher in the populations with an equal floral morph ratio than in the populations with a biased ratio. In the populations composed of one morph alone, seeds were not produced except in HN population. The parameter indicating the bias of floral morph composition was significantly correlated with fruit and seed set rates in the populations (Fig. 2-2).

Table 2-3 shows fruit and seed set rates of the inflorescences of long-styled and short-styled morphs sampled from nine populations. The fruit and seed set rates were similar between long-styled and short styled morphs in HG and HT with equal floral morph ratio. On the other hand, in HB, HC and HK, where the short-styled morph was dominant, the fruit and seed set rates were higher in the long-styled morph than in the short-styled morph. In HD and HE, where the long-styled morph was dominant, the reverse was true. Thus, the fruit and seed set rates of the rarer morph were generally higher than those of the common morph. In addition, in the populations showing a significant floral morph bias, fruit and seed set rates varied greatly among the inflorescences, which is reflected in the large values of SD in some populations as compared with the values of SD in the populations with equal floral morph ratio (Table 2-2).

2. 3. 2. Intermorph pollination rate among populations

Table 2-4 shows the pollen load and intermorph pollination rate in four populations. The mean of pollen load per stigma was 211.7, 202.7 and 144.8 for long-styled morph in HA, HD and HT, respectively. Intermorph pollination rate of the morph was significantly different among the three populations investigated ($P < 0.0005$). It was highest in HT (0.27 ± 0.19), where the composition of floral morph was 1:1. It was 0 in HA, where there were no short-styled morph, and 0.06 ± 0.12 in HD, where short-styled morph was rare.

The pollen load of short-styled morph was 58.9 and 40.6 in HT and HC, respectively. The intermorph pollination rate of the morph was significantly

higher in HT with 1:1 floral morphs (0.70 ± 0.13) than in HC (0.10 ± 0.19) with biased floral morph ($P < 0.0001$).

2. 3. 3. Spatial difference of the intermorph pollination rate and fruit and seed set within populations

The frequency distribution of the pollen load and the number of compatible pollens on the common morph were compared in Fig. 2-3. Compatible pollens on the common morph were remarkably limited with a few exceptions. In Fig. 2-4, intermorph pollination rates were shown for the stigma of each flower. Fig. 2-4 (a) shows the case of population where short-styled morph was dominant. In the quadrat HC-1 where both floral morphs occurred, intermorph pollination rate was 0.63 ± 0.32 (mean $\pm$ SD) in the rare, long-styled morph as compared with 0.24 ± 0.23 in common, short-styled morph. In the quadrats HC-2~4, where there only had common morphs, the intermorph pollination rate was significantly lower than that in HC-1 (Table 2-5; $P < 0.02$). There were not any significant differences in their intermorph pollination rates among the three quadrats.

The similar situation occurred in the population HD where long-styled morph was dominant (Fig. 2-4 b; Table 5). Intermorph pollination rate in HD-1 was 0.80 ± 0.13 in rare morph and 0.16 ± 0.16 in common morph. It was significantly lower in HD-2 and -3, where there occurred no rare floral morph, which was present in HD-1 ($P < 0.0001$).

The fruit and seed set rates of the quadrats were also shown in Table 2-5. In HC, they were significantly different and highest in HC-1 with two floral

morphs among the four quadrats (fruit set rate: $P < 0.0001$; seed set rate: $P < 0.0001$). Significant differences were not observed among the other three quadrats with a single floral morph.

Similarly, fruit and seed set rates was higher in HD-1 with two floral morphs than in HD-2 and -3 (fruit set rate: $P < 0.0001$; seed set rate: $P < 0.0001$). There was not significant difference between the other two quadrats composed of a single floral morph.

2. 3. 4. Artificial pollen addition and seed set

The results of artificial pollen addition are shown in Table 2-6. In HC, where short-styled morph was significantly dominant, addition of pollens from long-styled morph (intermorph pollination) improved fruit and seed sets. In other treatments neither fruit nor seed production was observed. In HD, where long-styled morph was dominant, fruits and seeds were produced in intermorph pollination, whereas in case of spontaneous, self and intramorph treatments, fruit and seed sets were not observed (fruit set rate: $P < 0.0001$; seed set rate: $P < 0.0001$).

2. 3. 5. Pollinators

Table 2-7 shows the insects observed in the present study that visited the flowers of *N.indica* in the five populations investigated. They were considered to be pollinators because the pollen was seen on their body. The common pollinators were different according to the populations.

The pollinators included Lepidoptera, Diptera, Hymenoptera and

Coleoptera. The most common pollinators were *Notiphila* sp. in HA and HG, *Lasioglossum mutilum* in HT, *Mesembrius flaviceps* in HC and *Apis mellifera* in HD. The numbers of pollinators visiting flowers during observation time of 13 and 9.5 hours were 19 and 35 in HC and HD, respectively. The frequency of flower visitation peaked from 10: 00 to 12: 00 in the two populations and only a few pollinators were observed in the afternoon when most of pollens were removed from the anthers.

The average flying distance from flower to flower was approximately 15cm in *M. flaviceps* and 17cm in *A. mellifera*. The flying distance of *P. guttata* was about 198 cm in HC and 53 cm in HD (Table 2-8).

2. 4. Discussion

2. 4. 1. Seed set and its limiting factors

The seed production of heterostylous plants is seen to be influenced by the number and spatial pattern of the compatible individuals because inter-morph pollination is required for seed set (Wyatt & Hellwig 1979; Ågren 1996; Nishihiro & Washitani 1998; Matsumura & Washitani 2000). *N. indica* is a typical heterostylous species with strict self-incompatibility and the seeds were successfully produced only in case of inter-morph pollination (Ornduff 1966; Reddy & Bahadur 1976; Barrett 1980; Shibayama & Kadono 2003 a)

In the present study, it was proved that the bias of floral morph ratio is negatively correlated with seed set rate and inter-morph pollination rate. In the populations with biased floral morph composition, the stigmas of the common

morphs were mostly pollinated by intra-morph and/or self pollens, which resulted in lower fruit and seed set.

The inter-morph pollination rate and the fruit and seed set rates of *N. indica* were highest when the two floral morphs were located in the near distance. This indicates that the success of inter-morph pollination was influenced by the occurrence pattern of the two floral morphs, in other words, neighbourhood effect (Feinsinger et al. 1991). Insects visiting on the flowers of *N.indica* was generalist as predicted from the morphology of flower. Insects such as Lepidoptera, Diptera and Hymenoptera visited the flowers. However, flying distance of common species from flower to flower was mostly less than 20 cm and it is thought that geitonogamous pollination occurs frequently.

When the pollens of opposite floral morph were artificially added, the fruit and seed set rates became significantly higher than spontaneous, intra-morph and self pollination. Considering these facts, it can be concluded that compatible pollen limitation is the most significant factor which limits the seed production in the populations of *N. indica*.

Mate distance is known to have a significant influence on female reproductive success, even if floral morph composition is balanced as a whole in a population. For example, in case of a heterostylous plant, *Persicalia japonica*, which reproduce vegetatively by creeping rhizome, the seed set is severely limited in the single clones compared with the congregated clones within the same population (Nishihiro & Washitani 1998). To the contrary, in case of the populations of *N.indica* with equal floral morph ratio, most flowers showed evenly high seed and fruit set, which is indicated by low values of

standard deviation. This suggests that the two floral morphs are spatially well mixed in the populations. Such spatial structure will be brought about by turions, a vegetative reproductive means of the species, which float freely apart from the parent plants (Hamashima 1985). In this respect *N. indica* would be more advantageous for maintaining mixed distribution of both floral morphs than other heterostylous clonal plants which propagate with rhizome or stolon as vegetative reproductive means because self- pollination and geitonogamy may frequently occur in the heterostylous plants with rhizome and stolon.

2. 4. 2. Implications for conservation of the populations

N. indica is effective in vegetative reproduction (Hamashima 1985). This is why many populations with one floral morph alone fail in seed production. However, seed set by sexual reproduction is needed to originate and maintain the genetic diversity of the populations and to balance the floral morph ratio, although a set of conditions are necessary for the seedlings to establish (Chapter 4). In addition, seeds are effective means of dispersal and colonization to new water bodies and support the metapopulation structure (Thomas 1994; Olivieri et al. 1995; Eriksson 1996; Husband & Barrett 1996; Groom 1998; Amarasekare 1998 a).

In recent times, successful seed production of heterostylous plant species has been threatened for various reasons including pollinator limitation as well as the limitation of compatible pollens caused by fragmentation and isolation of habitats (Washitani et al. 1991; Washitani et al. 1994; Nishihiro et al. 1998; Matsumura and Washitani 2000). The decline of populations with high

fecundity may threaten the long-term persistence of not only local populations but also metapopulation structure (Thomas 1994; Olivieri et al. 1995; Husband & Barrett 1996; Eriksson 1996; Groom 1998; Amarasekare 1998 a).

In *N. indica*, seed production differs from population to population according to the floral morph composition of the populations. Considering the present critical status of *N. indica* populations as an endangered species (Environment Agency of Japan 2000), a high priority for conservation may be given to the populations with high fecundity because these populations may have an ecological and evolutionary potential such as maintenance of genetic diversity required for adaptation to changes in environmental condition and the higher dispersal to found new populations. Such strategy may be supported by the study of genetic structure of the populations in relation to reproductive success conducted in the Higashi-harima area, Hyogo Prefecture. Furthermore, the study of genetic structure will tell me whether different fecundity of the populations gives an influence to metapopulation dynamics or not (Chapter 2).

CHAPTER 3

Reproductive success and the genetic structure of the populations of heterostylous clonal plant *Nymphoides indica* O.Kuntze (Menyanthaceae)

3.1. Introduction

Allozyme studies have been performed to know the population genetic structure of some aquatic macrophyte taxa (Triest 1991; Barrett et al. 1993). Genetic diversity within and among populations is generally low because aquatic macrophyte taxa are recruited by vigorous vegetative reproduction although sexual reproduction simultaneously occurs in many aquatic macrophytes (Les 1988; Aspinwall & Christian 1992; Laushman 1993; Barrett et al. 1993; Lokker et al. 1994; Philbrick & Les 1996). However, it is likely that the pattern of genetic diversity within and among populations changes according to the difference of reproductive modes of the species. So far, the studies on the reproductive mode have been limited in relation to genetic diversity (Hamrick & Godt 1990; Eckert & Barrett 1993; Piquot et al. 1996 ; Kudoh et al. 1999; Dorken & Eckert 2001).

The genetic diversity of *Eichhornia paniculata* (Glover & Barrett 1987; Husband & Barrett 1991; Husband & Barrett 1995) and *Decodon verticillatus* (L.) Ell. (Eckert & Barrett 1993; Dorken & Eckert 2001), which are both tristylous species, had been surveyed. Their allozyme study enabled them to

discuss the relationship between the genetic structure and the maintenance of floral morph ratio.

Nymphoides indica O.Kuntze is a typical of heterostylous plant with herkogamy and self-incompatibility (Ornduff 1966; Reedy & Bahadur 1976; Hamashima 1979; Barrett 1980; Shibayama & Kadono 2003 a) and reported as diploid (Harada 1952; Wada 1956; Ornduff 1970). The species has both clonal and sexual reproductive means. Sexual reproduction was confirmed to be limited by the bias of floral morph composition in the populations (Shibayama & Kadono 2003 b). Thus, the allozyme study of the populations with different fecundity will offer me the chance to know how it gives an influence to the genetic structure or balanced floral morph ratio in local populations in addition to metapopulation in *N.indica*.

Besides, *N. indica* is endangered species (Environment Agency of Japan 2000). The loss of local population by artificial influences to the habitats is going on in Japan. To conserve the existent populations or to restore the lost populations, the conservation of genetic diversity will be an important point to plan the most effective strategy. In this chapter, to elucidate the occurrence of genetic diversity and structures within and among populations in *N. indica*, I tried to reveal I) how the pattern of reproductive modes affects the genetic diversity in each population and II) how the metapopulation structure is formed on regional basis in *N.indica*.

3. 2. Materials and methods

3. 2. 1. Sampling and electrophoretic method

Fresh leaves of *N.indica* were sampled during flowering season in 23 populations in Hyogo Prefecture and two populations in Okayama Prefecture in Japan (Table 3-1). 21 ~ 35 (30 in principle) leaves were sampled at the interval of more than 1 m to avoid the sampling from the same individual. Leaves were ground in 400 μ l of Tris - HCl -PVP buffer including ion-changing resin (DOWEX) and 75 μ l of mercaptoetanohol (Soltis et al. 1983). They were centrifuged at 10,000 rpm for 10 minutes. Homogenate obtained by centrifuging was absorbed by paper wicks and loaded on 12% starch-gels (Sigma potato starch). Enzyme activity of 16 enzymes was preliminary surveyed and nine enzymes were active and well-resolved among them. Two buffer system, histidine-citrate, pH6.5 (System 1) and lithium-borate, pH 8.0 (System 2) were used to resolve the following nine enzymes (Soltis et al. 1983). System 1 was used in aconitate hydratase (ACO), malate dehydrogenase (MDH), phosphogluconate dehydrogenase (6PGD) phospho glucomutase (PGM), adenylate kinase (ADK) and shikimate dehydrogenase (SkDH). On the other hand, system 2 was applied to the other three enzymes, that is, glucose phosphate isomerase (PGI), triose-phosphate isomerase (TPI) and alcohol dehydrogenase (ADH). The period of electrophoresis was six hours at both systems. After 26 mA current for first 30 minutes, 40 mA constant current was continued until the sign marker reached the end of gel. The gel was sliced to six sheets horizontally and stained for each enzyme. The staining method of Wendel and Weeden (1989) modified by Akiyama (1991) was used. Photographs and hand-writing of all the gels were made to aid interpretation of zymograms.

3. 2. 2. Statistical analysis

For each enzyme system studied, genotype was identified and multilocus genotype (MLG) was determined. To know the relationship between the seed set rate and number of MLG representing genetic diversity in local population, Kendall's rank correlation test was performed. Furthermore, allele frequency and Wright's (1951) F statistics (F_{st} and F_{is}) and G_{st} was calculated by FSTAT 2.9.3.2 (Goudet 2002), and the levels of significance were obtained by randomization procedures [see Goudet (2002) for details]. The parameters of F_{st} and G_{st} show genetic differentiation among populations and F_{is} is an inbreeding coefficient, which means the deviation from Hardy –Weinberg equilibrium showing random- mating population.

To know the migration among populations, genetic similarity was calculated. Individuals determined as multilocus genotype (MLG) were scored for the presence (1) or absence (0) in each population. A similarity was calculated for pairwise comparison of each population. Dendrogram was obtained by Ward Method based on standardized squared Euclidean distance.

3. 3. Results

3. 3. 1. The relationship between genetic diversity and life history

Three enzyme loci were polymorphic and six ones were monomorphic among nine enzymes investigated. There were three genotypes for PGM, SkDH and PGI, respectively. Genotype was determined for each three locus and I defined multilocus genotype (MLG) based on the combination of genotype of three polymorphic loci (Table 3-2).

MLGs determined were 11 in total (Table 3-2). The relationship between seed set rate and the number of MLGs was positively significant ($\tau=0.542$, $P<0.001$; Kendall rank correlation test; Fig. 3-1.). In addition, in populations with equal floral morph ratio, F_{is} showed only minor deviations from Hardy-Weinberg equilibrium in one or two loci (Table 3-4). On the other hand, in populations with biased floral morph composition, randomization tests detected significant deficits of heterozygotes relative to Hardy-Weinberg expectations in the HK and HL populations (Table 3-4). In HW, significant excess of heterozygotes relative to Hardy-Weinberg expectations (Table 3-4) was shown. Monomorphic populations for floral morph were consisted of only one MLG except a population (HM). (Table 3-5; Fig. 3-3). Furthermore, nine out of observed 11 MLGs appeared in the two populations, HI and HV, with equal floral morph ratio (Table 3-5). However, genetic differentiation among populations with equal floral morph ratio (HG, HI, HT and HV) occurred (Fig. 3-2).

3. 3. 2. The metapopulation structure

Allele frequency of PGI and SkDH was limited among populations (Table 3-3). The frequency of each MLG in 25 populations was shown in Table 3-5 and Fig. 3-3. The frequency of MLG (e) was the highest in all the MLGs and widespread all over the populations in Hyogo Prefecture. On the other hand, there were six rare MLGs within one or two populations in Hyogo Prefecture. MLG(b) occurred in HU and HV, MLG (c) in HV, MLG(d) in HI and HK, MLG(f) in HV and HW, MLG(h) in HI and HJ and MLG(k) in HK alone (Table

3-5).

According to the cluster analysis, two population groups were roughly recognized in Hyogo Prefecture (Fig. 3-2 ; Fig. 3-3). F_{st} of each enzyme locus shows a large value and the value of G_{st} (0.51) showed that genetic differentiation was great among populations (Table 3-6). In two groups based on dendrogram, F_{st} of Group A was 0.21 and that of Group B was 0.52 (Table 3-7).

3. 4. Discussion

3. 4. 1. The relationship between genetic diversity and life history

I used multilocus genotype (MLG) as the genotype of each individual and the number of MLG was regarded as an indicator of genetic diversity in each population of *N.indica*.

According to the floral morph ratio, genetic diversity was different among populations. The number of MLG in populations with equal floral morph ratio was higher than that of the rest of populations. The correlationship between the number of MLG and seed set rate in each population was positively significant (Kendall's rank correlation test). Furthermore, minor deviation from Hardy-Weinberg equilibrium showed that random mating occurred in populations with equal floral morph ratio and nine of the observed eleven MLGs occurred in HI and HV populations with high fecundity of equal floral morph ratio. Contrary to Husband and Barrett (1995) and Eckert et al. (1996), populations with equal floral morph ratio in *N.indica* have not been maintained by gene flow because genetic differentiation among populations

with equal floral morph ratio was large according to the cluster analysis, which also supported the possibility that the establishment of seedlings occurred in each population. Thus, these results showed that the successful recruitment from seedlings contributed to the maintenance of genetic diversity and balanced floral morph ratio.

In populations with biased floral morph ratio, two populations showed the deficit of heterozygotes and the excess of heterozygotes were confirmed in one population. These results indicated that random mating has not occurred. It was thought that only a vegetative reproduction has been occurring although some clonal lineage had succeeded in colonizing.

The genetic structure such as *N.indica* was reported in *Decodon verticillatus* (Eckert & Barrett 1993; Dorken & Eckert 2001) and *Rubus saxatilis* (Eriksson & Bremer 1993). It was documented that some populations with frequent sexual reproduction were genetically diverse and others with no fruit production were less diverse.

On the contrary, monomorphic populations were composed mostly of one MLG. There are two possible explanations for that phenomenon. One possibility is that the populations had clonally propagated after immigration of some propagules from other populations (Sculthorpe 1967; Hutchinson 1975; Cook 1988). Another possibility is that several genotypes including different floral morph types occurred but demographic stochasticity eventually eliminated all but one from the population. To verify these hypotheses, the comparison of genetic structure between adult plants and seed bank will help me to dissolve (Mccue & Holtsford 1998).

3. 4. 2. Metapopulation structure and conservation

Allele frequency of PGI and SkDH showed a great difference among populations. According to Hamrick & Godt (1996), G_{st} (0.50) of *N.indica* approximately corresponded to that of selfing species though this species was self-incompatible. This value showed that gene flow was a rare event. The two possible reasons for large genetic differentiation in *N.indica* were suggested. The one of possible reasons is that gene flow may be limited in populations because irrigation ponds were thought to form a discrete island in a 'terrestrial landscape' if hydrographic connections is absent (Barrett et al. 1993 ; Piquat et al. 1996). Another possible reason is that the difference of reproductive mode among populations may contribute to the genetic differentiation.

However, there were six rare MLGs which occurred only within one or two populations in Hyogo Prefecture. MLGs (d, h, k) occurred in HI, HJ and HK. Also, MLGs (b, c, f) occurred in HU, HV and HW. Three populations, HI, HJ and HK, were situated in the closed range and the populations, HU, HV and HW, were also located nearby.

Owing to the cluster analysis, geographic differentiation in the genetic structure was observed. Group A was composed of the populations located in the western part of Higashi-harima area and the populations of Group B were scattered in the eastern part of Higashi-harima area. F_{st} of Group A was 0.21. Based on Wright (1978), it showed that gene flow rarely occurred within Group A. HV with high fecundity of equal floral morph ratio may work as a source of genetic diversity within a region. It was regarded as " source- sink "relationship

(Pulliam 1988; Pulliam & Danielson 1991; Howe et al. 1991; Eriksson 1996 ; Pulliam 1996). Group A showed that gene flow by seeds and /or turions had rarely occurred in a closely situated populations. The present study provided us an interesting aspect that metapopulation in *N.indica* was brought about in scattered irrigation ponds if the spatial range was limited.

On the other hand, F_{st} of Group B was 0.52. Genetic differentiation was great and some subgroups occurred in Group B. Subgroup 1 (HK), Subgroup 2 (HI and HJ) and Subgroup 3 (populations except HI, HJ and HK) were recognized. Geographical location and the difference of life history attributes were thought to contribute to the genetic differentiation in Group B.

Populations with high fecundity which have a potential to bring about gene flow and adapt to environmental changes should be given a priority of conservation for the long-term persistence of *N.indica*. In addition, the restoration of populations with high fecundity may promote more effective gene flow among populations than ever. Furthermore, genetic differentiation between Group A and Group B showed the absence of historical gene flow. Therefore, Group A and Group B should be separately managed as distinct evolutionary significant units (ESUs; Moritz 1994; Crandall et al. 2000).

3. 4. 3. The limitation of the allozyme analysis

Allozyme studies so far conducted have shown that genetic diversity within and among populations of some aquatic macrophyte taxa is lower than that of terrestrial macrophyte taxa (Triest 1991; Barrett et al. 1993). Genetic diversity of even outcrossing species such as dioecious sea grasses is low

(Waycott et al. 1996; Schlueter & Guttman 1998). This can be caused by extensive clonal reproduction, limited sexual reproduction and an isolation of populations (Les 1988; Aspinwall & Christian 1992; Laushman 1993; Barrett et al. 1993; Lokker et al. 1994; Hollingsworth et al. 1996; Philbrick & Les 1996)

The number of genotypes was low in *N.indica*. All the MLGs observed were 11 types in Hyogo Prefecture and Okayama Prefecture. Polymorphic enzymes detected were three out of nine enzymes, and thus I may underestimate the number of the correct genotypes in each population. In closely-related heterostylous *Nymphoides peltata*, the level of genetic diversity analyzed by microsatellite was higher than that of allozyme analysis (R. Uesugi, personal communication, Tokyo University).

Accordingly, to evaluate genetic structure in local populations and metapopulation structure and also to determine dispersal ability of *N.indica*, analyzing of genetic diversity by using powerful genetic marker such as microsatellite (Ouborg et al. 1999; Reuch et al. 2000; Sun et al. 2001) may provide me more useful genetic information.

CHAPTER 4

Germination ecology in relation to water level fluctuation in *Nymphoides indica* O. Kuntze (Menyanthaceae)

4. 1. Introduction

The seed phase of life history is important because the genetic diversity for adaptation to changes in environmental conditions is maintained and seeds may be means by which vacant habitats are colonized. Seeds may also play a role of escape in time and in space from unfavorable environmental conditions (Baskin & Baskin 1998).

The relationship between the seed germination and water depth (Keddy & Ellis 1985) and between the occurrence of seed bank and vegetation (e.g. Grillas et al. 1993; Leck & Simpson 1995; de Winton et al. 2000; Chang et al. 2001) had been solved in wetland community. Furthermore, recent studies of seed production in a population and seed bank within aquatic macrophyte species suggest that seeds may be important for colonization (McMillan 1988; Terrados 1993 ; Orth et al. 1994; Lokker et al. 1997) and maintenance of the populations (McMillan 1988; Smits et al. 1990; Terrados 1993; Kimber et al.1995). However, these hypotheses were proposed by a field survey or cultivated experiment. To verify these hypotheses, intensive field survey combined with a laboratory- based study is thought to be more effective. However, field survey has been limited in aquatic macrophytes. The most

probable reason for it is that many populations in aquatic macrophytes are recruited by vigorous vegetative reproduction except annual plants although sexual reproduction simultaneously occurs in most of aquatic macrophytes (Barrett et al. 1993; Philbrick & Les 1996). So far, the exact ecological role of sexual reproduction has still remained to be adequately elucidated in aquatic macrophytes (Philbrick & Les 1996).

I had already showed that the genetic diversity of local populations in *N.indica* was positively correlated with the seed set rate (Chapter 3). This result suggests that the degree of establishment of seedlings may vary greatly among populations. I expected *N.indica* to offer us the ecological significance of sexual reproduction for aquatic macrophytes.

N. indica is also an endangered species in Japan (Environmental Agency of Japan 2000). It is indispensable to reveal the seed dynamics from seed dispersal to the establishment of seedlings to maintain genetic diversity and balanced floral morph ratio (Chapter 3) and restore the extinct populations.

One of the major factors controlling seed germination in aquatic macrophytes is thought to be water level fluctuation since it consequently affects the light, temperature and /or an oxygen condition required for germination (Baskin & Baskin 1998). It has been already reported that the seed germination in *N.indica* had been observed on mudflats in monsoonal wetlands of north-central India (Mason & van der Valk 1992). However, intensive ecological and laboratory-based studies have not been conducted sufficiently for the seed germination and establishment of seedlings in *N.indica*.

Thus, the primary object of this study was to determine how water fluctuation affects seed germination and establishment of seedlings in *N.indica*. The spatial distribution of seed bank was also studied.

4. 2. Materials & Methods

4. 2. 1. Spatial distribution of dispersed seeds and the emergence of seedlings

To determine the spatial distribution of seed bank as a consequence of seed dispersal, a transect survey to sample bottom soil was performed in the population of *N. indica* in Heisou-cho, Kakogawa City, Hyogo Prefecture (HG) in June, 2001. Soil sampling was performed at the interval of 50cm along 12 m line from the offshore to shore in three transects. The soil was collected by Eckman – Birge dredge. The collected soil was brought back to the agricultural field in Kobe University. The soil was even spread out in the plastic container (442 mm×322 mm×70 mm). Water was sprinkled every two days in order that the soil is always filled with water since seed germination of *N.indica* occurs under moist condition. The disturbance was performed once a week to promote germination of all the seeds in the collected soil. The seeds germinated from collected soil was observed from June to November in 2001. Since I wanted to know the germinable portion of seeds in the sediment, I directly observed the germinated seeds (=seedlings) from soil (Gross 1990). Accordingly, the minimum germinable seeds in each sediment were recorded.

In the filed condition, from 2001 to 2003, spatial distribution of seedlings was determined by the occurrence of quadrats (50cm ×50cm) established on

upper and lower side along the elevation of the ground to know the border where seedlings occurred.

4. 2. 2. Seed burial experiment

I conducted a seed burial experiment to confirm whether persistent seed-bank was formed or not. Seeds prepared for burial experiment were collected from HG in fall, 2000. 50 seeds after cold treatment were enclosed in a cotton mesh envelope. Three envelopes were set on the exposed surface (water depth: 0cm). 18 envelopes packed in a meshed bag of nylon with plummet were submerged at the bottom of water (water depth: about 70cm) in spring, 2001. The germinated seeds in each treatment were confirmed in June and October, 2001. In fall, from 2001 to 2003, three envelopes placed at the water bottom were withdrawn and seeds were washed under running water. They were sorted whether the content of seeds remained or not. The seeds with capability of germination were placed in $\phi 55$ mm Petri dishes for germination test under an alternating temperature course of 12 C°(10 h: dark) / 25 C°(14 h: light) for one month. The ungerminated seeds were dyed by 1 % (w/v) 2, 3, 5-triphenyl tetrazolium chloride solution (Downs and Cavers 1995) to ascertain the further potential for germination.

4. 2. 3. Germination experiment

4. 2. 3. 1. Observation of the conditions required for germination in the field

To know the environmental conditions required for seed germination in the

field, the seasonal change of water depth was measured at the point where germination occurred in Hayashida-cho, Himeji City, Hyogo Prefecture (HV) from 2001 to 2003. The diurnal change of water temperature was simultaneously measured by setting data logger (Optic StowAway Temperature, Onset computer corporation) from 2002 to 2003. Besides, vegetation around the point where germination was observed was also investigated to understand the feature of habitat in Heisou-cho, Kakogawa City (HG), Uji-cho, Ono City (HI) and Hayashida-cho, Himeji City (HV), Hyogo Prefecture.

4.2.3.2. Germination test in the laboratory

Germination test to examine the traits of seed responses to light and temperature was performed. Seeds used for each treatment were collected from Heisou-cho, Kakogawa City (HG) and Uji-cho, Ono City (HI), Hyogo Prefecture in September, 2001. Germination test was performed with three replicates of 50 seeds for the following four regimes; (I) light with alternating temperature, (II) light with constant temperature, (III) dark with alternating temperature and (IV) dark with constant temperature. Seeds were placed in $\phi 55$ mm Petri dishes containing filter papers moistened with distilled water. Petri dishes were placed in the temperature-controlled incubators illuminated with white fluorescent lamp for 14h per day ($120\mu\text{mol}^{-2} \text{s}^{-1}$) during the high temperature periods of the alternating temperature course ($25^\circ\text{C}/12^\circ\text{C}$). The same light condition was also applied to the constant temperature course (20°C). The treatment for darkness was simultaneously carried out in the same incubator. Petri dishes for dark treatment were placed in the untransparent

box. Germinated seeds under the treatment for darkness were observed under the green light to block red and far-red wavelength influence to seed dormancy (Downs & Cavers 2000).

Furthermore, to know the degree of seed dormancy, moist chilling treatments for control (without cold treatment), 4 and 8 weeks were performed with treatment described above.

Germinated seeds were removed and distilled water was appropriately added to keep moist condition. Germination was confirmed by the appearance of radicle through seed coat. The period for observation of seed germination was one month in each treatment.

4. 2. 4. Development of seedlings

To know the effect of water depth to the growth of seedlings, seedlings at some developing stages were submerged at the different water fluctuation. Seeds were collected from HG in fall, 2000. Seedlings used for treatments were obtained under the temperature-controlled incubator after cold treatment of seeds for 2 months. Geminated seeds were transplanted into $\phi 6\text{cm}$ pots filled with soil derived from an irrigation pond and were placed in the plastic container ($656\text{ mm} \times 456\text{ mm} \times 326\text{ mm}$). The water in the plastic container was circulated by air-supplying machine to stabilize the water quality. Developing stage of seedlings was classified into three stages ; (A) cotyledon stage (Biomass: $<0.0001\text{ g}$; $N=5$), (B) 4~5 foliage leaved stage (Biomass: $0.004 \pm 0.001\text{ g}$; $N=5$) and (C) 7~8 foliage leaved stage with petiolate elongated

leaves (Biomass: 0.043 ± 0.020 g; N=5). Seedlings for these developing stage were grown under the moist soil in the glasshouse for a while. 300 seedlings were prepared for each developing stage. Experiments were performed from June 25 to August 27 for stage A, from July 11 to September 11 for stage B and from August 1 to October 3 for stage C in 2001. Four patterns of water fluctuation were tried. Seedlings of each developing stage were exposed to the following four water fluctuation patterns, respectively ; (I) 0cm~5cm~15cm, (II) 5cm~15cm~40cm, (III) 15cm~40cm~60cm and (IV) 40cm~60cm~90cm. 6~8 seedlings were retrieved at each water depth after three weeks and the rest of seedlings were transferred into the following water depth. Leaves of the retrieved seedlings were counted. Seedlings were dried at 80°C and weighed to the nearest milligram. The statistical analysis was performed by one-way ANOVA and Games –Howell test was utilized as Post hoc test.

4. 2. 5. The fate of seedlings

To know the fate of seedlings in the field, the investigation of seedlings was performed in HV every two weeks from 2001 to 2003. The marking for seedlings was performed as soon as germination occurred in the maximum eleven quadrats (50cm×50cm). The bars used for marking were made from steel and vinyl tape with serial number was attached to the top of bars. The survival of all the seedlings emerged in each quadrat was monitored and the stage and number of leaves were recorded to know the growth of each seedling.

4. 3. Results

4. 3. 1. Spatial distribution of dispersed seeds and the emergence of seedlings

Fig. 4-1 showed the spatial distribution of dispersed seeds from the shore to offshore in three transects. The germinated seeds in the collected soil were from 0 to 43 (15cm×15cm) in three transects.

In the field condition, seedlings which occurred in quadrats were limited to the water margin in 2001 and 2003. However, spatial distribution of seedlings became larger when drought occurring in 2002 (Fig. 4-2).

4. 3. 2. Seed burial experiment

All the seeds could germinate on the exposed surface. On the other hand, seeds placed at the water bottom did not germinate at all (Table 4-1).

In seeds burial experiment at the water bottom, the rate of viable seeds was approximately 71% in the first year, 40% in the second year and 28% in the third year and that of germinable seeds was 65 % in the first year, 38 % in the second year and 23 % in the third year in the incubator with light –temperature controlled (Table 4-2).

4. 3. 3. Germination experiment

4. 3. 3. 1. The environmental condition required for germination in the field

Seed germination was observed at HV from 2001 to 2003. The emergence of cotyledon was observed when water was receded every year (Fig. 4-3).

Seed germination was observed when diurnal fluctuation of temperature was brought about by exposure of the surface of the ground (Fig. 4-4).

Vegetation around the safe site for germination was composed of amphibious species such as *Myriophyllum ussuriense* Maxim. and *Scirpus lineolatus* Franch et Savat. and uliginous species such as *Lindernia procumbens* (Knock.) Philcox., *Eriocaulon atrum* Nakai and *Deinostema violaceum* (Maxim.) Yamazaki (Table 4-3).

4.3.3.2. Germination test in the laboratory

Effect on cumulative germination rate under light- alternative temperature regime by cold treatment was significant (Table 4-4). The cumulative germination rate rose up when the period of cold treatment was longer in both of populations (Fig. 4-5).

Light and temperature significantly influenced to the cumulative germination rate after 0, 4 and 8 weeks of cold treatment in both of populations (Table 4-5). The highest cumulative germination rate of four treatments without cold treatment was light-alternative regime (HG: 0.56 ± 0.02 ; HI: 0.59 ± 0.02) (Fig. 4-5).

Furthermore, in the light- alternative temperature regime, the cumulative germination rate reached almost 100% after one month of cold treatment in HG and after two months of cold treatment in HI (Fig. 4-5). Also, I got the maximum cumulative germination rate after two months of cold treatment under the light - constant temperature regime in both of populations (Fig. 4-5).

N.indica could germinate in darkness. However, the cumulative germination rate was significantly higher in the light condition than that in the dark condition (Table 4-5 ; Fig. 4-5).

4. 3. 4. Development of seedlings

In the field condition, the growth of seedlings was promoted under the moist condition. Seedlings could grow to the developing stage with floating leaves (Fig. 4-6 ; Table 4-6). In the cultivated experiment, the remarkable difference of the growth of seedlings in *N.indica* was observed at the earlier developing stage (Fig. 4-7). The growth of cotyledon was promoted when the change of water fluctuation was smaller (Fig. 4-7 a ; Fig.4-7 b ;Biomass: $P<0.01$; the number of floating leaves: $P<0.001$). In addition, the growth of seedlings increased under the treatment of I (0 cm~5cm~15cm) in case of 4~5 foliage leaved stage (Fig. 4-7 a; Fig.4-7 b; Biomass: $P<0.0001$; the number of floating leaves: $P<0.0001$). However, the difference of water level fluctuation did not give an influence to the growth when seedlings developed to the latest stage (Fig. 4-7 a; Fig.4-7 b; Biomass: $P=0.77$; the number of floating leaves: $P=0.10$).

4. 3. 5. The survival of seedlings

The seedlings were died because of drought in 2001 (Fig. 4-3). However, seed germination occurred again after heavy rain but all the seedlings could not survive over to the following year (Fig. 4-3). Though the most of seedlings died in 2002 as was the case in 2001, 21 seedlings could survive over to winter in 2002. Consequently, 51 individuals originated from seedlings succeeded in the establishment in 2003 (Fig. 4-3).

4. 4. Discussion

4. 4. 1. Seed dispersal

Dispersed seeds were observed from the shore to offshore at HG although the density of seeds was different along three transect. I also got the similar results from three transects. This suggested that some seeds fell around their parents and others were dispersed to the shore. Since the turions (=reproductive shoot) with undehisced fruits were often observed at the water margin, turions will contribute to disperse seeds as buoyant vegetative organs. Similar observation has been made in *Zostera mariana* (Philips & Backman 1983; Orth et al. 1994).

However, the emergence of seedlings was limited to the exposed ground at the water margin or bare space after drought. In seed burial experiment, seeds stored on the surface of ground could immediately germinate, however, seeds stored at the water bottom could not germinate and stayed in dormant state. It was proved that seeds buried in the deeper water played a role of seed bank although the number of viable and germinable seeds was decreasing every year.

Colonization hypothesis of three adaptive significances for seed dispersal (Howe & Smallwood 1982) emphasizes that seeds are dispersed to the disturbed and relatively noncompetitive habitats and that seed bank is formed to germinate after disturbance occurred. The features of seeds in colonizing species are small and dispersed on a massive scale. In case of *N.indica*, dispersal ability of seeds is high as described above and the seed size are small (seed mass: Mean $\pm$ SE: 0.25 $\pm$ 0.01 mg : N=100). Therefore, *N.indica* is typically

thought to be a colonizing species taking into account of these features (Rees 1995; Jacobsson & Eriksson 2000 ; Ehrlén & Eriksson 2000).

Furthermore, environmental condition required for growth was remarkably different between seedlings and rootstocks or turions as vegetative organs which can grow in the deeper water as described below. This feature suggested that seeds in *N.indica* may escape from interference by rootstocks and/or sib competition with respect to time and space. This trait of seeds in *N. indica* showed the escape hypothesis proposed by Janzen (1970).

Therefore, intermingling of colonization and escape hypotheses showing adaptive significances for seed dispersal may be applied to the seed dispersal of *N.indica*.

4. 4. 2. Germination traits

The moist-chilling treatment promoted seed germination in *N.indica*. This result suggested that the condition of dormancy may prevent seeds of *N.indica* from germinating in late autumn and winter.

Effects on cumulative germination rate by light and temperature were significant. Light-alternating temperature condition of four treatments promoted seed germination in every period for cold treatment. Furthermore, without previous moist-chilling treatments, approximately 56% (HG) and 59% (HI) seeds could germinate under the light- alternating regime. This result implies that fluctuating temperature with light is more important than moist-chilling in determining seed germination in *N.indica*. Germination also began as soon as diurnal change of water temperature occurred in the field

condition. These strategies can be prepared for detecting open spaces such as gap without dense vegetation. This was also supported by the fact that germination occurred at the water margin and bare space after occasional water drawdown. Thus, the species with these features are thought to be disturbance - dependent (Grime 1979; Westboy et al. 1996; Araki & Washitani 2000; Ikeda & Itoh 2001).

4. 4. 3. Growth of seedlings

Unsubmerged condition was required for the growth of seedlings at the early developing stage for a while. The growth of cotyledon under submerged condition was suppressed, whereas the growth under the moist condition was promoted. When the seedlings with foliage leaves were submerged, floating leaves were continuously produced. In the field condition, the growth of seedlings was also promoted under the moist condition as was the case with cultivated experiments. Furthermore, some seedlings could survive even in drought and the establishment of seedlings perfectly succeeded in 2003. On the other hand, the individuals originated from rootstocks and/or turions could sufficiently grow at the water depth of about 1 m (Shibayama, observation). Thus, this result showed that environmental condition required for growth was remarkable different between seedlings and rootstocks and /or turions for the early stage of development. The difference of environmental condition required for growth between rootstocks and seedlings may be regarded as an adaptation to the variable environment of wetland, which would be possible to escape from extinction of rootstocks and/or turions by severe disturbance.

In short, I am sure that the ecological role of seeds in *N.indica* was not only colonization and population maintenance (Mcmillan 1988; Smits et al. 1990; Terrados 1993; Lokker et al. 1997) but also re-establishment from seed bank after disturbance such as drought (Kimber et al.1995).

4. 4. 4. Conservation for safe site in *N.indica*.

Destruction of safe site for seed germination in aquatic macrophytes is going on in wetland environment in Japan. Environmental condition required for the establishment of seedlings following the seed germination in *Nymphoides peltata* is being lost by high splashed wave caused by artificial habitat alteration in Lake Kasumigaura in Japan (Nishihiro et al. 2001). This phenomenon is true of *N. indica*.

In addition, genetic diversity in local population was significantly correlated with the seed set rate in *N.indica* (Chapter 3). In the present study, it was clear that water margin with water fluctuation could lead the seeds to germinate in *N.indica*. Though irrigation for rice crops brings about seasonal water fluctuation in the ponds, the water margin was artificially constructed. More and more irrigation ponds were abandoned in the present circumstance and water fluctuation was hard to occur.

Furthermore, exotic species such as *Myriophyllum aquaticum* (Vellozo) Verdc. and *Elodea nuttallii* (Planch.) St. John were extensively forming community in water bodies in Japan (Kadono 1989; Kadono et al. 1997). They also invaded into the water margin in the populations of *N.indica*. Invasion of these species may give a negative influence to the growth of seedlings of

N.indica because of competitive exclusiveness and/or by inhibiting irradiation. These facts suggested that some of artificial events such as the bank protection, abandonment of water management and invasion of exotic species may give an interference to the establishment of seedlings in *N.indica*. As long as the environment where establishment of seedlings in *N.indica* is difficult is continued, deterioration of genetic diversity will occur in *N.indica* in the future.

CHAPTER 5

Computer simulation of the relationship between the establishment of seedlings and floral morph composition of the populations in a heterostylous plant *Nymphoides indica* O.Kuntze (Menyanthaceae)

5. 1. Introduction

Floral polymorphism such as heterostyly, dioecy and gynodioecy gives an strong influence to the mating patterns and consequently ecological and evolutionary dynamics (Cox 1981; Bierzychudek & Eckhart 1988; Eckert & Barrett 1992; Eckert & Barrett 1995; Eppley et al. 1998) .

Floral polymorphism in heterostylous plants was genetically controlled (Ganders 1979; Lewis & Jones 1992). Floral morph of distylous species was basically determined by two alleles in one locus. Regeneration from only seeds through disassortative pollination theoretically brings about equal floral morph ratio. It had also been known that disassortative mating among each style morph in tristylous plant populations should lead to the equilibrium with the three morphs at equal frequencies by theoretical analysis of computer simulation (Heuch 1979 ; Heuch & Lie 1985).

However, it has been reported that morph frequencies in natural populations frequently show significant deviations (e.g. Barrett et al. 1983;

Morgan & Barrett 1988; Eckert & Barrett 1992, 1995 ; Ågren 1996). This variation has been thought to be resulted from the ecological characters of species, especially their life history and reproductive traits. In fact, Eckert and Barrett (1992) explained about the causes bringing about each floral morph composition in two tristylous species with different life history traits by the computer simulation and survey of floral morph frequencies in natural populations. The floral morph composition of *Decodon verticillatus* with self-compatible and clonality is tend to maintain uneven floral morph ratio, whereas that of *Lythrum salicaria* with self-incompatible and unclonality maintain equality. However, heterostylous plants generally regenerate with sexual and vegetative reproduction and it is likely that the balance of reproductive mode within a species changes in accord with the environmental condition (i.e. the characteristics of habitat, the extent of disturbance and the rate of habitat succession) (Sebens & Thorne 1985; Philbrick & Les 1996; Damman & Cain 1998; Mandujano et al. 1998; Barsoum 2002 ; Winkler & Fisher 2002), which will bring about the further variation of floral morph composition within a heterostylous species.

In addition, the understanding of the balance of reproductive mode within one species may give a benefit to an endangered species. Basically, the deterioration of regeneration from sexual reproduction means the inferiority of genetic diversity and may give a limitation to the geographical distribution of the target species because of rare chances of seed dispersal. Therefore, I thought that floral morph ratio representing the balance of reproductive mode for heterostylous species may be indicator for planning of a conservation

strategy.

The floral morph composition of *Nymphoides indica* was remarkably different from equal floral morph ratio to monomorphic in a given range (Shibayama & Kadono 2003 b). The correlation between the seed set rate and genetic diversity was positively significant (Chapter 3). Regeneration from seeds severely depends on the water level fluctuation. Seeds can germinate under the moist condition and the growth of seedlings at the earlier developing stage also required for the moist condition for a while, whereas the vegetative reproduction occurred under the stable water level. Thus, environmental condition required for growth was remarkably different between sexual and vegetative reproduction in *N.indica* (Chapter 4).

The aim of this chapter is to elucidate the causes bringing variety of floral morph composition in *N.indica*. I investigated 1) the establishment of seedlings in two populations 2) the bias of floral morph composition in 36 populations and the change of floral morph composition for six years in populations dominated by a vegetative reproduction and 3) I performed a computer simulation to reveal the effect of the establishment of seedlings on floral morph composition in the populations of *N.indica*.

5. 2. Materials and Methods

5. 2. 1. Cultivated experiments

To confirm the genetic traits of floral morph in *N.indica*, mature fruits were sampled in Heisou-cho, Kakogawa City, Hyogo Prefecture (HG) and O uji-cho, Ono City, Hyogo Prefecture (HI) in late autumn in 2001. Ten fruits at

the interval of 5 m were collected from both of floral morphs in each population. Seeds derived from each family were germinated under the light (Light : 14 hours ; Dark: 10 hours) and alternative temperature (25 C° / 12 C°) condition in a controlled incubator after cold treatment for two months. Ten seeds germinated from each family were transplanted to the pot (φ6cm) filled with soil collected from an irrigation pond and fertilized by commercial fertilizer (MAGAMP K, Hyponex Japan). They were cultivated under the moist condition in the glasshouse. Individuals with four or five foliage leaves were submerged under the plastic container (600 mm×300 mm×191 mm) in June, 2002. Twenty pots were randomly arranged in each plastic container. Floral morph of each plant was determined as soon as they bloomed. This observation was continued from summer in 2002 to early-autumn in 2003.

5. 2. 2. Establishment of seedlings in two populations

To know the environmental condition in which equal floral morph ratio was brought about, the establishment of seedlings was investigated in two populations. Seedlings with floating leaves were regarded as the established ones.

The number of seedlings and individuals originated from rootstocks or turions was counted and the size of community was measured in Nishi-ku, Kobe City, Hyogo Prefecture (HB-1). Marking for seedlings was done as soon as seed germination occurred and the fate of seedlings was followed. The stuff for marking was referred to methods in Chapter 4.

The number of regeneration from seedlings and rootstocks or turions and

seed germination was counted from 1997 to 2003 in Hayashida-cho, Himeji City, Hyogo Prefecture (HV). The data of rainfall obtained from AMeDAS (Meteorological Agency) from 1997 to 2003 in Himeji district was utilized as one of environmental indicators. Drought was regarded as less than 1000mm per year of rainfall. The seedlings with floating leaves were defined as the establishment in HV as was the case in HB-1 and the fate of seedlings was simultaneously followed. To know the difference of the water fluctuation between the two populations, water depth was measured at the deepest and shallowest points where seedlings occurred every two weeks.

5. 2. 3. Survey of floral morph ratio

To know the floral morph ratio of *N.indica* in Higashi-harima, Hyogo Prefecture, Japan, the floral morph ratio of 36 populations was investigated from 1997 to 2003 in summer season. Flowering inflorescences according to the population size from a wide area of each population at intervals of more than 1 m to avoid sampling from the same plant was collected and floral morph composition was determined in each population. Furthermore, to know the change of floral morph ratio in populations dominated by vegetative reproduction during six years, floral morph ratio of 21 populations surveyed in 1997 was re-evaluated in 2001 and 2003 by the same method.

The bias of floral morph ratio was represented by $|L-S| / (L+S)$ (Nishihiro et al. 1998), which shows that 0 is monomorphic and 1 is equal floral morph ratio.

5. 2. 4. Monte Carlo Simulation

To know the effect of the establishment of seedlings to the floral morph composition, I applied the computer model $\{NI + N(1-I)(C+S) = N, C+S=1\}$ formulated by Eckert and Barrett (1992) to the distylous species. One of assumptions for the model is that regeneration from vegetative reproduction randomly occurs in both of floral morphs because there is no difference of the fitness between the floral morphs. Another assumption is that equal floral morph number is brought about according to the genetic trait if sexual reproduction occurs successfully.

The floral morph composition of populations was determined by two integers (0 or 1) representing each floral morph because each floral morph in distylous species was determined by two alleles in one locus (Fig. 5-1). Population size (N) was held constant every year. (I) of the survival rate was randomly selected from the population of the previous year. Either clonal (C) or sexual (S) reproduction compensated for lost floral morph $\{N \times (1-I)\}$. Floral morph regenerated from clonal reproduction was randomly selected from the population of the previous year. On the other hand, equal floral morph number was brought about according to the genetic traits in case of sexual reproduction ($C+S=1$).

The bias of floral morph composition was represented by $|L-S| / (L+S)$ (Nishihiro et al. 1998).

According to the patterns of establishment of seedlings in each local population, two types of simulation were performed. The one case of simulation was that the same reproductive mode is replicated every year. Three

types with different bias of floral morph composition were prepared as founding populations [$|L-S| / (L+S) = 0$; $|L-S| / (L+S) = 0.4$; $|L-S| / (L+S) = 0.8$] and the simulation was performed in each founding population. The other case of simulation was that only a vegetative reproduction occurs when water level is high and sexual reproduction occurs simultaneously with vegetative reproduction in case of occasional water drawdown. The parameter was set as follows. I made the rate of clonal reproduction decrease in proportion to survival rate and that of sexual reproduction increase. Occasional water drawdown is thought to be stochastic event and I made water drawdown occur randomly every 10 years and thirty years during 100 years. Seeds required for sexual reproduction were also supplied from the seed bank in this simulation because seed bank in *N.indica* was persistently formed (Chapter 4). In case of this simulation, founding population was regarded as equal floral morph ratio. Computer simulation in different reproductive modes was performed about 100 populations for 100 years. This program was performed by Visual Basic for Applications of Excel 2000 (Microsoft).

5. 3. Results

5. 3. 1. Cultivated experiments

Floral morph type originated from seeds by disassortative pollination was determined in the cultivated experiment (Fig. 5-1). Seeds derived from 10 families in HG were confirmed to bring about an equal floral morph ratio by G-test (Long- styled morph :60 ; Short-styled morph:73; $G = 1.27$, $P > 0.1$). In the case of HI, the result was obtained (Long- styled morph : 56 ; Short-styled

morph: 53; $G=0.08$, $P>0.9$).

5. 3. 2. Establishment of seedlings

In HB-1, many seedlings with some floating leaves succeeded in the establishment along the water margin alone (Fig. 5-2). Community of *N.indica* was formed at 30m off from the shore (Fig. 5-2). 62 seedlings could survive in 2003 (Fig. 5-3). In HV, field survey was performed from 1997 to 2003. The establishment of seedlings failed when the rainfall was normal though the seed germination occurred (Fig. 5-4). On the other hand, the number of regeneration from turions or rootstocks was increasing (Fig. 5-4). However, the establishment of 21 seedlings succeeded in 2002 when the rainfall was much less than the rest of years. Seedlings which occurred in 2003 also grew to the stage of some floating leaves (Fig. 5-4). However, adult plants died and the production of turions was prevented because of severe drought in 2000 and 2002 (Fig. 5-4).

Compared with HV, water fluctuation between the shallowest and deepest point where seedlings occurred was small in HB-1 (Fig. 5-5).

5. 3. 3. Survey of floral morph ratio

In most of populations, the bias of floral morph ratio was remarkable and the value of $|L-S| / (L+S)$ was more than 0.5 (Fig. 5-6). There were no populations of 0.2 to 0.6 in the value of $|L-S| / (L+S)$ except two populations (Fig. 5-6). Populations with equal floral morph ratio were only a few (Fig. 5-6). The bias of floral morph ratio with only a vegetative reproduction became more

notable (Fig. 5-7). Five populations out of nine populations with biased floral morph ratio turned into monomorphic (Table 5-1). In spite of sampling effort, the number of rare morph was decreasing among some populations. Even populations with equal floral morph ratio in 1997 began to show a bias of floral morph ratio (Table 5-1). Population size was severely decreasing in two of 21 populations and three populations have been extinct (Table 5-1).

5.3.4. Computer simulation

The results of the computer simulation showed that the equal floral morph based on *G*-test will be brought about if sexual reproduction with a low frequency ($I=0.6$ or $I=0.8$, $C=0.8$ and $S=0.2$; $N=100$ and $N=500$) constantly occurs even in the populations with biased floral morph ratio (Fig. 5-8 a ; Fig. 5-8 b). However, if the population size is small ($N=50$), the higher sexual reproduction ($I=0.8$, $C=0.6$ and $S=0.4$) will be needed to be brought about equal floral morph ratio (Fig. 5-8 a ; Fig. 5-8 b). This type of simulation was roughly based on the establishment of seedlings with adult plants highly surviving in HB-1. Furthermore, if sexual reproduction occurs over vegetative reproduction in case of occasional water drawdown, equal floral morph ratio will be brought about in populations ($I=0.4$, $C=0.4$ and $S=0.6$; $N=50$ and $N=100$), which corresponded to the establishment of seedlings in HV (Fig. 5-9 a). When the frequency of water drawdown is less (Fig. 5-9 b), the bias of floral morph composition will be improved if the higher sexual reproduction is brought about ($I=0.2$, $C=0.2$ and $S=0.8$; $N=50$ and $N=100$).

On the other hand, the computer simulation showed that the bias of floral

morph ratio became more remarkable after 100 years in case of only a vegetative reproduction. However, the rare morph has not been lost for 100 years even in populations with remarkable bias of floral morph composition ($C=1.0$ and $S=0$; Fig. 5-8 a ; Fig. 5-8 b). Only a regeneration from vegetative reproduction did not give a large influence to the floral morph ratio in case of the large population size of $N=500$ (Fig. 5-8 ; Fig. 5-9).

5. 4. Discussion

5. 4. 1. The maintenance of floral dimorphism in *N.indica*.

Cultivated experiment showed that floral morph of *N.indica* was genetically determined and floral morph ratio was significantly equal when regenerated from seeds (*G*-test).

Although seedling recruitment has been thought to be of minor importance in some clonal plants (Soukupova 1992; D'Antonio 1993), the present study showed that seedling recruitment played an important role to the maintenance of equal floral morph ratio, which will result in persistence of populations in *N. indica*.

In population with equal floral morph ratio, spatial and /or temporal environmental heterogeneity such as the occurrence of ecotone and occasional water drawdown was maintained.

In HB-1, the community of *N.indica* was formed at the 30m from a water edge. On the other hand, the occurrence of seedlings was limited at the range of 3 m from a water edge. Wetlands developed around the water bodies, where *Juncus wallichianus*, *Juncus alatus*, *Spiranthes sinensis* var. *amoena* and

Utricularia bifida inhabited. Gentle spatial and /or temporal environmental gradient was always formed from a shore to the offshore, which allowed the seedlings of *N.indica* to establish. Furthermore, computer simulation showed that the establishment of seedlings with high survival rate of rootstocks and turions will also lead to the equal floral morph ratio even in population with biased floral morph ratio.

In HV, seedlings could also establish after extreme water drawdown occurred. HV was constantly utilized as an irrigation pond. When rainfall was much less in 2002, water use for rice crops remarkably increased and the pond almost dried out. Consequently, adult plants died and formation of turions was completely prevented. On the other hand, open space uncovered with water due to the extreme drought has been kept to the next year, which gave a chance for seeds to germinate and for seedlings to establish and spatial distribution of seedlings became larger than the rest of years (Chapter 4). Though seed germination occurred in HV every year, the establishment of seedlings could not succeed in the years except 2002 and 2003. It was possible that a period of moist condition required for the establishment of seedlings (Chapter 4) was not continued. The maintenance of equal floral morph ratio caused by the process of an occasional water drawdown was also supported by the computer simulation, which showed that equal floral morph ratio will be brought about if sexual reproduction occurs more than vegetative reproduction in case of occasional water drawdown. Furthermore, in case that the frequency of occasional water drawdown is less, the bias of floral morph composition will be improved if the higher sexual reproduction is brought about.

This result in the field survey showed that seed bank played an important role as not only in re-establishment of a population but also in the increase of effective population size (Barrett & Husband 1997). This balance of reproduction modes confirmed in *N.indica* will correspond to Aphid –Rotifier model in the plant, which means that advantage in sexual reproduction is frequency dependent in a way that will explain the relative frequencies of sexual and vegetative reproduction in life cycles such as those of aphids, rotifers, and many parasites (Williams 1975).

The difference of the establishment of seedlings confirmed in two populations may be caused by the size and utility of irrigation ponds because the size of HV was about one-third as large as HB-1, what is more, HV was used for irrigation, which made water fluctuation in HV drastic.

Which case of the establishment of seedlings is adaptive for *N.indica*? If the probability of seedling establishment is higher, theoretical analysis shows that the fitness will basically increase even though the degree of fitness changes according to the trade-off between the sexual and vegetative reproduction (Winkler & Fisher 2002). The further theoretical analysis shows that the high level of genetic diversity caused by the establishment of seedlings in the clonal plants (Sebens & Thorne 1985) and the maintenance of the sex in the dioecious liverwort *Marchantia inflexa* (McLetchie et al. 2002) are thought to be brought about by the intermediate disturbance. However, empirical analysis to examine these hypotheses has not been performed so far. In addition to the theoretical analysis, the experiment of modulating water fluctuation to change the degree of establishment of seedling may provide me an empirical data for representing

an adaptive trait in populations of *N.indica*.

On the other hand, floral morph ratio severely became skewed in the most of populations without the occurrence of ecotone and/ or occasional water drawdown. The floral morph composition in each local population dominated by vegetative reproduction remarkably became skewed for six years. Rare floral morph has been lost in some populations. The speed of the bias of floral morph ratio was much faster than computer prediction. Furthermore, compared with the tristylous clonal plants *Decodon verticillatus* (Eckert & Barrett 1995), the magnitude of the change of the bias of floral morph composition was large in *N.indica*. As the population size was severely decreasing in a few populations (HC, HW), environmental stochasticity such as the occurrence of eutrophication by inflow of polluted water from a basin may have occurred. In fact, I often observed that *Trapa japonica* and/or *Trapa natans* that preferred to eutrophicated water dominated in 2001 and 2003 (Kadono 1998). However, the number of rare floral morph became less in some populations with bias of floral morph ratio even though population size did not severely decrease. The possible reason for it may be the occurrence of trade-off between sexual and vegetative reproduction. Rare floral morph had much more remarkable fruit and seed set rates than dominant morph in each local population with biased floral morph ratio (Shibayama & Kadono 2003 b). According to the cultivated experiments, floral morphs with pollinated (= rare floral morph) tended to produce significantly small size of turions and decrease dry weight of rootstocks compared to unpollinated floral morphs(=dominant floral morph) (Appendix 5-1). Thus, this result suggests that it may be difficult for turions

and rootsracks originated from rare morphs to regenerate in each population because of competition with the dominant morph, which means that sexual reproduction in *N.indica* may be selectively excluded under the stable deep water level whereas the establishment of seedlings occurred in the environment with a remarkable water fluctuation. Dorken & Barrett (2003) also pointed out that greater allocation to ramet and corm size at the expense of propagation in dioecious plant populations of *Sagittaria latifolia* is likely to have important implications for the balance of between sexual and clonal reproduction in the permanent wetlands in which dioecious populations exist. To the contrary, plants from monoecious populations which occur in the highly disturbed habitat had higher investment in both flowering and clonal reproduction than dioecious populations. These results suggest that divergence in life history traits through ecological selection by water may occur (Schluter 2000).

In summary, environmental stochasticity and /or trade-off between sexual and vegetative reproduction may have brought about a remarkable bias of floral morph ratio in populations dominated by vegetative reproduction in *N.indica*. The improvement of the model including the fluctuation of population size and trade -off between sexual and vegetative reproduction will be needed in the future.

Furthermore, three populations had been extinct. The reasons for extinction were due to the reclamation, eutrophication and the discharging of *Ctenopharyngodon idellus* (Grass Carp) from my observation and interview.

I concluded that the difference of the establishment of seedlings caused by

environmental condition among irrigation ponds led to the remarkable variation of floral morph composition in *N.indica*.

5. 4. 2. The maintenance of *N.indica* at the metapopulation level

In the present study, the traits of life history combined with a habitat character were shown to give a great influence to floral morph composition and seed production in *N.indica*. Habitats with spatial and /or temporal environmental heterogeneity such as the occurrence of ecotone and occasional water drawdown give a chance to bring about equal floral morph in local population and serve to maintain a metapopulation. On the other hand, the prevention of establishment of seedlings by artificial bank construction and/or no remarkable water fluctuation (Chapter 4) lead to skewed floral morph composition and seed set rate will severely decrease. Unfortunately, in Japan, those populations have been increasing. The genetic structure also showed that *Gst* was very large in Higashi- harima area in *N.indica*, which indicated that gene flow was rare event (Chapter 3). Local extinction is generally thought to be hard to occur because of recurrent migration among populations in many species (Harrison & Taylor 1997). In case of *N.indica*, however, the deterioration of colonizing ability resulted from the severe decrease of seed production may give a damage to the persistence of metapopulation and raise the possibility of local extinction though the availability of the seed bank is needed to be investigated in populations where seed production is limited (Williams 1975; Holsinger & Vitt 1997; Warburton et al. 2000).

The further theoretical analysis and constant field survey will tell me how

life history attributes combined with a habitat character gives an influence to long-term habitat occupancy in a heterostylous plant *N.indica* (Quintana-Ascencio et al. 1996; Amarasecare 1998 b 2001; Brachet et al. 1999; Crowley & McLetchie 2002; Dupré & Ehrlén 2002 ; Ellner & Fussmann 2003).

5. 4. 3. The population management for *N.indica*

This result showed that the bias of floral morph composition in *N.indica* is rapidly going on in most of populations because stable deep water level is maintained and regeneration from seeds is difficult to occur.

Irrigation ponds for rice crops have been originally managed by human beings in Japan. However, there are many irrigation ponds without the appropriate managements and water quality is recently being damaged by agricultural inflow and human sewage.

The one of traditional managements for irrigation ponds was to make the bottom of ponds dried in winter. To remove excessive nitrogen and phosphorous by utilizing mud as fertilizer was supposed to suppress the eutrophication and succession of ecosystem in the past years (Hamashima et al. 2001). This management in irrigation ponds may be also effective for *N.indica* to give a positive influence to regenerate from seed bank. Thus, the long - term population persistence of *N.indica* may be achieved by adding an occasional water drawdown to water bodies considering the frequency and intensity of water level fluctuation (Lovett Doust & Lovett Doust 1995; Christensen et al. 1996).

Conclusion

Some distinct traits of heterostyly were shown in *N.indica* in Japan. Long-styled and short-styled morph was clearly discriminated. The difference of pollen size between both of the floral morphs was apparent and self-incompatibility was normally functioned. These facts quantitatively supported the result of the previous studies. Traits of heterostyly shown in *N.indica* were also functioned in the field condition.

The seed set rate of *N.indica* is limited by the shortage of compatible pollens and the success of legitimate pollination was the highest when two floral morphs were located in the near distance. These phenomenon supported the fact that the fruit and seed set rates of populations with equal floral morph composition were higher than those of any other types of floral morph composition. Considering the present critical status of *N.indica* populations as an endangered species, a priority of conservation may be given to the populations with equal floral morph ratio which can have an evolutionary and ecological potential such as maintenance of genetic diversity required for adaptation to changes in environmental condition and the higher dispersal ability to found populations.

The number of MultiLocus Genotypes (MLGs) representing genetic diversity was positively correlated with the seed set rate in each population and Hardy-Weinberg equilibrium showed that random mating has occurred in populations with equal floral morph ratio, which indicated that sexual reproduction including the establishment of seedlings contributed to the maintenance of genetic diversity and balanced floral morph ratio. Moreover,

the fact is that the value of G_{st} in the whole range was large, which showed that gene flow was rare event and the isolation among populations was going on. As mentioned above, these results also supported that sexual reproduction from seed production to the establishment of seedlings was indispensable event to persist and found *N.indica* populations.

How can the establishment of seedlings occur in *N.indica*? The field survey and laboratory-based experiment indicated that the environmental condition required for seed germination was the exposed ground with diurnal change of temperature and for the establishment of seedlings was a period with water uncovered. The habitat which demonstrated these traits showed the water margin of water bodies and bare ground brought about by the occasional water drawdown. In fact, the successful establishment of seedlings was observed in such habitats. Though the successful establishment of seedlings was confirmed in a few populations with ecotone and occasional water fluctuation, the bank construction and abandonment of water management prevented seed germination from occurring in most of populations. As the maintenance of genetic diversity had relevance to the establishment of seedlings in *N.indica*, artificial habitat alternation may bring about the inferiority of genetic diversity.

Furthermore, computer simulation showed that equal floral morph composition will be brought about if the establishment of seedlings constantly occurs even in a low frequency or the sexual reproduction occurs more than vegetative reproduction in case of occasional water drawdown. These results showed that balanced floral morph ratio is maintained by the success of establishment of seedlings under the environmental condition with ecotone or

occasional water drawdown.

On the contrary, the field survey showed that the bias of floral morph composition dominated by vegetative reproduction had become more notable for six years, which was also supported by computer simulation. However, the speed of the bias of floral morph composition was faster than the prediction by the computer. The increase of populations with severely skewed floral morph composition caused by an artificial habitat alternation may give a negative influence to the persistence of endangered species *N.indica*. Since local extinction is thought to be hard to occur because of recurrent migration among populations in many species, the deterioration of colonizing ability resulted from the severe decrease of seed production in *N.indica* may raise the possibility of local extinction.

Based on such ecological traits of *N.indica*, conservation of *N.indica* showed that the maintenance of water level fluctuation and ecotone is effective.

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Table 1-1. Locality of the investigated populations.

Locality (Population code)
Hyogo Prefecture
1.Kande-cho, Nishi-ku, Kobe City (HB)^{a)}
2.Hiraoka-cho, Kakogawa City (HC)
3.Higashikanki-cho, Kakogawa City (HD)
4.Nishikanki-cho, Kakogawa City (HE)
5.Heiso-cho, Kakogawa City (HG)
6.Ouji-cho, Ono City (HI)
7.East, Aono-cho, Kasai City (HO)
8.Kuchisami, Hayashida-cho, Himeji City (HV)
^{a)} Boldface indicates the populations where SCD (Staminate-Carpellate distance) was investigated.

Table 1-2. Fruit and seed set rate following three pollination treatments in *N. indica*.

Treatment	No. of pollination	% fruit set	Fruit set rate (%)	Seed set rate Mean $\pm$ SD (%)
Long-styled (Maternal)				
intermorph	17	100	100	74.3 $\pm$ 25.2
intramorph	18	0	0	0
self	18	0	0	0
Short-styled (Maternal)				
intermorph	17	100	100	76.0 $\pm$ 18.9
intramorph	18	0	0	0
self	22	0	0	0

Table 2-1. Locality of the investigated populations and the frequency of long-styled and short-styled morphs.

Locality (Population code)	No. of plants (Proportion)		G ^{a)}
	Long-styled	Short-styled	
Hyogo Prefecture			
1.Uozumi-cho, Akashi City (HA)	72 (0.95)	4 (0.05)	74.02**
2.Kande-cho, Nishi-ku, Kobe City (HB) ^{b)}	5 (0.09)	53 (0.91)	46.34**
3.Hiraoka-cho, Kakogawa City (HC)	4 (0.07)	55 (0.93)	52.54**
4.Higashikanki-cho, Kakogawa City (HD)	56 (0.93)	4 (0.07)	53.79**
5.Nishikanki-cho, Kakogawa City (HE)	61 (0.97)	2 (0.03)	69.60**
6.Kanno-cho, Kakogawa City (HF)	30 (1.00)	0 (0.00)	-
7.Heiso-cho, Kakogawa City (HG)	61 (0.52)	57 (0.48)	0.14
8.Shikata-cho, Kakogawa City (HH)	30 (1.00)	0 (0.00)	-
9.Ouji-cho, Ono City (HI)	33 (0.54)	28 (0.46)	0.41
10.Kuboki-cho, Ono City (HJ)	0 (0.00)	35 (1.00)	-
11.Yato, Yashiro-cho, Kato-Gun (HK)	8 (0.11)	63 (0.89)	48.43**
12.Hiraike Park, Yashiro-cho, Kato-Gun (HL)	10 (0.32)	21 (0.68)	3.99*
13.Kamimiyagi-cho, Kasai City (HM)	81 (1.00)	0 (0.00)	-
14.North, Aono-cho, Kasai City (HN)	0 (0.00)	56 (1.00)	-
15.East, Aono-cho, Kasai City (HO)	55 (0.92)	5 (0.08)	48.76**
16.West, Aono-cho, Kasai City (HP)	0 (0.00)	30 (1.00)	-
17.Nishikasahara-cho, Kasai City (HQ)	0 (0.00)	30 (1.00)	-
18.Kami-ike, Aono-cho, Kasai City (HR)	30 (1.00)	0 (0.00)	-
19.Hourai-ike, Aono-cho, Kasai City (HS)	0 (0.00)	30 (1.00)	-
20.Befu-cho, Kasai City (HT)	53 (0.42)	72 (0.58)	2.9
21.Shimoise, Hayashida-cho, Himeji City (HU)	10 (0.22)	36 (0.78)	15.6**
22.Kuchisami, Hayashida-cho, Himeji City (HV)	16 (0.52)	15 (0.48)	0.03
23.Okusami, Hayashida-cho, Himeji City (HW)	5 (0.17)	25 (0.83)	14.56**
Okayama Prefecture			
24. Sakane, Seto-cho, Akaiwa-Gun (OA)	0 (0.00)	30 (1.00)	-
25. Nishikubota, Seto-cho, Akaiwa-Gun (OB)	0 (0.00)	30 (1.00)	-

a) G-value is also shown as a parameter of the significance of the bias of floral morphs.

* $P < 0.05$; ** $P < 0.005$

Table 2-2. Fruit and seed set rates of *N.indica* in 20 populations.

Type of floral morph composition	Population	No. of inflorescences	Fruit set rate*	Seed set rate*
Long- and short-styled morphs with equal ratio	HG	26	0.55±0.24	0.60±0.24
	HI	38	0.59±0.24	0.74±0.28
	HT	46	0.53±0.18	0.87±0.10
	HV	24	0.44±0.32	0.72±0.36
Long- and short-styled morphs with biased ratio	HA	29	0.24±0.27	0.26±0.28
	HB	28	0.09±0.12	0.23±0.31
	HC	27	0.07±0.18	0.08±0.23
	HD	30	0.28±0.22	0.28±0.20
	HE	31	0.12±0.20	0.09±0.15
	HK	42	0.08±0.18	0.12±0.26
	HO	28	0.32±0.15	0.36±0.18
	HU	46	0.35±0.42	0.34±0.40
Monomorphic	HW	27	0.19±0.24	0.18±0.26
	HM	30	0	0
	HN	24	0.03±0.05	0.08±0.12
	HQ	30	0	0
	HR	30	0	0
	HS	30	0	0
	OA	30	0	0
	OB	30	0	0

*Mean±SD

Table 2-3. Fruit and seed set rates of long-styled and short-styled morphs in each type of population.

Type of floral morph composition	Population	Fruit set rate ^{a)}		Seed set rate ^{a)}	
		Long styled	Short styled	Long styled	Short styled
Long- and short-styled morph with equal ratio	HG	0.54±0.26(14)	0.55±0.23(12)	0.67±0.23(14)	0.52±0.24(12)
	HT	0.57±0.14(23)	0.50±0.21(23)	0.89±0.09(23)	0.85±0.09(23)
Long- and short-styled morph with biased ratio	HB	0.87±0.15(5)^{b)}	0.11±0.22(64)	0.92±0.03(5)	0.16±0.33(64)
	HC	0.64±0.13(2)	0.02±0.08(25)	0.82±0.12(2)	0.03±0.08(25)
	HK	0.68±0.16(8)	0.13±0.21(49)	0.92±0.26(8)	0.26±0.39(49)
	HD	0.13±0.23(53)	0.57±0.22(4)	0.13±0.24(53)	0.53±0.21(4)
	HE	0.09±0.16(29)	0.59±0.07(2)	0.06±0.11(29)	0.50±0.02(2)
Monomorphic	HR	0(30)	-	0(30)	-
	HS	-	0(30)	-	0(30)

^{a)} Mean±SD^{b)} Boldface shows the fruit and seed set rates of rare morph.

Table 2-4. Comparison of intermorph pollination rate of each floral morph between populations dominated by either of floral morphs and populations composed of equal floral morph. Different alphabets indicate statistically significant differences.

(A) Long-styled flowers

Population	Floral morph [*]	Sample size	Pollen load	Intermorph pollination rate
			Mean±SD (Range)	Mean±SD
HA ^{**}	Long alone	40	211.7±74.2 (20-375)	0 ^a
HD	Long > Short	93	202.7±103.5 (30-500)	0.06±0.12 ^b
HT	Long = Short	23	144.8±87.7 (38-460)	0.27±0.19 ^c

(B) Short-styled flowers

Population	Floral morph	Sample size	Pollen load	Intermorph pollination rate
			Mean±SD (Range)	Mean±SD
HC	Long < Short	103	40.6±32.9 (0-234)	0.10±0.19 ^a
HT	Long = Short	22	58.9±30.9 (16-134)	0.70±0.13 ^b

^{*}Floral morph composition in the population.

^{**}HA was monomorphic in 1998.

Table 2- 5. Comparison of intermorph pollination rate, fruit and seed set rates of common morph among quadrats in two populations (HC and HD). Short- and long-styled morphs were dominant in HC and HD, respectively. Different alphabets indicate statistically significant differences.

(a) HC

Quadrat	Pollen load	Intermorph pollination rate	Fruit set rate	Seed set rate	Distance from HC-1 (m)
	Mean±SD (Range)	Mean±SD (N)	Mean±SD (N)	Mean±SD (N)	
HC-1 (two morphs)*	46.9±48.3 (2-234)	0.24±0.23 (28) ^a	0.32±0.24 (16) ^a	0.30±0.22 (16) ^a	0
HC-2 (short alone)	27.5±18.1 (0-72)	0.07±0.18 (29) ^b	0.05±0.08 (20) ^b	0.07±0.12 (20) ^b	8
HC-3 (short alone)	43.6±26.4 (1-119)	0.01±0.04 (29) ^b	0 (19) ^b	0 (19) ^b	10
HC-4 (short alone)	37.7±26.6 (0-99)	0.03±0.12 (27) ^b	0.02±0.05 (21) ^b	0.01±0.02 (21) ^b	40

(b) HD

Quadrat	Pollen load	Intermorph pollination rate	Fruit set rate	Seed set rate	Distance from HD-1 (m)
	Mean±SD (Range)	Mean±SD (N)	Mean±SD (N)	Mean±SD (N)	
HD-1 (two morphs)*	178.1±84.0 (60-343)	0.16±0.16 (28) ^a	0.42±0.20 (15) ^a	0.43±0.23 (15) ^a	0
HD-2 (long alone)	187.0±79.0 (67-379)	0.02±0.07 (28) ^b	0.00±0.02 (19) ^b	0.01±0.03 (19) ^b	5
HD-3 (long alone)	214.0±117.6 (30-500)	0.00±0.01 (35) ^b	0 (19) ^b	0 (19) ^b	10

*Composition of floral morphs in the quadrats.

Table 2- 6. Fruit and seed set after artificial pollination in two populations (HC and HD).
Different superscripts indicate significant differences.

Population	Intermorph	Intramorph	Self	Spontaneous
	Mean±SD (N)	Mean±SD (N)	Mean±SD (N)	Mean±SD (N)
HC				
Fruit set rate	1 (21) ^a	0 (18) ^b	0 (18) ^b	0 (18) ^b
Seed set rate	0.97±0.38 (21) ^a	0 (18) ^b	0 (18) ^b	0 (18) ^b
HD				
Fruit set rate	1 (20) ^a	0 (20) ^b	0 (20) ^b	0 (20) ^b
Seed set rate	0.87±0.35 (20) ^a	0 (20) ^b	0 (20) ^b	0 (20) ^b

Table 2- 7. Visiting insects of the flowers of *N.indica* observed in five populations.

+++ : common + : rare

Insect visitors	Population				
	HA	HC	HD	HG	HT
Lepidoptera					
<i>Parnara guttata</i> Bremer & Grey		+	+		+
Diptera					
<i>Notiphila</i> sp.	+++			+++	
<i>Mesembrius flaviceps</i> Matsumura		+++			
Hymenoptera					
<i>Apis mellifera</i> Linnaeus			+++		
<i>Lasioglossum mutilum</i> Vachal					+++
Coleoptera					
<i>Bagous</i> sp.				+++	

Table 2-8. The number of visiting insects and their flying distance observed in two populations.

(a) HC

Insect species	Number	Flying distance (cm)
<i>Mesembrius flaviceps</i>	14	15.6±16.0 (85)*
<i>Parnara guttata</i>	5	198.2±340.0 (25)

(b) HD

Insect species	Number	Flying distance (cm)
<i>Apis mellifera</i>	24	17.1±9.3 (123)
<i>Parnara guttata</i>	11	53.2±95.3 (38)

* The number of flights observed.

Table 3-1. Type of floral morph composition, locality, sampling number, the number of multilocus genotype (MLG) and seed set rate in each population.

Type of floral morph composition	Locality (Population code)	N	MLG	Seed set rate*
Equal floral morph ratio	1.Heiso-cho, Kakogawa City (HG)	34	3	0.60
	2.Ouji-cho, Ono City (HI)	30	5	0.74
	3.Befu-cho, Kasai City (HT)	27	3	0.87
	4.Kuchisami, Hayashida-cho, Himeji City (HV)	30	4	0.72
Biased floral morph ratio	5.Uozumi-cho, Akashi City (HA)	21	1	0.26
	6.Kande-cho, Nishi-ku, Kobe City (HB)	30	3	0.23
	7.Hiraoka-cho, Kakogawa City (HC)	30	1	0.08
	8.Higashikanki-cho, Kakogawa City (HD)	30	1	0.28
	9.Nishikanki-cho, Kakogawa City (HE)	29	2	0.09
	10.Yato, Yashiro-cho, Kato-gun (HK)	35	1	0.12
	11.HiraikePark, Yashiro-cho, Kato-gun (HL)	29	3	-
	12.East, Aono-cho, Kasai City (HO)	30	1	0.36
	13.Shimoise, Hayashida-cho, Himeji City (HU)	30	2	0.34
	14.Okusami, Hayashida-cho, Himeji City (HW)	30	3	0.18
Monomorphic	15.Kanno-cho, Kakogawa City (HF)	29	1	-
	16.Shikata-cho, Kakogawa City (HH)	30	1	-
	17.Kuboki-cho, Ono City (HJ)	30	1	-
	18.Kamimiyagi-cho, Kasai City (HM)	30	2	0
	19.Aono-cho, Kasai City (HN)	30	1	0.08
	20.West, Aono-cho, Kasai City (HP)	30	1	0
	21.Nishikasahara-cho, Kasai City (HQ)	30	1	0
	22.Kami-ike, Aono-cho, Kasai City (HR)	30	1	0
	23.Hourai-ike, Aono-cho, Kasai City (HS)	30	1	0
	24.Sakane, Seto-cho, Akaiwa-gun (OA)	30	1	0
	25.Nishikubota, Seto-cho, Akaiwa-gun (OB)	30	1	0

* Shibayama & Kadono (2003 b)

Table 3 - 2. Multilocus genotype (MLG) based on the combination of genotype of three polymorphic loci.

Locus	PGM	PGI	SkDH	MLG
	aa	bb	bb	a
	aa	aa	bb	b
	aa	ab	bb	c
	aa	bb	ab	d
	ab	bb	bb	e
	ab	aa	bb	f
	ab	ab	bb	g
	ab	bb	ab	h
	bb	bb	bb	i
	bb	aa	bb	j
	bb	bb	aa	k

Table 3- 3. Allele frequency of PGM, PGI and SkDH.

Enzyme locus	PGM		PGI		SkDH	
Population	a	b	a	b	a	b
HA	1.00	0.00	0.00	1.00	0.00	1.00
HB	0.95	0.05	0.00	1.00	0.00	1.00
HC	0.00	1.00	0.00	1.00	0.00	1.00
HD	0.50	0.50	0.00	1.00	0.00	1.00
HE	0.93	0.07	0.00	1.00	0.00	1.00
HF	0.50	0.50	0.00	1.00	0.00	1.00
HG	0.66	0.34	0.00	1.00	0.00	1.00
HH	0.50	0.50	0.50	0.50	0.00	1.00
HI	0.73	0.27	0.00	1.00	0.07	0.93
HJ	0.50	0.50	0.00	1.00	0.50	0.50
HK	0.14	0.86	0.00	1.00	0.93	0.07
HL	0.48	0.52	0.00	1.00	0.00	1.00
HM	0.82	0.18	0.00	1.00	0.00	1.00
HN	0.00	1.00	0.00	1.00	0.00	1.00
HO	1.00	0.00	0.00	1.00	0.00	1.00
HP	0.00	1.00	0.00	1.00	0.00	1.00
HQ	0.50	0.5	0.50	0.50	0.00	1.00
HR	0.50	0.5	0.00	1.00	0.00	1.00
HS	0.50	0.5	0.00	1.00	0.00	1.00
HT	0.67	0.33	0.00	1.00	0.00	1.00
HU	0.97	0.03	0.97	0.03	0.00	1.00
HV	0.82	0.18	0.80	0.20	0.00	1.00
HW	0.50	0.50	0.47	0.53	0.00	1.00
OA	0.50	0.50	1.00	0.00	0.00	1.00
OB	0.50	0.50	1.00	0.00	0.00	1.00

Table 3-4. Wright's fixation indices (F) of ten populations obtained by randomization procedure for polymorphic loci .

a) Equal floral morph ratio

Locus	HG	HI	HT	HV
PGM	-0.10	0.16	0.02	-0.21
PGI	-	-	-	-0.23
SkDH	-	-0.06	-	-
Overall	-0.10	0.11	0.02	-0.22

b) Biased floral morph ratio

Locus	HB	HE	HK	HL	HU	HW
PGM	0.66	-0.06	1.00*	0.46	-0.02	-
PGI	-	-	-	-	-0.02	-0.73
SkDH	-	-	-0.06	-	-	-
Overall	0.66	-0.06	0.63*	0.46*	-0.02	-0.73*

* Significant deviation of F from zero ($P < 0.05$).

Table 3-5. The pattern of MLG occurred in Hyogo Prefecture.

Population	Multi Locus Genotype (MLG)									
	a	b	c	d	e	f	g	h	i	k
HA	▲									
HB	▲				▲				▲	
HC									▲	
HD					▲					
HE	▲				▲					
HF					★					
HG	●				●				●	
HH							★			
HI	●			●	●			●	●	
HJ								★		
HK				▲						▲
HL	▲				▲				▲	
HM	★				★					
HN									▲	
HO	▲									
HP									★	
HQ							★			
HR					★					
HS					★					
HT	●				●				●	
HU		▲					▲			
HV		●	●			●	●			
HW					▲	▲	▲			
Sum of each MLG occurred in 23 populations	9	2	1	2	12	2	5	2	8	1
Frequency of each MLG occurred in 23 populations	0.4	0.1	0	0.1	0.5	0.1	0.2	0.1	0.3	0

(●):Equal floral morph ratio; (▲):Biased floral morph ratio;(★): Monomorphic

Table 3-6. F_{st} calculated by Weir and Cockerham's(1984) estimation in each enzyme locus and G_{st} .

Enzyme locus	F_{st} (S.E.)
PGM	0.39(0.10)*
PGI	0.68(0.14)*
SkDH	0.91(0.32)*
G_{st}	0.51

*Significance for F statistics obtained by randomization procedures ($P < 0.001$).

Table 3-7. F_{st} between groups based on a dendrogram.

Group	F_{st}
A	0.21
B	0.52

Table 4-1. Seed burial experiment at different water depth in HG.

Water depth(cm)	Cumulative germination rate	
	Jun.	Oct.
0	97.0±1.0%*	97.0±1.0%
70	0%	1.0±2.0%

*Mean ± SD

Table 4-2. The rate of viable and germinable seeds in the seed burial experiment at the water bottom.

The trait for seed	2001	2002	2003
Viable seeds	71.0±5.0%*	40.0±5.0%	28.0±5.0%
Germinable seeds	65.0±6.0%	38.0±7.0%	23.0±5.0%

*Mean ± SD

Table 4-3. Other plants inhabited with seedlings of *N.indica* .

Population	Species	Family
HG	<i>Beckmannia syzigachne</i> (Steud.) Fern.	Poaceae
	<i>Alopecurus aequalis</i> Sobol.	Poaceae
	<i>Phragmites communis</i> Trin.	Poaceae
	★ <i>Myriophyllum aquaticum</i> (Vellozo) Verdc.	Haloragaceae
	<i>Myriophyllum ussuriense</i> Maxim.	Haloragaceae
	<i>Limnophila sessiliflora</i> Blume	Scrophulariaceae
HV	<i>Ottelis alismoides</i> (L.) Pers.	Hydrocharitaceae
	★ <i>Elodea nuttallii</i> (Planch.) St. John	Hydrocharitaceae
	<i>Potamogeton distinctus</i> A.Benn.	Potamogetonaceae
	<i>Juncus tenuis</i> Willden	Juncaceae
	<i>Eriocaulon atrum</i> Nakai	Eriocaulaceae
	<i>Isachne globosa</i> (Thunb.) O.Kuntze	Poaceae
	<i>Pseudoraphis ukishiba</i> Ohwi	Poaceae
	<i>Cyperous haspan</i> L.	Cyperaceae
	<i>Scirpus lineolatus</i> Fr. et Sav.	Cyperaceae
	<i>Lythrum anceps</i> (Koehme) Makino	Lythraceae
	<i>Myriophyllum ussuriense</i> Maxim.	Haloragaceae
	<i>Lysimachia fortunei</i> Maxim.	Primulaceae
	<i>Lindernia procumbens</i> (Knock.) Philcox	Scrophulariaceae
	<i>Deinostema violaceum</i> (Maxim.) Yamazaki	Scrophulariaceae
HI	<i>Juncus tenuis</i> Willden	Juncaceae
	<i>Aeschynomere indica</i> L.	Leguminosae
	★ Exotic species.	

Table 4-4. Effect of the cold treatment on cumulative germination rate under light-alternative regime by one-way ANOVA .

(a) HG						(b) HI					
Source of variation	df	SS	MS	F	P	Source of variation	df	SS	MS	F	P
Cold treatment	2	1.65	0.83	90.77	<.0001	Cold treatment	2	0.49	0.25	26.15	0.001
Residue	6	0.06	0.10			Residue	6	0.06	0.10		

Table 4-5. Effects of light , temperature and interaction between light and temperature on cumulative germination rate by two-way ANOVA. Weeks show the period for cold treatment.

(a)HG						(b)HI					
Source of variation	df	SS	MS	F	P	Source of variation	df	SS	MS	F	P
0 week						0 week					
Light	1	0.07	0.07	17.0	0.003	Light	1	0.14	0.14	14.27	0.005
Temperature	1	0.07	0.07	18.6	0.003	Temperature	1	0.39	0.39	40.53	0
Light×Temperature	1	0.01	0.01	3.20	0.11	Light×Temperature	1	0.05	0.05	5.46	0.48
Residue	8	0.03	0			Residue	8	0.08	0.01		
4 weeks						4 weeks					
Light	1	1.17	1.17	188.60	<.0001	Light	1	0.35	0.35	29.91	0.0006
Temperature	1	1.27	1.27	204.80	<.0001	Temperature	1	0.33	0.33	28.32	0.0007
Light×Temperature	1	0.24	0.24	38.70	0.0003	Light×Temperature	1	0.05	0.05	4.32	0.07
Residue	8	0.05	0			Residue	8	0.09	0.01		
8weeks						8weeks					
Light	1	1.7	1.72	64.51	<.0001	Light	1	1.69	1.69	133.85	<.0001
Temperature	1	0.16	0.16	5.90	0.04	Temperature	1	0.08	0.08	6.25	0.04
Light×Temperature	1	0.07	0.07	2.69	0.14	Light×Temperature	1	0.02	0.02	1.73	0.22
Residue	8	0.21	0.02			Residue	8	0.1	0.01		

Table 4-6. This result shows the relationship between the water depth (cm) and developing stage of seedlings.

The number of living individuals is also shown.

Date	Cotyledon	1~2 foliage leaves	3~4 foliage leaves	5~6 foliage leaves	1~2 floating leaves	Living individuals	Water depth(cm)
14-Jan	0	0	0	0	0	0	31
11-Feb	0	0	0	0	0	0	35
11-Mar	0	0	0	0	0	0	33
9-Apr	0	0	0	0	0	0	34
23-Apr	0	0	0	0	0	0	37
8-May	0	0	0	0	0	0	38
21-May	0	0	0	0	0	0	31
2-Jun	0	4	0	0	0	4	10*
20-Jun	16	4	0	0	0	20	0
2-Jul	93	81	1	0	0	175	0
16-Jul	0	135	115	2	0	252	13
30-Jul**	20	13	265	1	5	304	7
8-Aug	3	42	147	0	1	193	1
20-Aug	0	46	15	0	0	61	0
3-Sep	0	45	2	0	0	47	0
17-Sep	0	33	6	1	0	40	0
1-Oct	0	9	20	1	0	30	0
15-Oct	0	4	20	1	0	25	0
28-Oct	0	2	22	1	0	25	0
12-Nov	0	1	19	1	0	21	0

* The water depth at which seedlings were intially observed.

**The date when individuals with floating leaves were initially observed.

●—● The period required for the establishment of seedlings.

↔ Drought occurred.

Table 5-1. This result indicates that the change of the number of rare morph, frequency of rare morph, bias of floral morph composition calculated by $|L-S| / (L+S)$ and the sampling number in each population for six years. The values of initial investigated year (0), 4 years later (4) and 6 years later (6) are shown.

Population	N of rare morph(0)	N of rare morph(4)	N of rare morph(6)	Frequency(0)	Frequency(4)	Frequency(6)	Bias(0)	Bias(4)	Bias(6)	Sampling N (0)	Sampling N (4)	Sampling N (6)
Monomorphic**												
HF	0	0	0	0	0	0	1.00	1.00	1.00	30	52	69
HH	0	0	0	0	0	0	1.00	1.00	1.00	30	15	5
HJ	0	0	0	0	0	0	1.00	1.00	1.00	35	102	100
HM	0	0	0	0	0	0	1.00	1.00	1.00	81	75	52
HN	0	0	2	0	0	0.02	1.00	1.00	0.98	56	58	102
HP	0	0	0	0	0	0	1.00	1.00	1.00	30	72	90
HQ	0	0	0	0	0	0	1.00	1.00	1.00	30	32	80
HR ^{b)}	0	0	-	0	0	-	1.00	1.00	-	30	58	0
HS ^{b)}	0	-	-	0	-	-	1.00	-	-	30	0	0
Biased floral morph ratio												
HA	4	32	25	0.05	0.18	0.14	0.89	0.65	0.71	76	185	174
HB	5	2	3	0.09	0.03	0.03	0.83	0.95	0.94	58	82	95
HC ^{*a)}	4	0	0	0.07	0	0	0.86	1.00	1.00	59	100	3
HD*	4	2	0	0.07	0.03	0	0.87	0.97	1.00	60	70	69
HE*	2	0	0	0.03	0	0	0.94	1.00	1.00	63	70	72
HK*	8	3	0	0.11	0.03	0	0.77	0.94	1.00	71	106	43
HL	10	21	17	0.32	0.18	0.14	0.35	0.65	0.72	31	120	120
HU	10	15	18	0.22	0.11	0.13	0.57	0.77	0.74	46	131	138
HW ^{*a)}	5	0	0	0.17	0	0	0.67	1.00	1.00	30	50	5
Equal floral morph ratio												
HG	57	52	48	0.48	0.46	0.41	0.03	0.09	0.17	118	114	116
HI	28	49	55	0.46	0.45	0.4	0.08	0.10	0.20	61	104	138
HT ^{b)}	53	-	-	0.42	-	-	0.15	-	-	125	0	0

** The floral morph composition based on *G*-test in 1997.

* Rare morph was lost.

^{a)} Population size remarkably decreased.

^{b)} Extinction

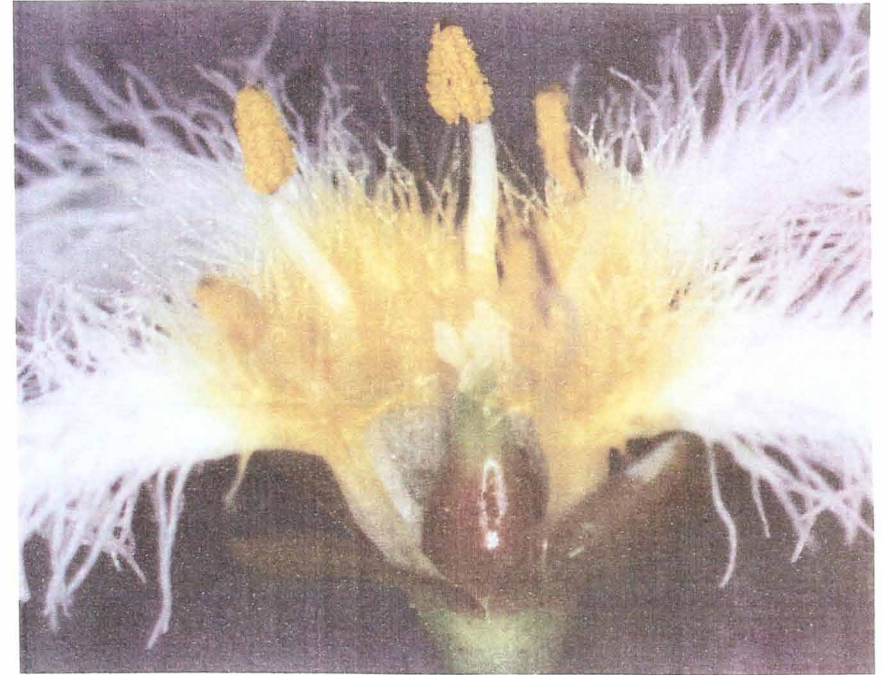
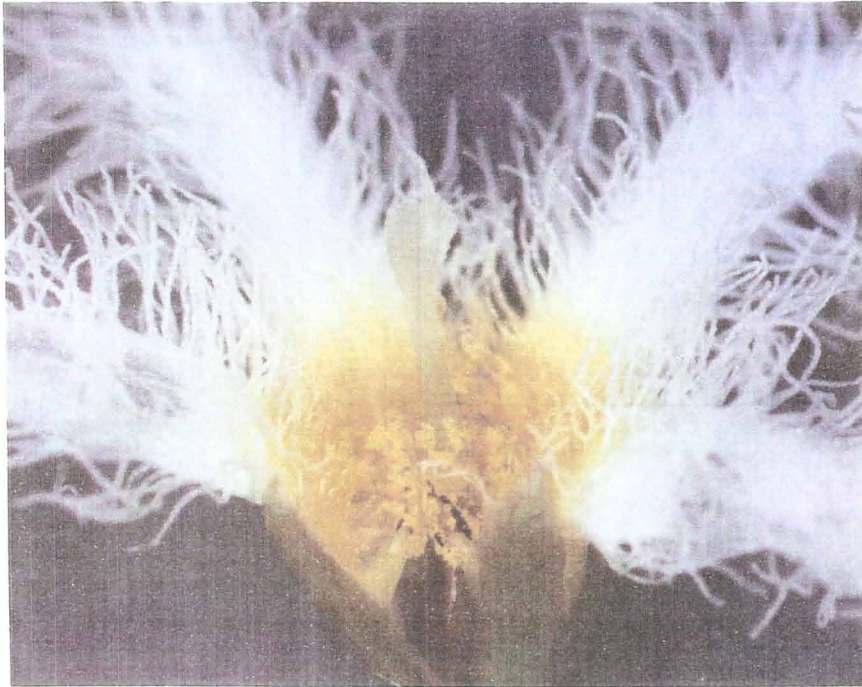


Fig. 1-1. Long - styled (left) and short-styled morph (right).

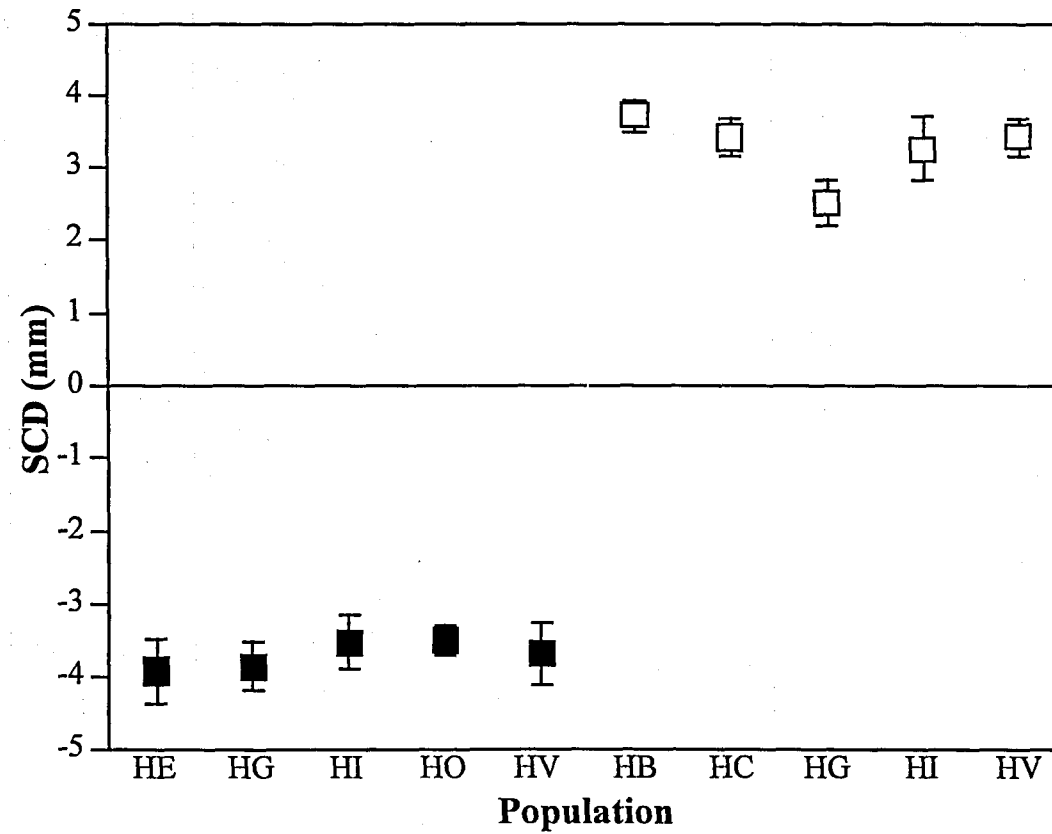
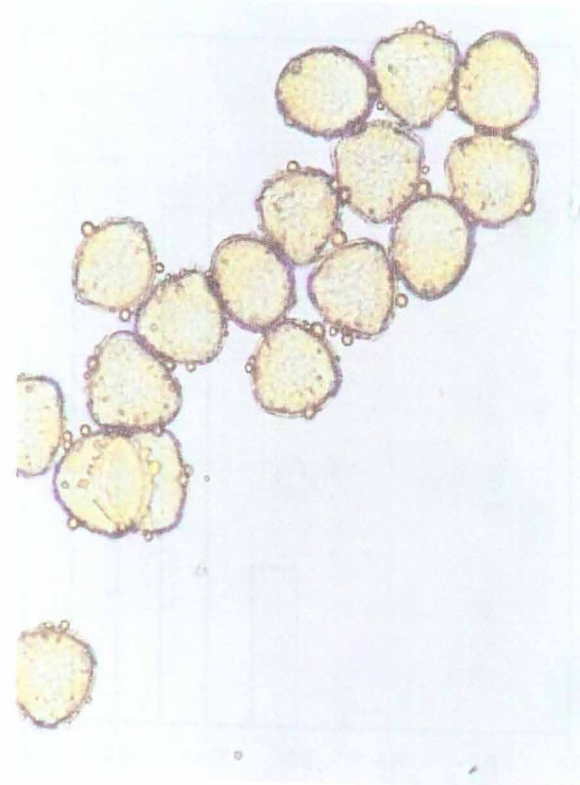


Fig.1-2. Staminate –carpellate distance (SCD) of long-styled (closed) and short-styled (open).Morphs collected from seven populations.The mean and SD are shown. For population Codes, see Table 1-1.



× 400

Fig.1-3. Pollens of short -styled (left) and long-styled morph (right).

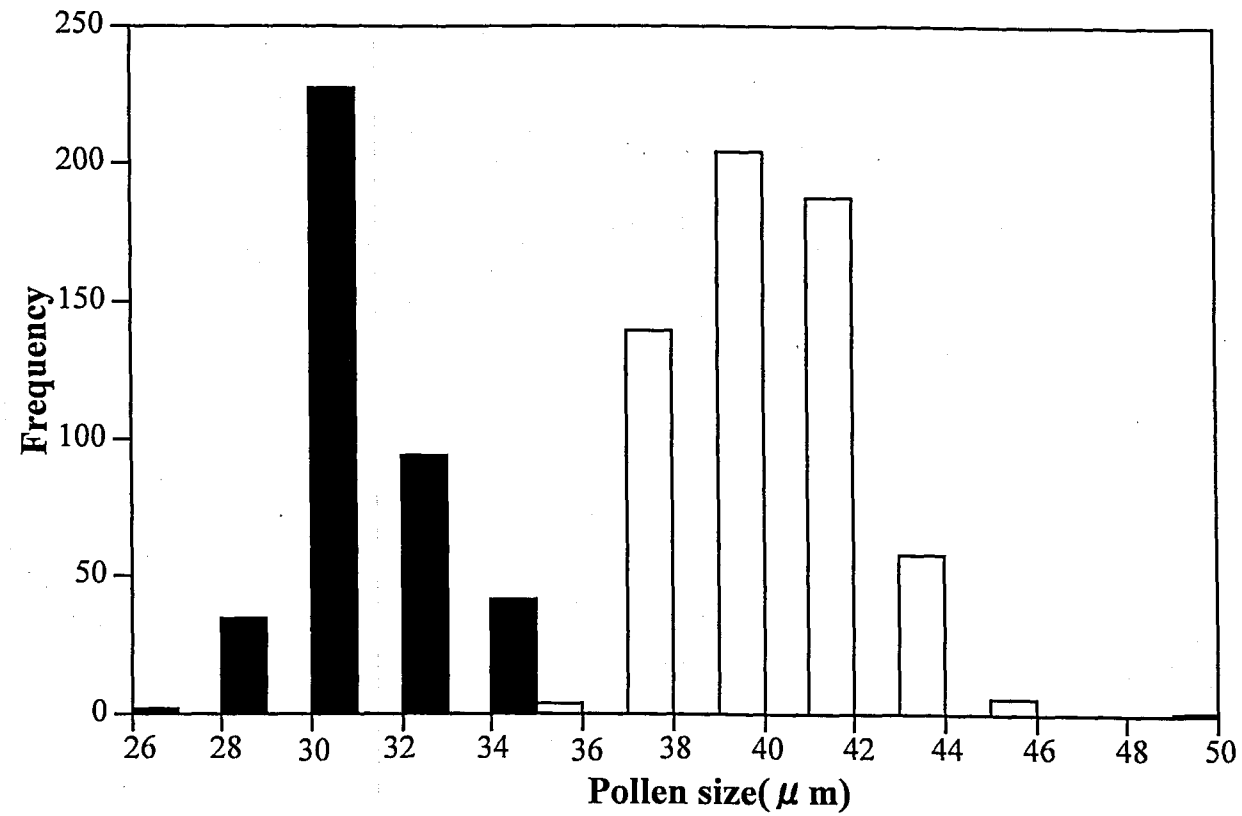


Fig. 1-4. Comparison of pollen size between long-styled (closed) and short-styled (open) flowers.

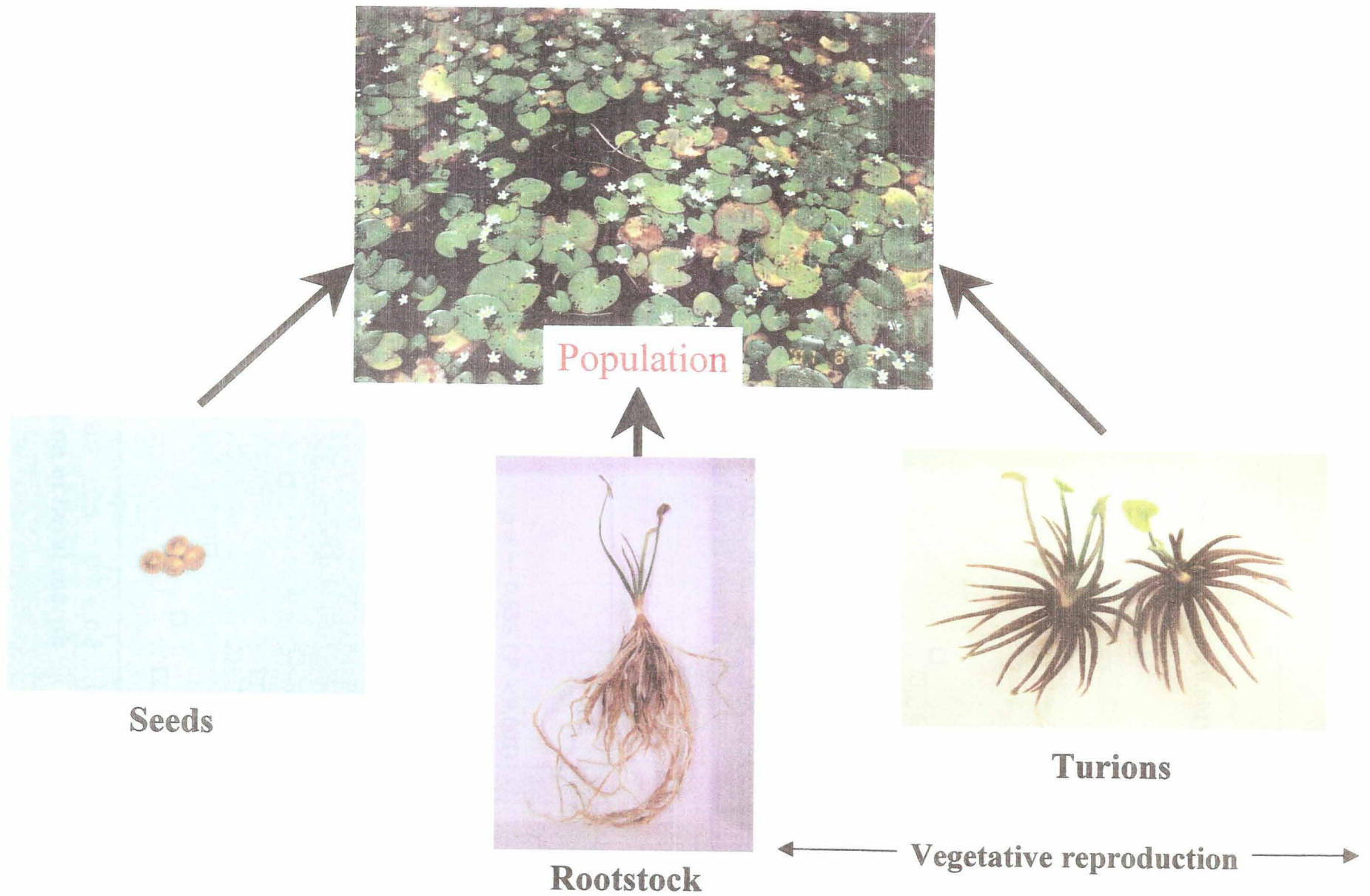


Fig. 2-1. Reproductive means in *N. indica*.

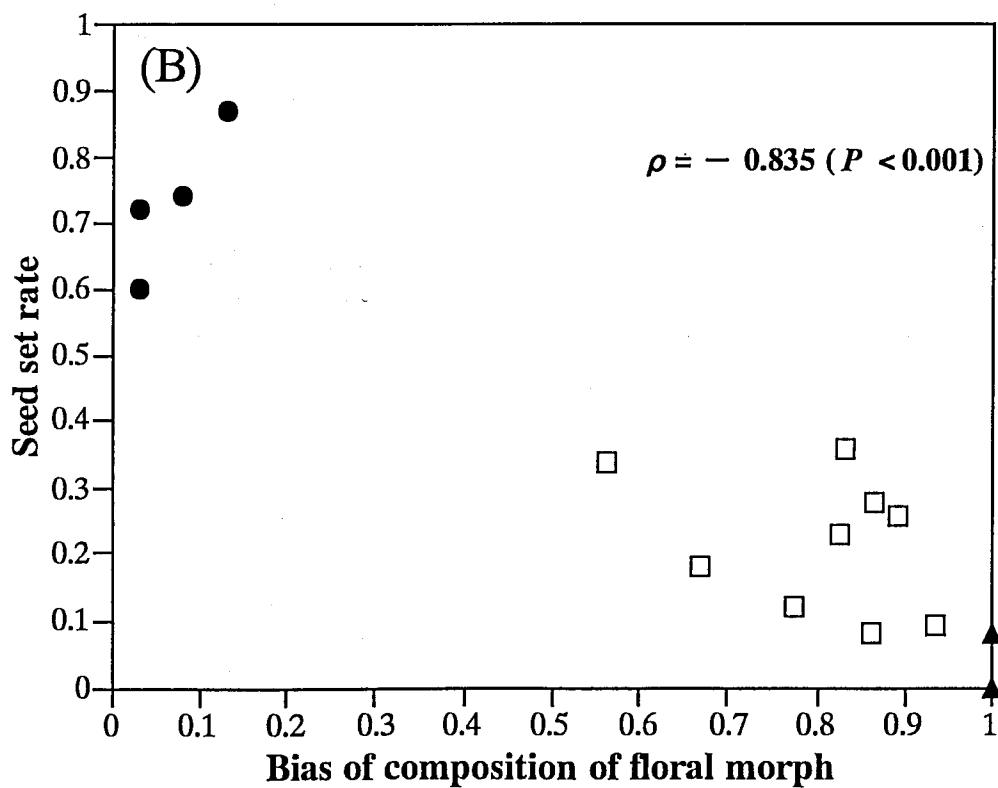
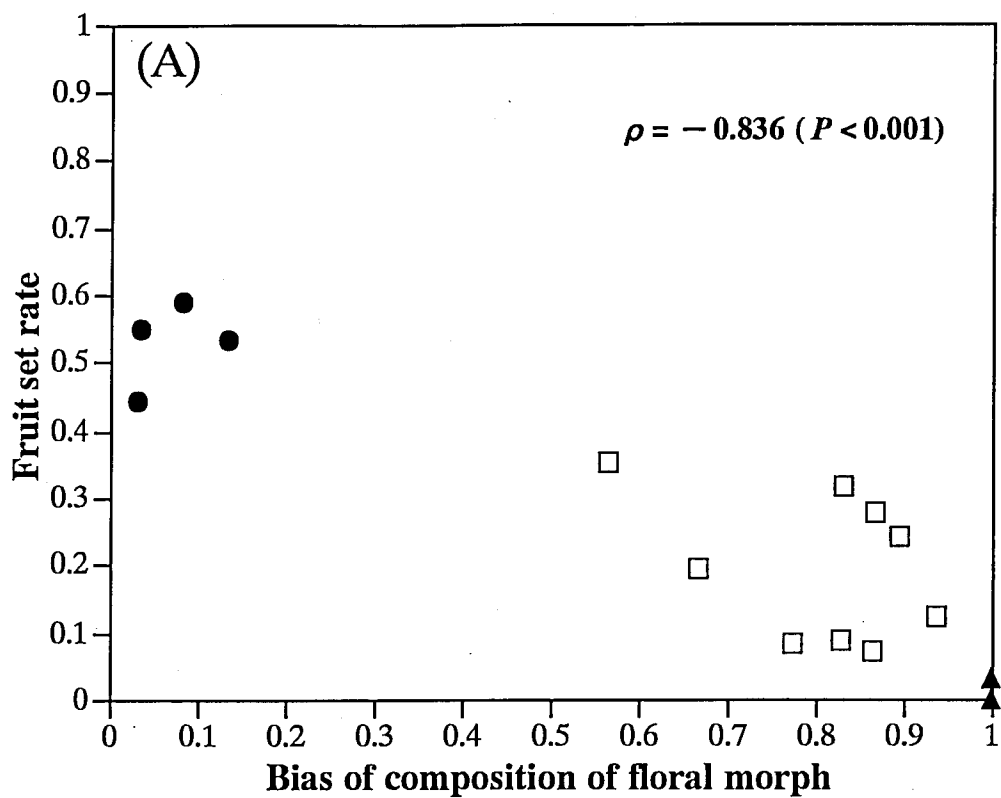


Fig. 2-2. Spearman's rank correlation between the bias of composition of floral morph and fruit set (A) and seed set rate (B) in the populations of *N.indica*. (●): populations with equal ratio ; (□): populations with biased ratio; (▲): monomorphic.

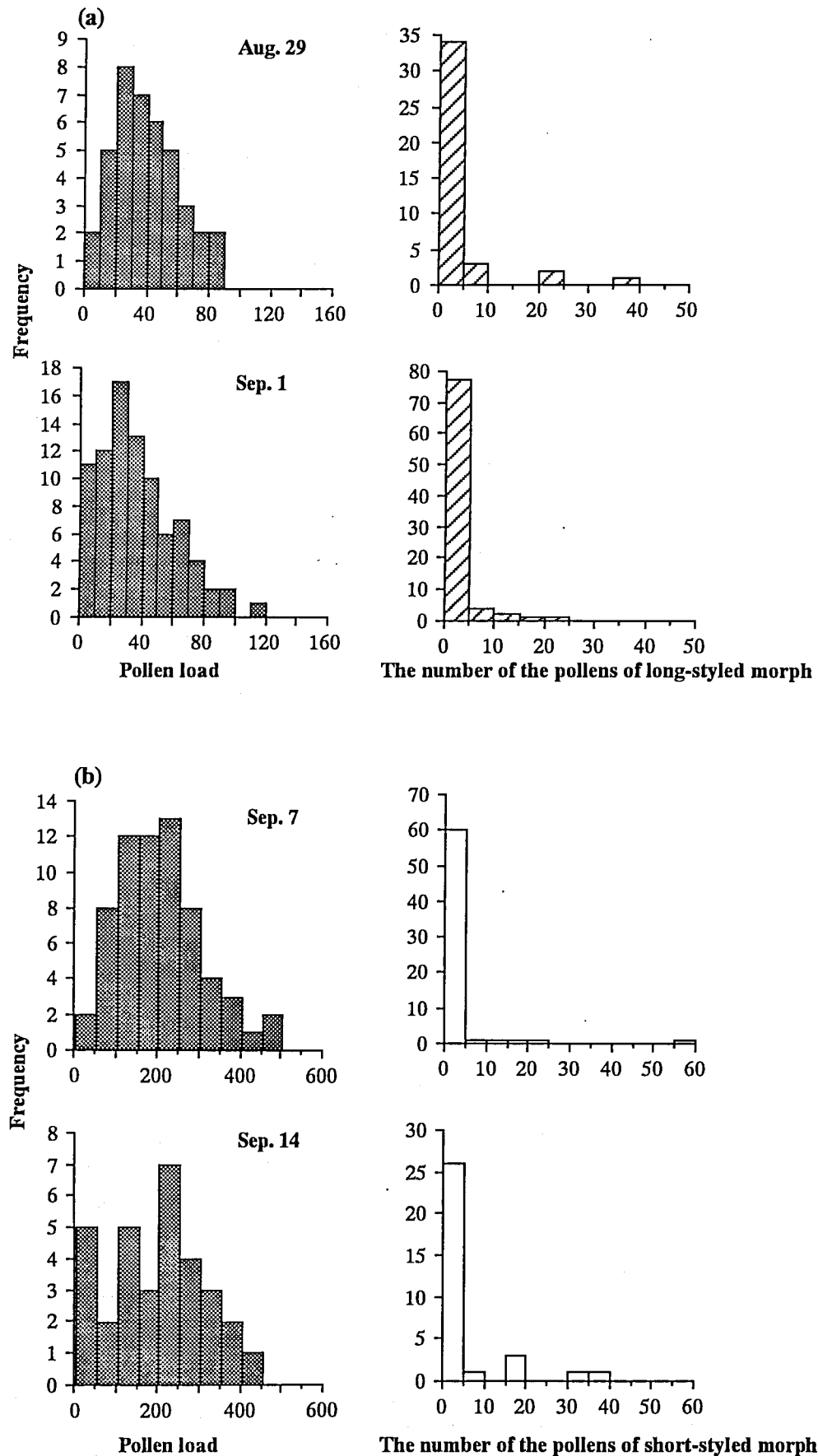


Fig. 2-3. Frequency of total pollen load on all the stigmas (left) and the number of the pollens of opposite floral morph (right) on the stigmas of common morph in two populations observed on two days. (a) HC; (b) HD

S0.74	S0.44	L0.73	S*
S0.43			
	S0	S0 S0.45 S0	<u>L0.73</u>
		S0.46	
S0 S0	S0 S0.46	S0.76	
	S0.05 S0		
S0 S0		S0.20	S0.42
	S0 S0.39		
	S0.15		
S0.36	S0.3	<u>L0.16</u>	
		<u>L0.90</u>	
			S0.44
		S0.33	
		S0.22	S0.20

S0
 S0.3 S0 S0.82
 S0 S0.29
 S0
 S0
 S0 S0 S0
 S0 S0
 S0.23
 S0 S0.35
 S0 S0 S0 S0
 S0 S0
 S0 S0
 S0 S0

[illegible]

\$0					
\$0		\$0			
\$0.38				\$0	\$0
\$0		\$0		\$0	\$0
		\$0			\$0
		\$0			
			\$0		
\$0.5	\$0 \$0 \$0				
\$*	\$0	\$*	\$*	\$0	
\$0			\$0	\$0	
	\$0			\$0	
	\$*				
\$0			\$0		

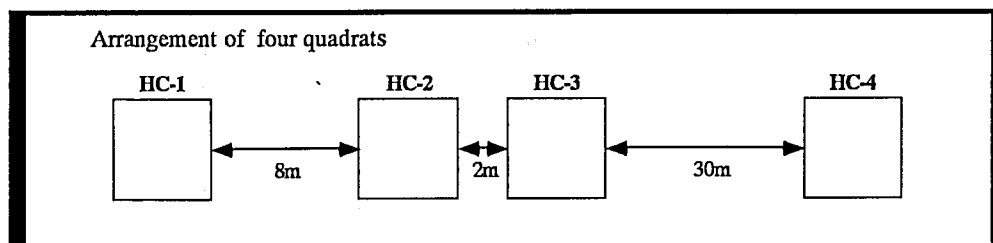


Fig. 2-4 (a). Positions of each floral morph and its intermorph pollination rate in each quadrat (1m × 1m) in HC. Arrangement of each quadrat is shown schematically below. L: long-styled; S: short-styled; Numerals indicate the intermorph pollination rate of each flower. *: data unavailable.

L0
L0.17 L0.01 L0.15 L* L*
L0.27 L* L0.27 L0.22
L0.04 L* L0.24 L0.27
L0.18 L0.06
L0.23 L0.56 S0.76
S0.88
L0.27 L0.08 S0.81
L* L0.63
L0.05 L0.14 L0.07
L0 S0.94 L0.20
L0 L0.19
L0.09
L0 S0.64 L0.14
L0

[illegible]

L0	L0.01	L*	L0.04	L0
	L0		L0 L0	L*
	L*	L0		L*
L0				
L0 L0.02	L0			
	L0			
	L0	L0 L0 L0 L0		
L0.01 L*		L0		L0
	L0			L0
L0	L0		L0 L0	
	L0		L0 L0	
L0				L0
L0				L* L0
L0				
				L0

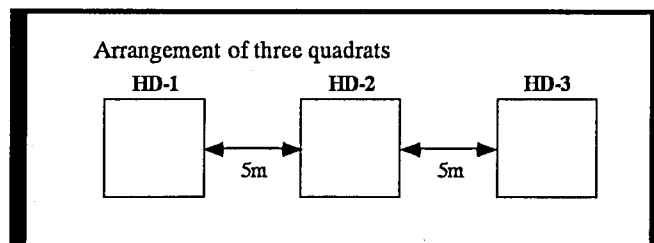


Fig. 2-4 (b). (continued) The same in HD

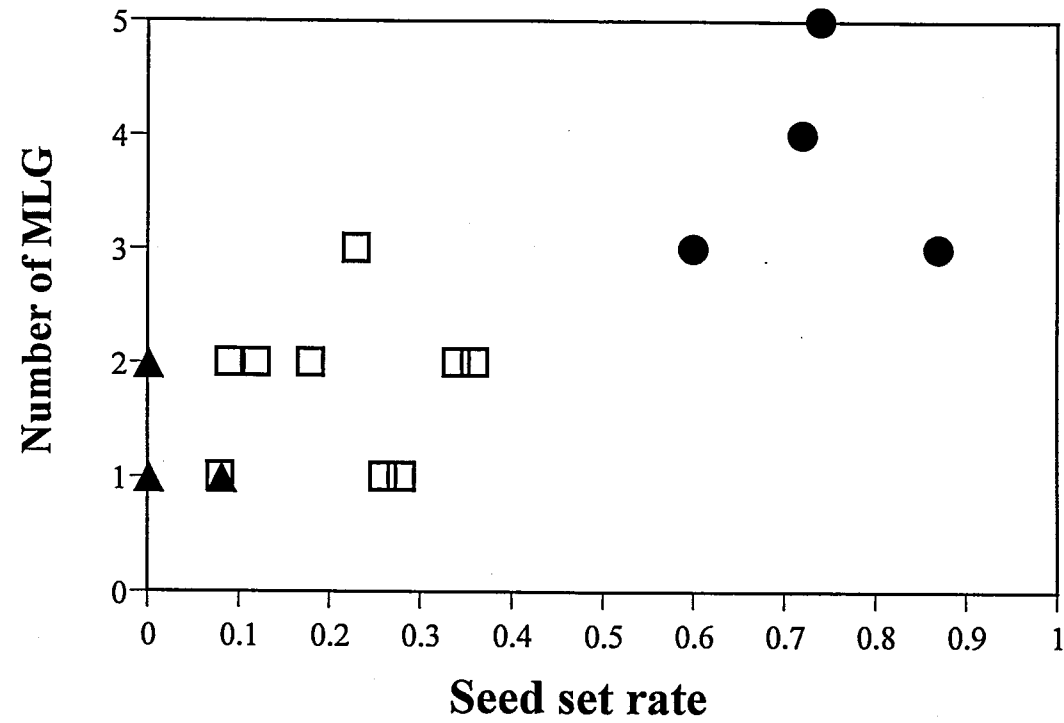


Fig. 3-1. The correlation between the seed set rate and number of MLG.
 (●): Equal floral morph ratio; (□): Biased floral morph ratio; (▲): Monomorphic.

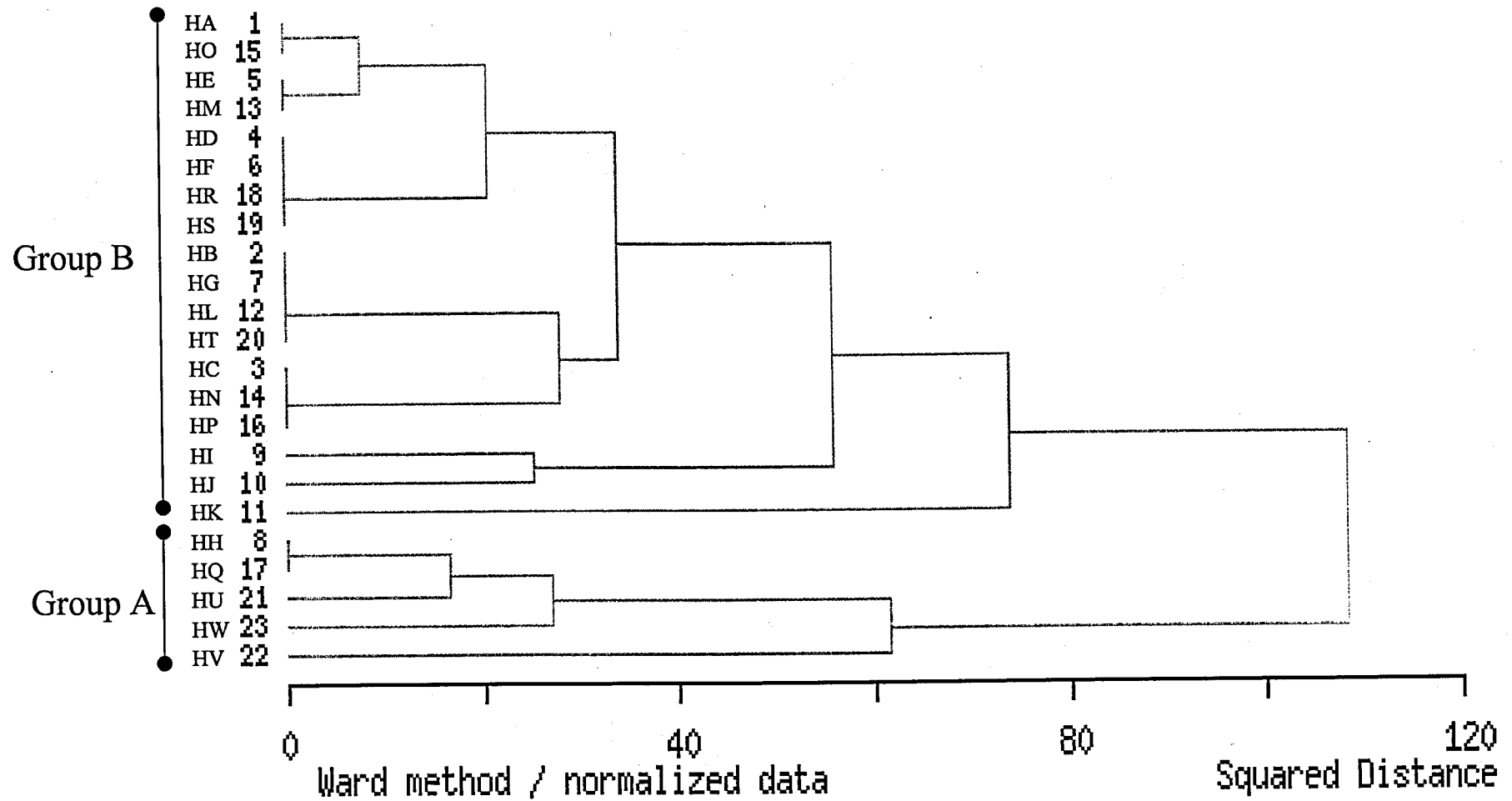
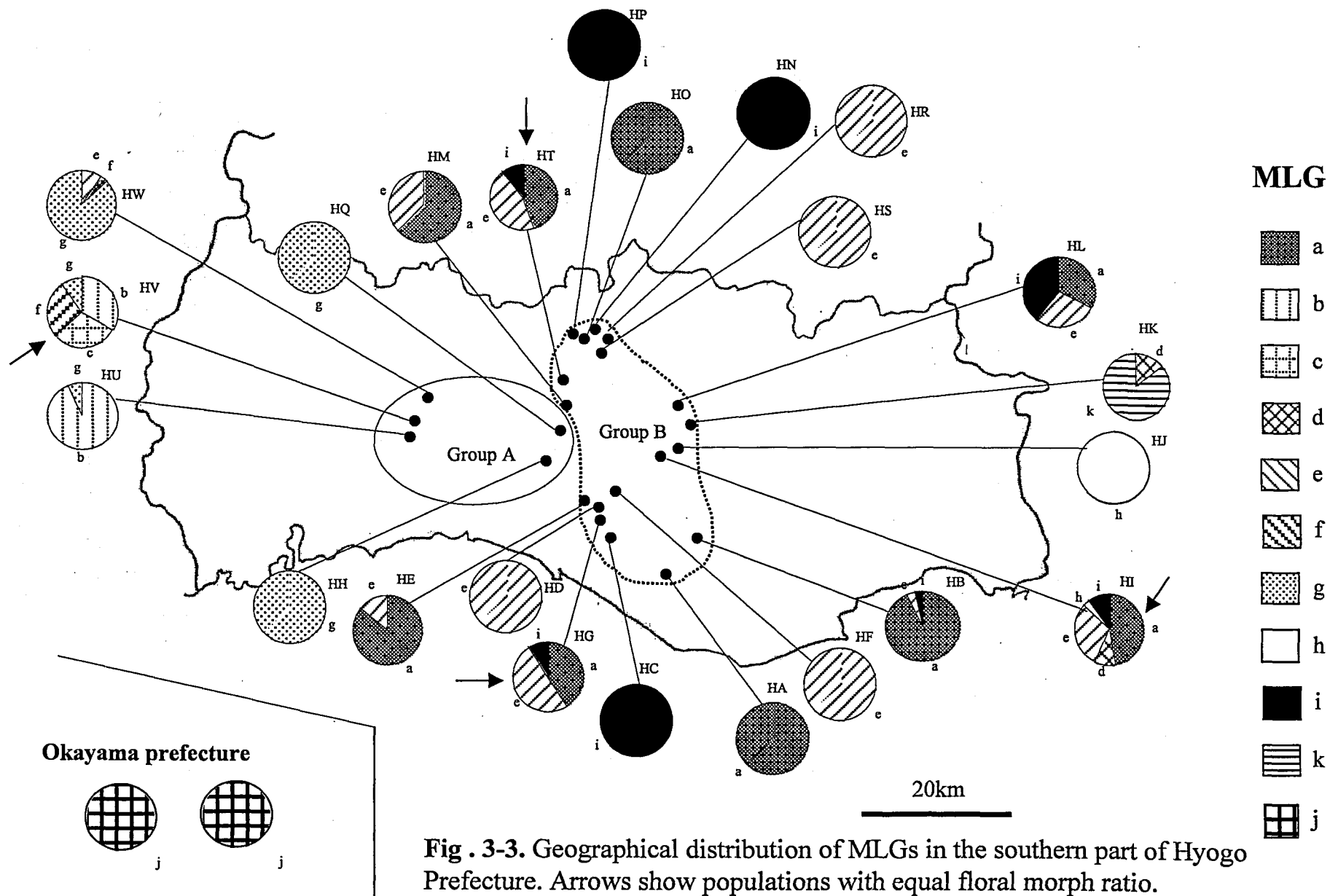


Fig.3-2. Dendrogram obtained from Ward method based on standardized squared Euclidean distance.



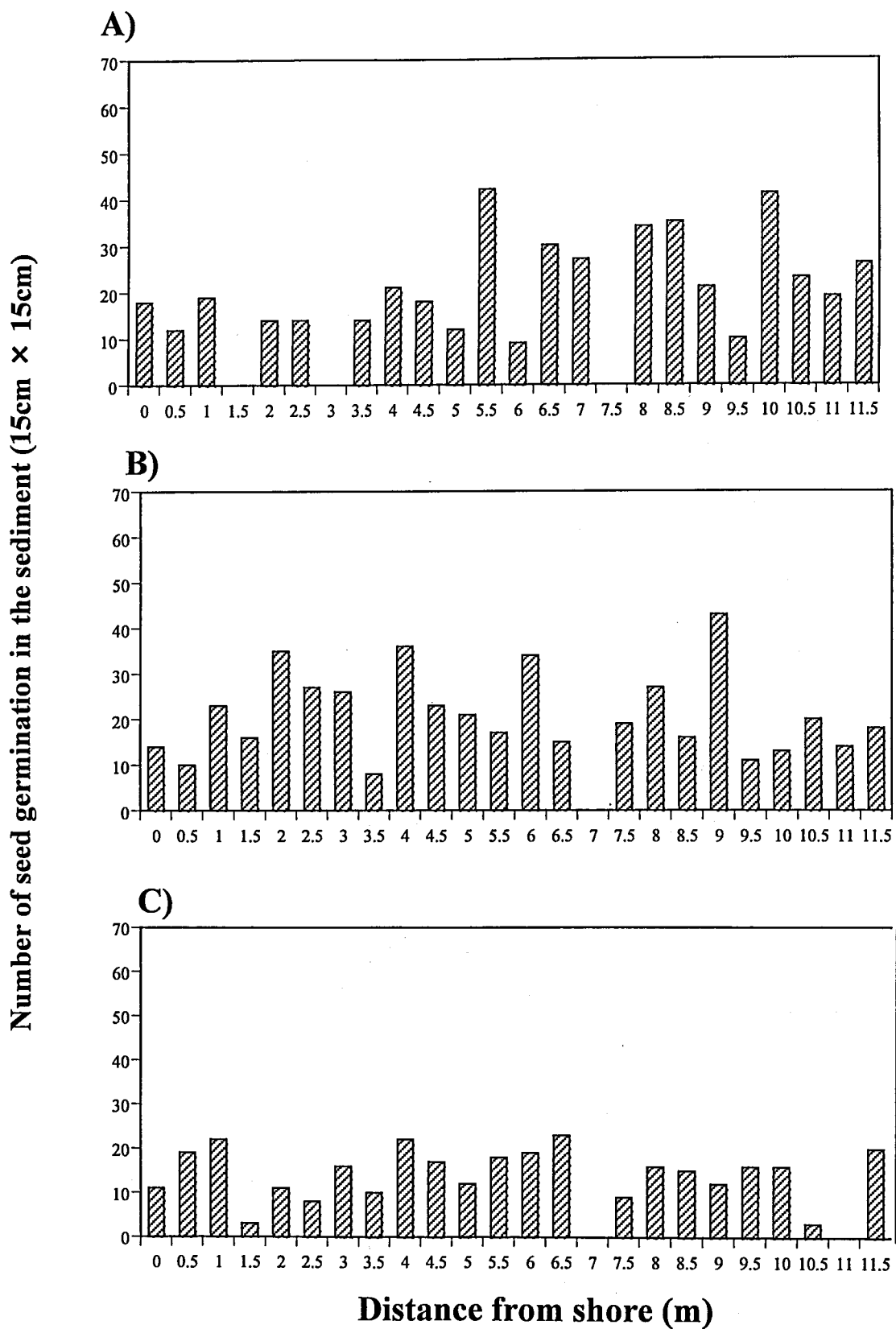
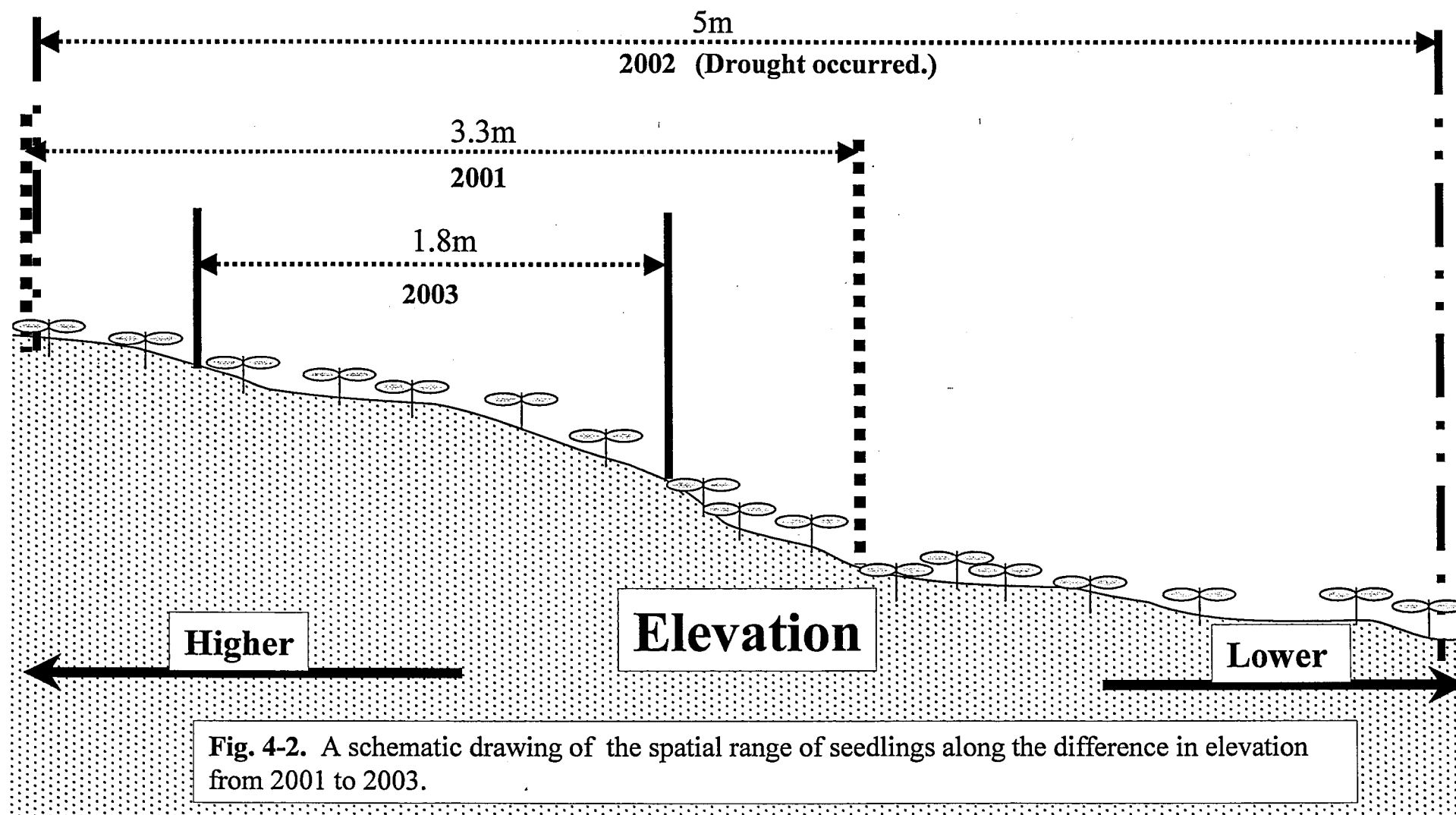


Fig. 4-1. Spatial distribution of dispersed seeds in three transects. Shaded bar: the number of seed germination.



Number of cumulative seed germination and surviving seedlings

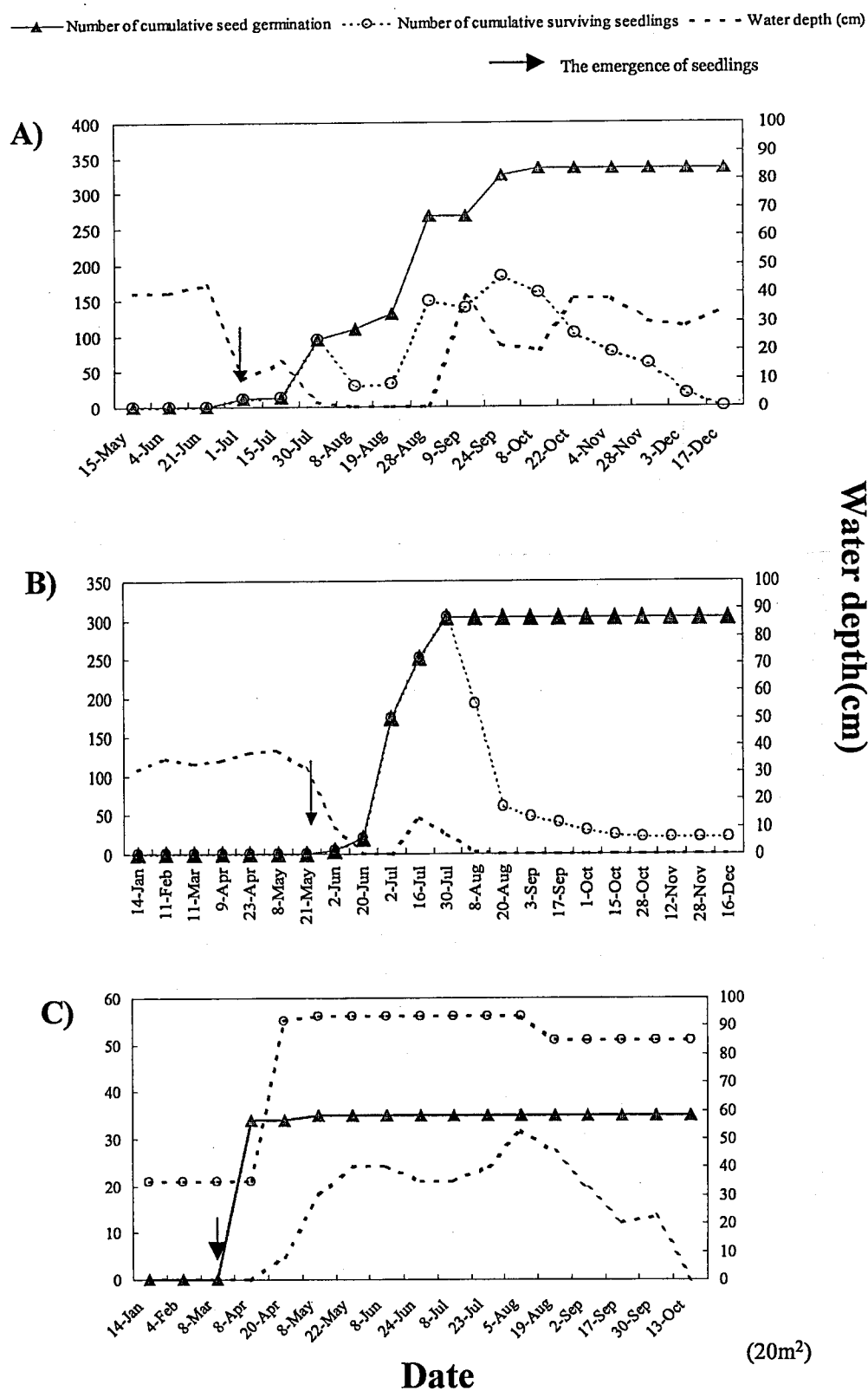


Fig. 4-3. The relationship between the timing of seed germination and water depth (cm). The number of surviving seedlings is also shown. A): 2001; B): 2002; C): 2003.

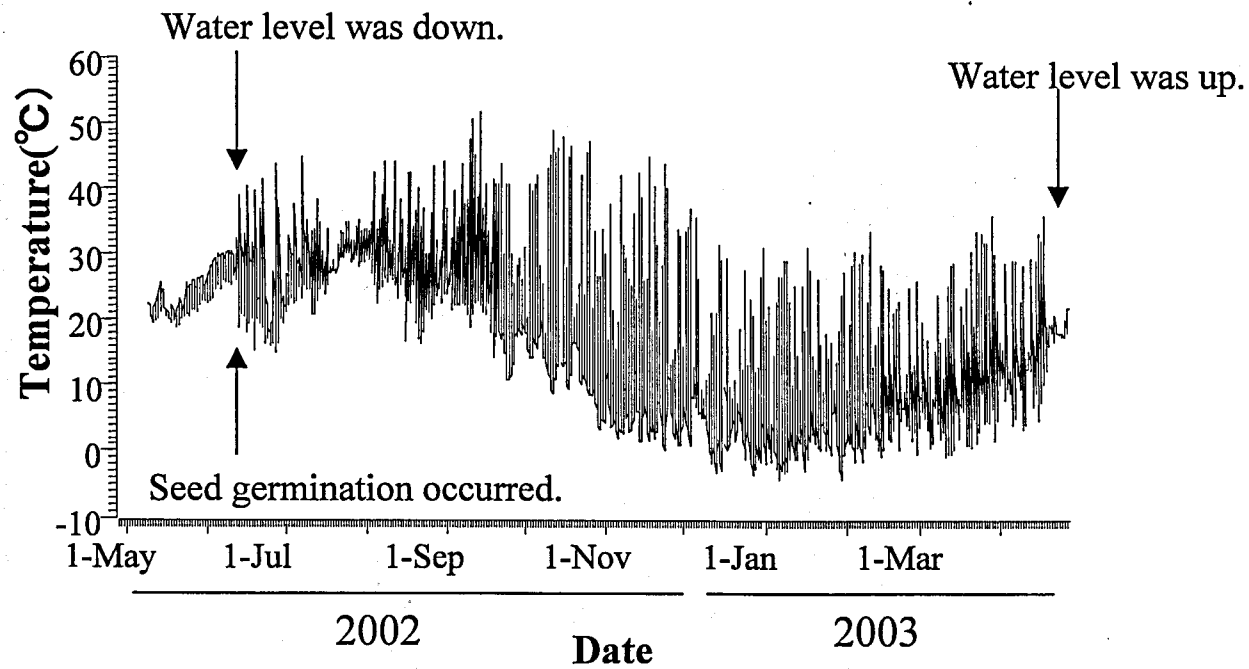


Fig. 4-4. Diurnal change of water temperature at the water margin.

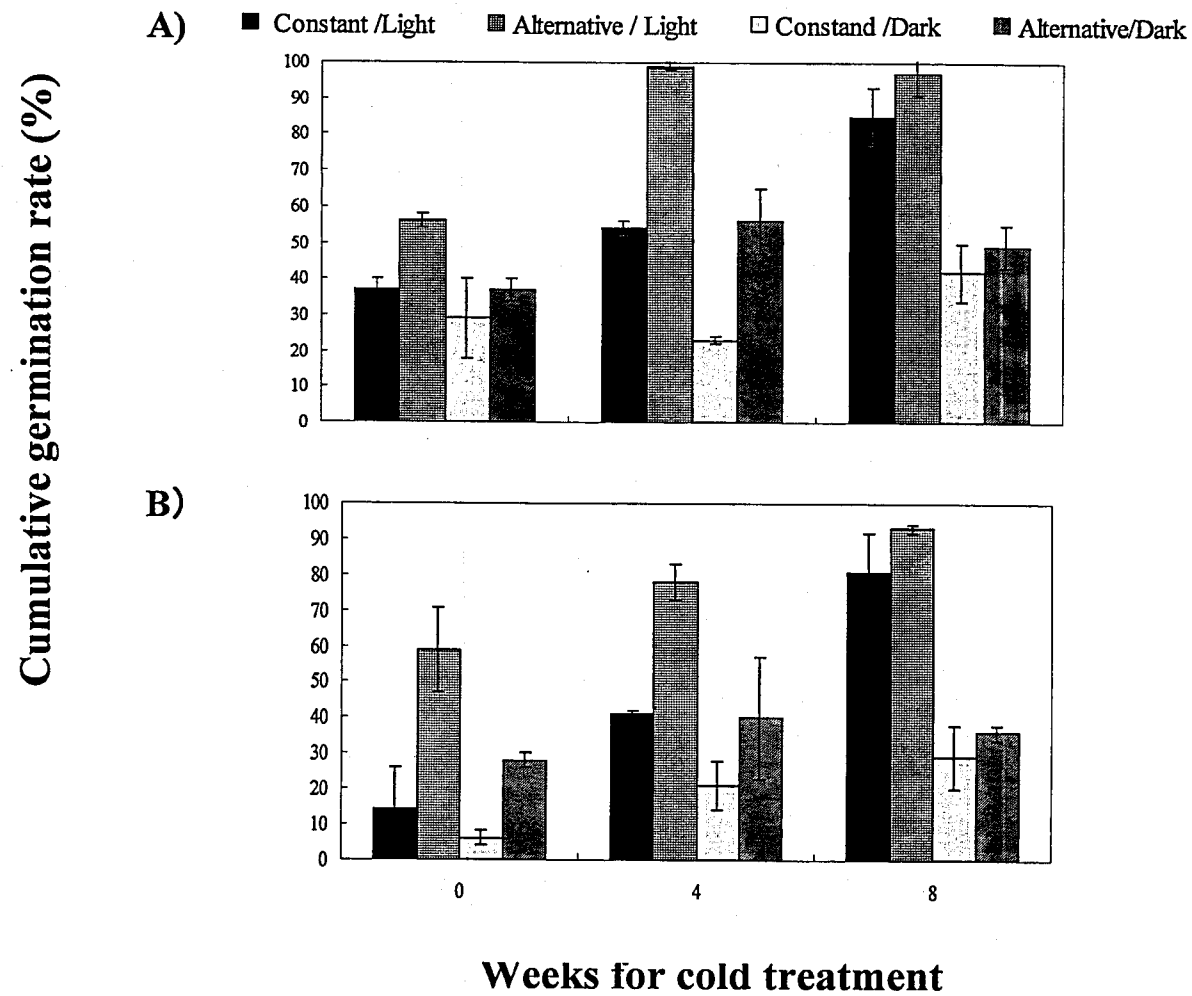


Fig. 4-5. The effect of light (Light / Dark) and temperature (Constant /Alternative) on cumulative germination rate in each cold treatment in two populations. Error bar: SD. A) HG; B) HI.

A)



C)



B)

Fig. 4-6. An example showing the process of successful establishment of seedlings.
A) Cotyledon stage ; B) Foliage leaf- stage; C) Successful establishment.

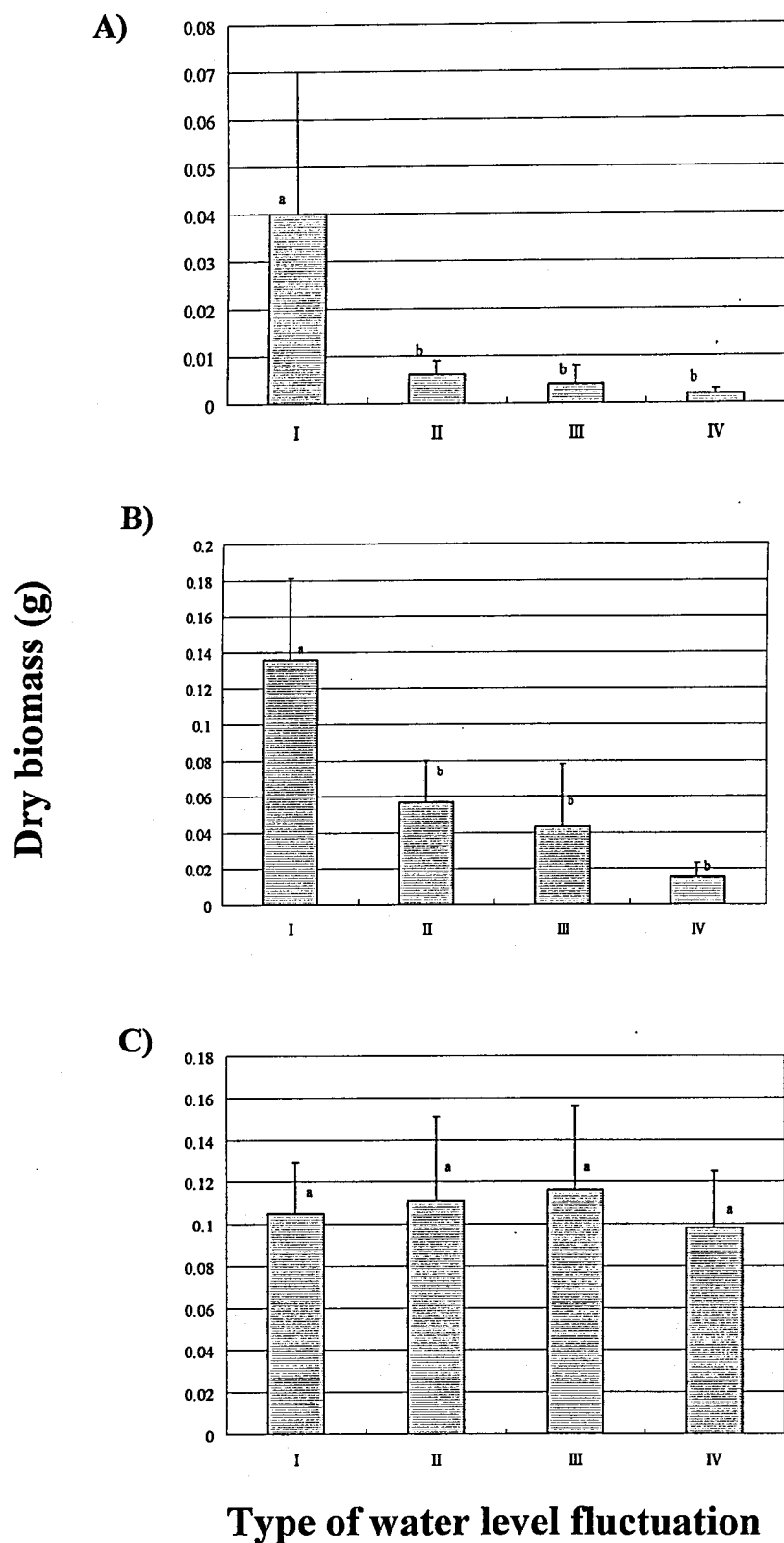


Fig .4-7(a). Effect of water level fluctuation on dry biomass of three developing stage in seedlings. A) Cotyledon stage; B) 4~5 foliage leaved stage; C) 7~8 foliage leaved with petiolate elongated leaves. Alphabets indicate statistical significance. Error bar: SD.

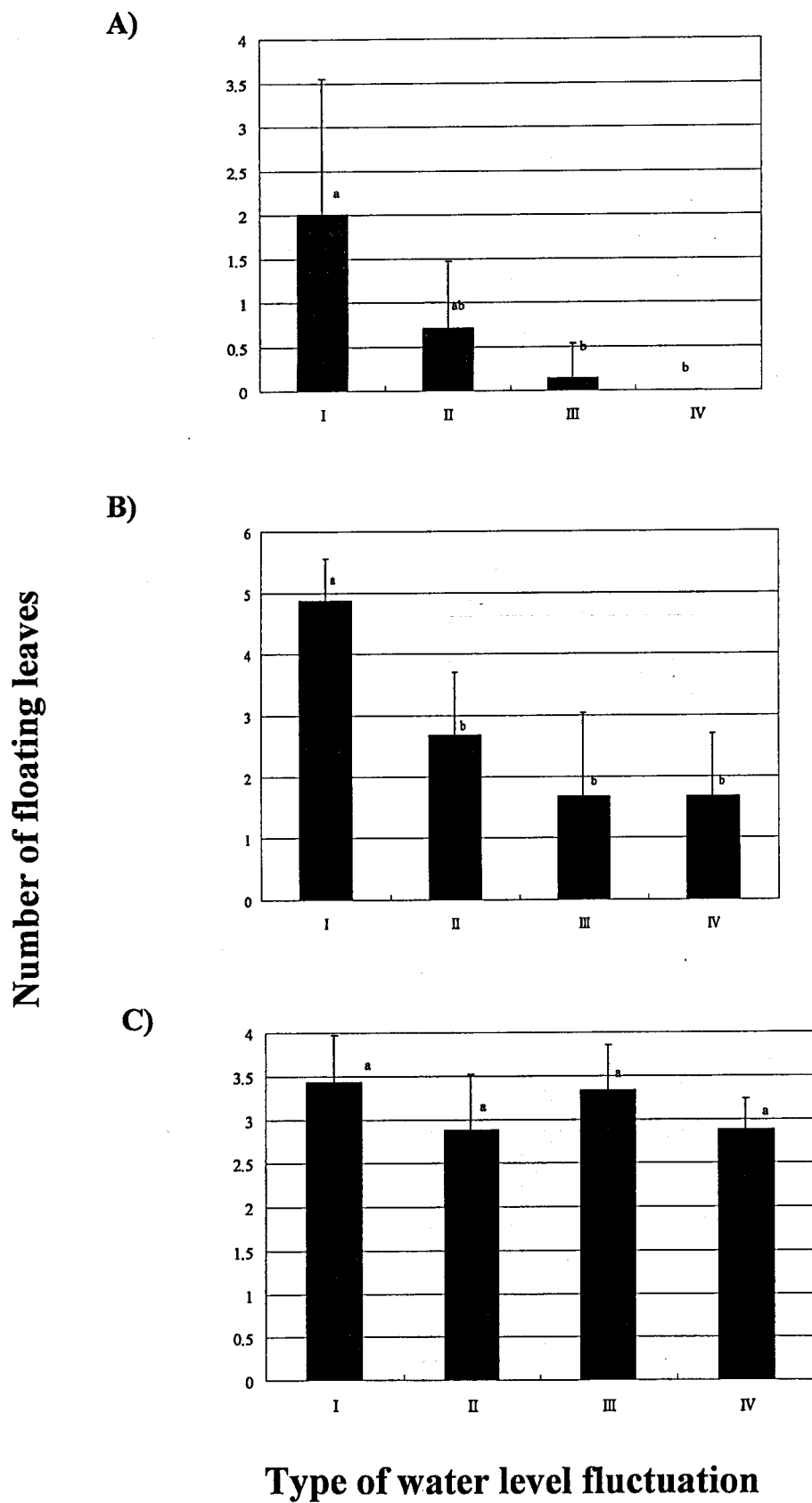


Fig .4-7(b). Effect of water level fluctuation on number of floating leaves of three developing stage in seedlings. A) Cotyledon stage; B) 4~5 foliage leaved stage; C) 7~8 foliage leaved with petiolate elongated leaves. Alphabets indicate statistical significance. Error bar: SD.

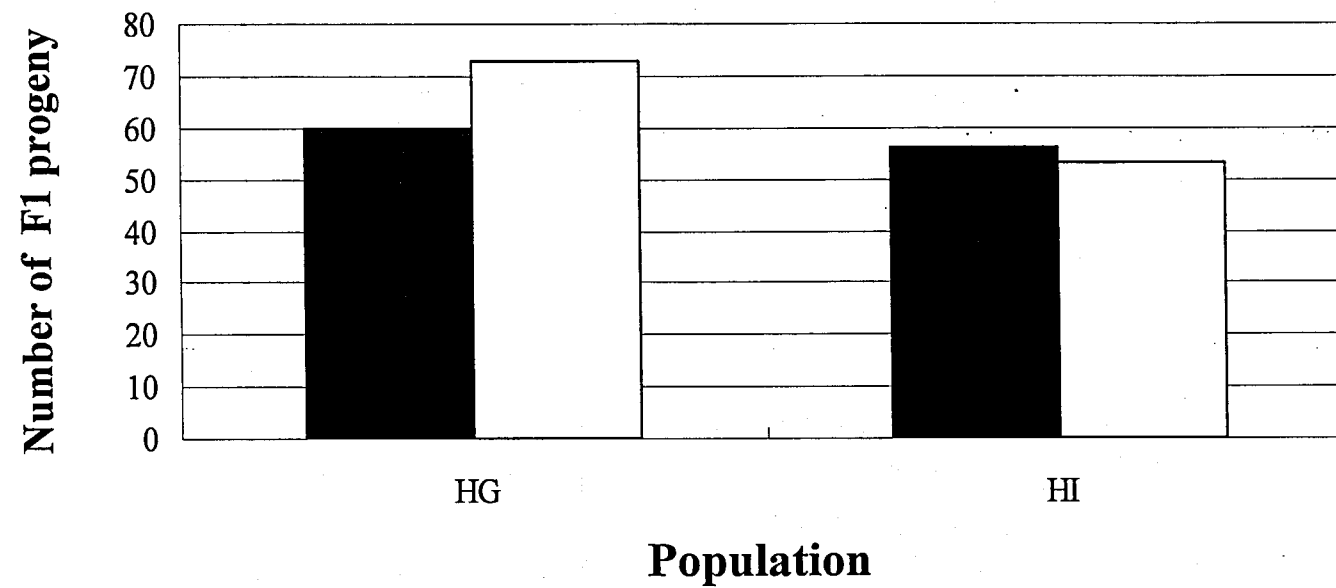


Fig. 5-1. The floral morph ratio of F1 progeny originated from HG and HI.
(■): Long-styled morph; (□): Short-styled morph.

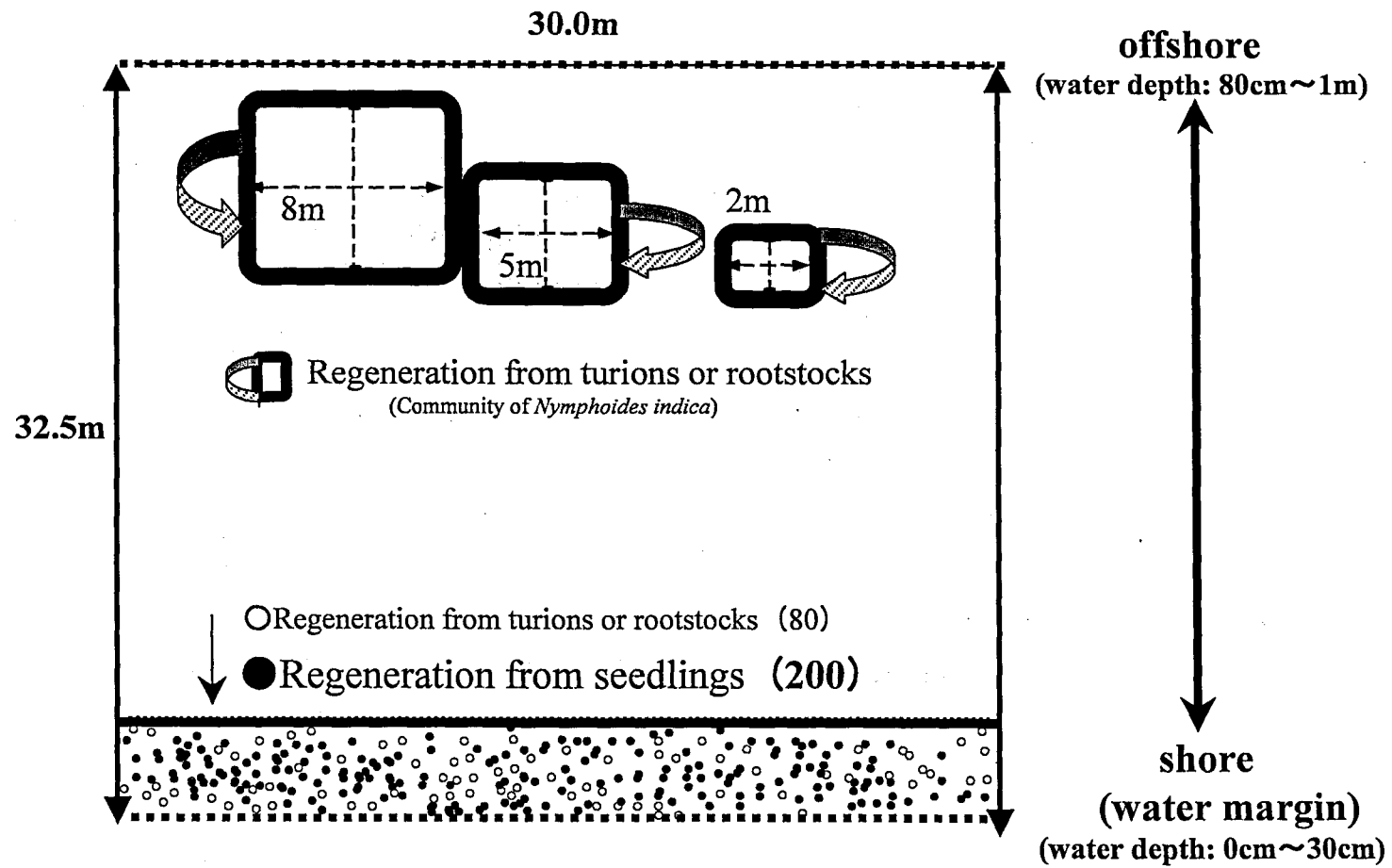


Fig. 5-2. Spatial distribution of the establishment of seedlings and regeneration from rootstocks or turions in HB-1.

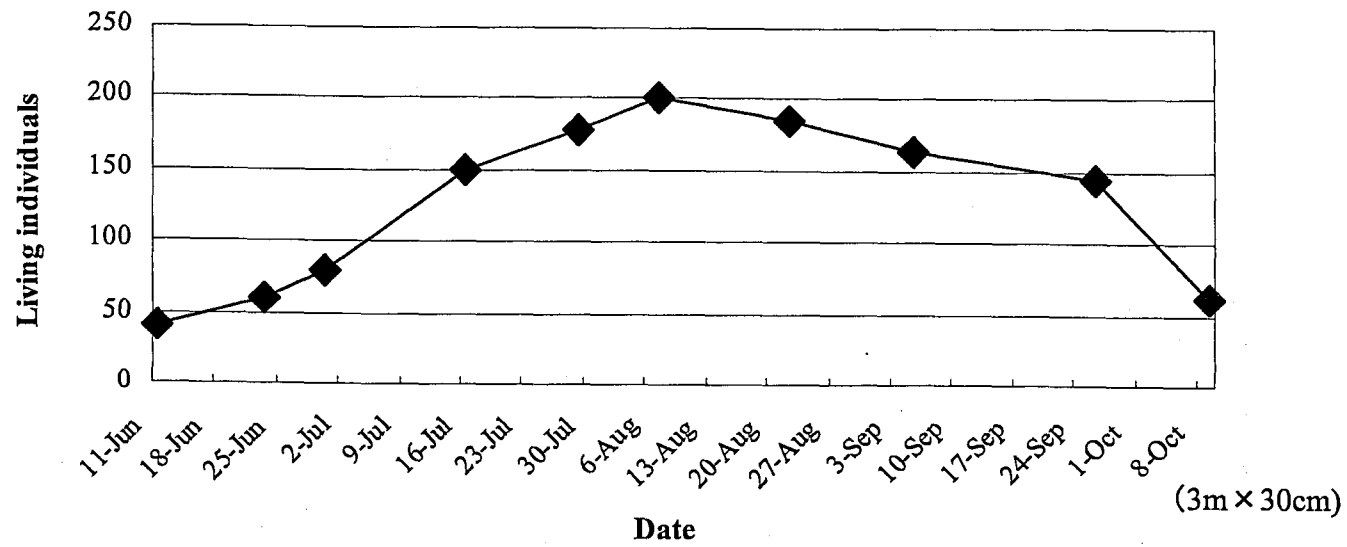


Fig. 5-3. The seasonal change of the number of seedlings in HB-1.

■ Regeneration from rootstocks or turions

□ Cumulative establishment of seedlings

○ Seed germination

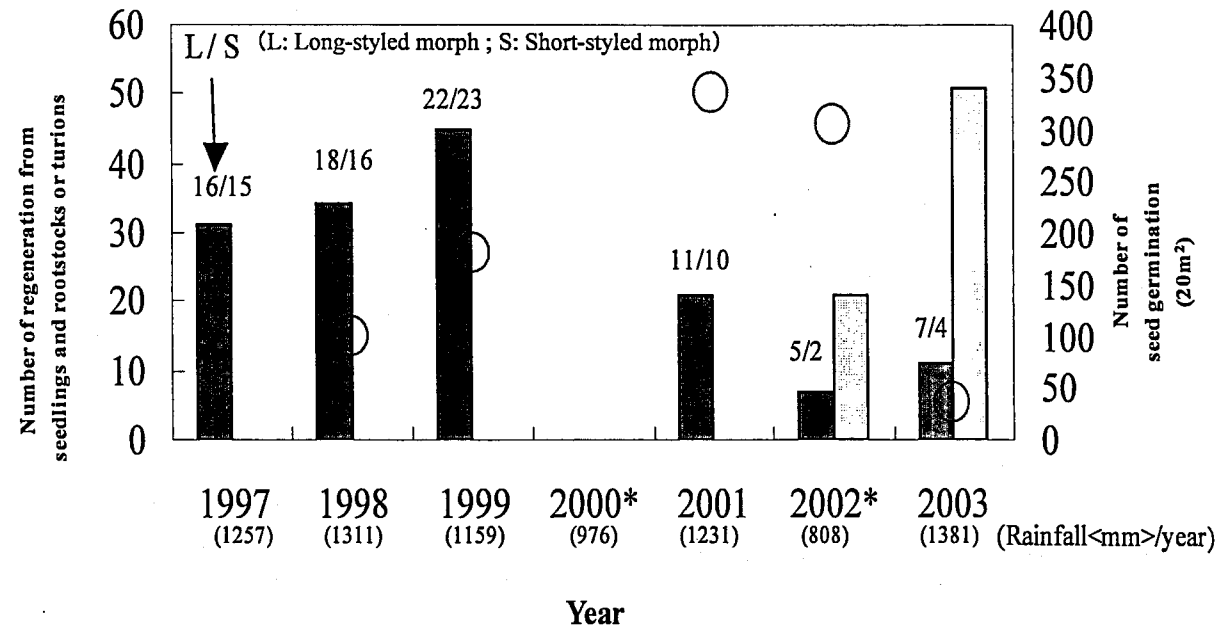


Fig. 5-4. The number of regeneration from vegetative reproduction, establishment of seedlings and seed germination for seven years. Drought was defined as less than 1000mm per year of rainfall. * Drought occurred.

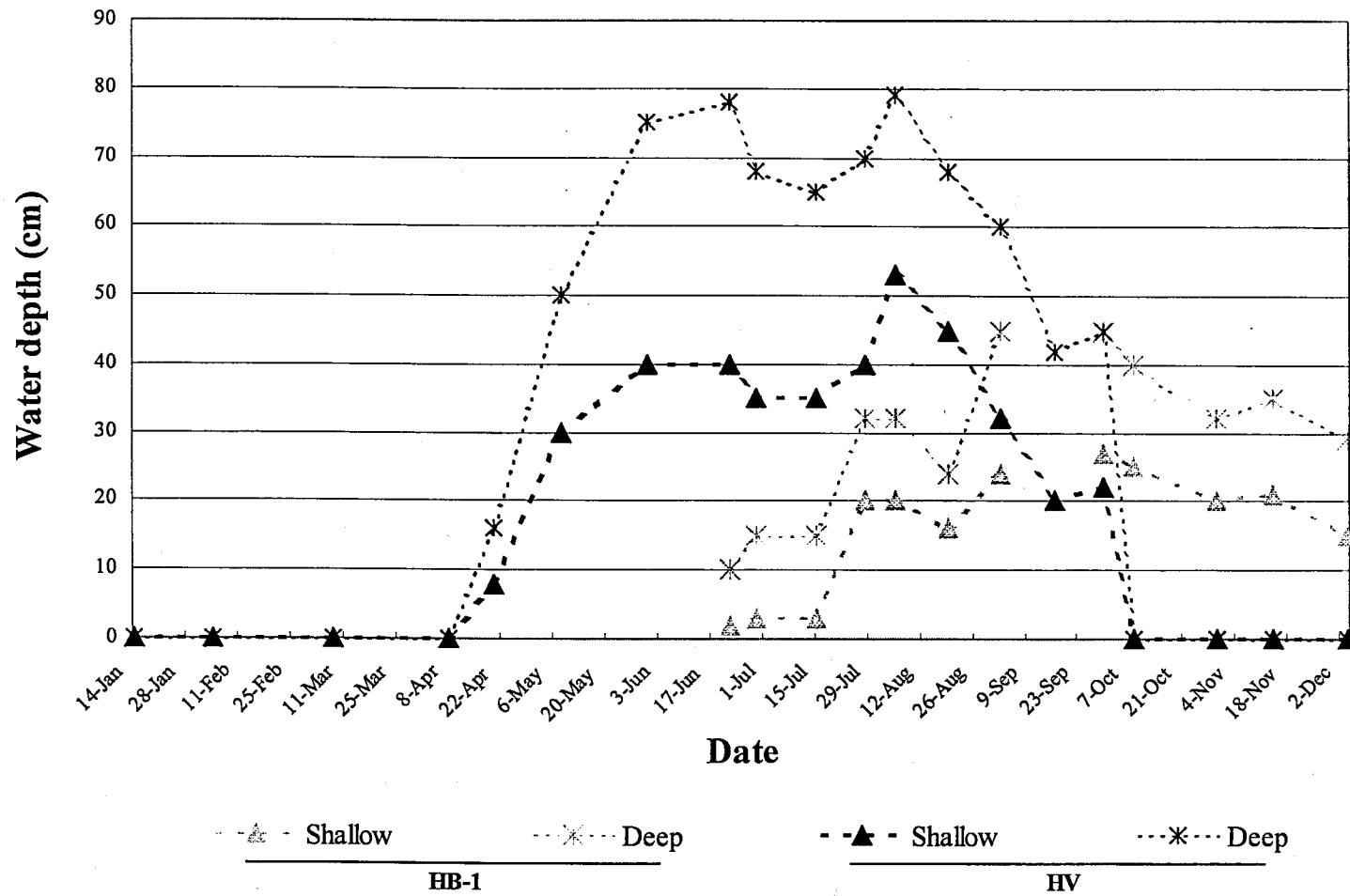


Fig. 5-5. The seasonal change of water depth (cm) between the shallowest and deepest point where seedlings occurred.

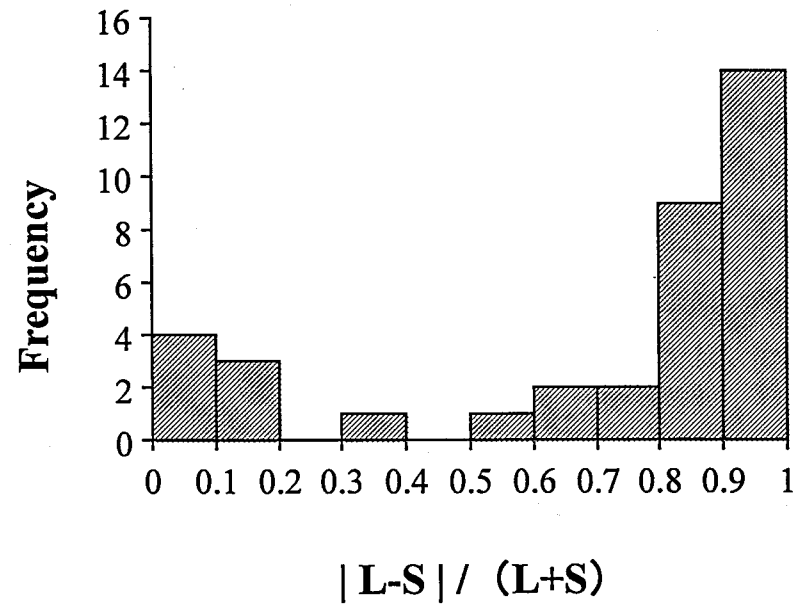


Fig. 5-6. Frequency distribution of the bias of floral morph composition in 36 populations.

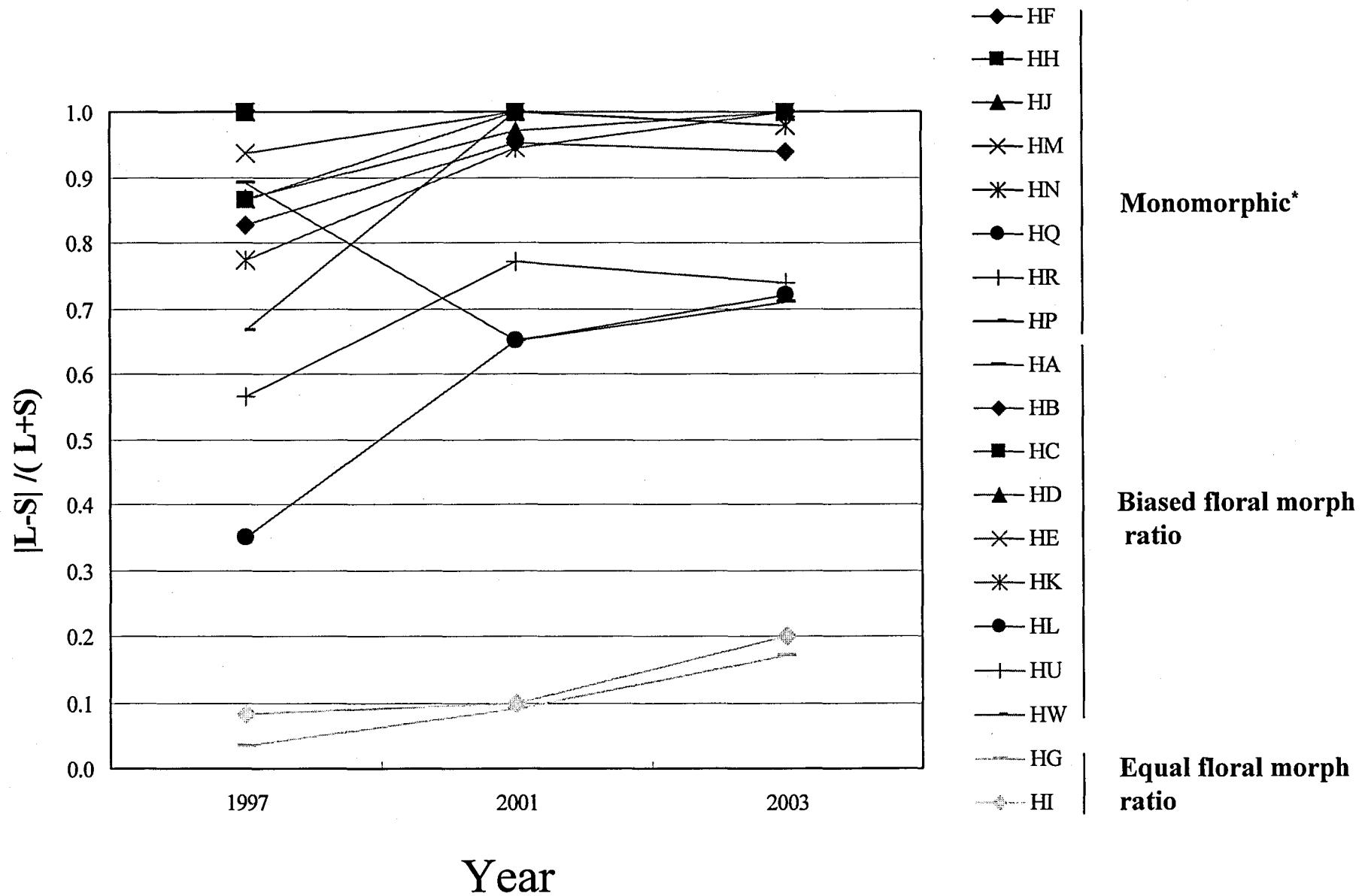


Fig. 5-7. The change of floral morph ratio in populations with only a vegetative reproduction for six years. * Floral morph ratio based on *G*-test in 1997.

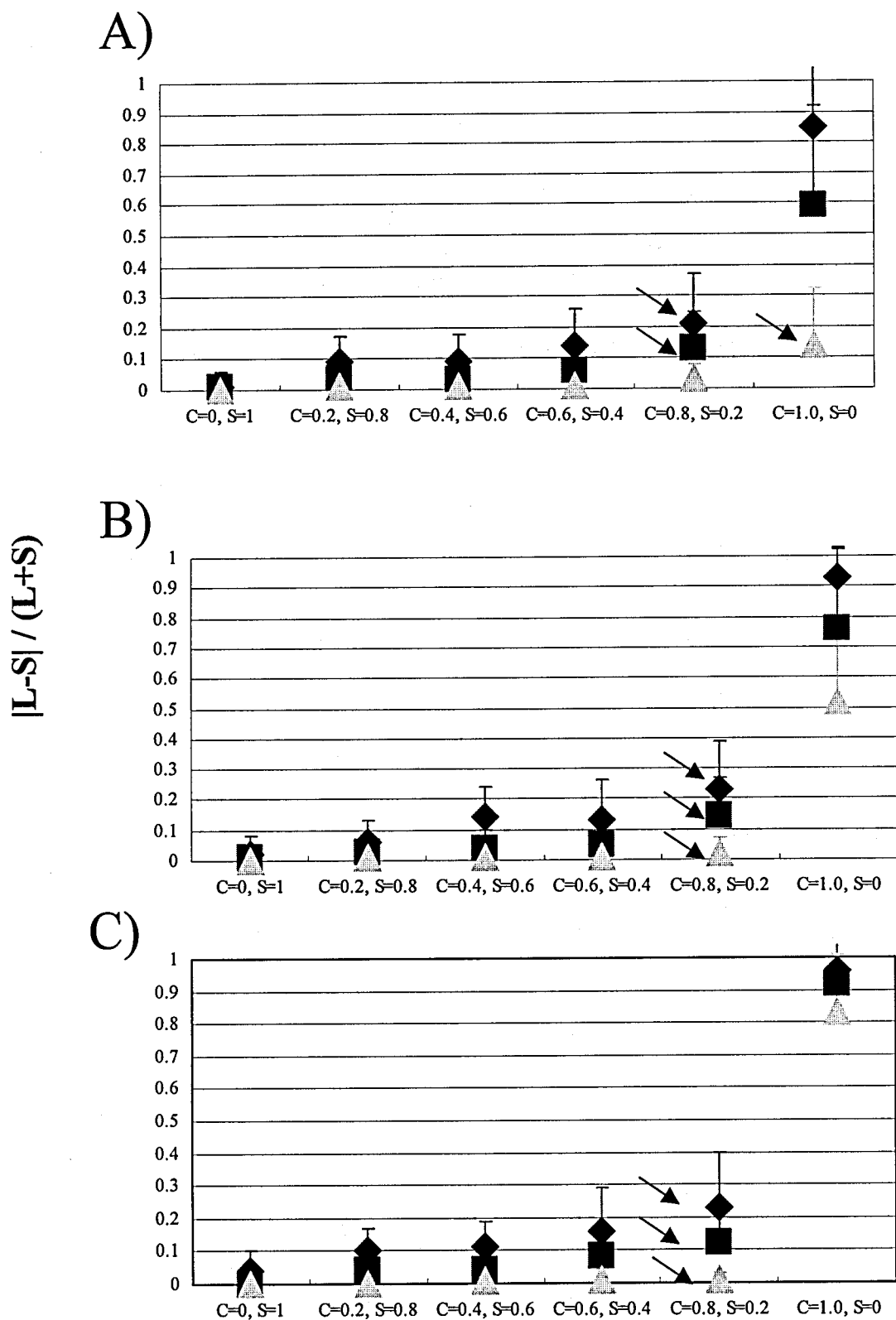


Fig. 5-8 (a). This case of simulation shows that the same reproductive mode as $I=0.6$ is replicated every year. The value was calculated by $|L-S| / (L+S)$. A): $|L-S| / (L+S)=0$; B): $|L-S| / (L+S)=0.4$; C): $|L-S| / (L+S)=0.8$. Arrows indicate the proximate value showing equal floral morph ratio based on G -test. $\blacklozenge$: $N=50$; $\blacksquare$: $N=100$; $\blacktriangle$: $N=500$.

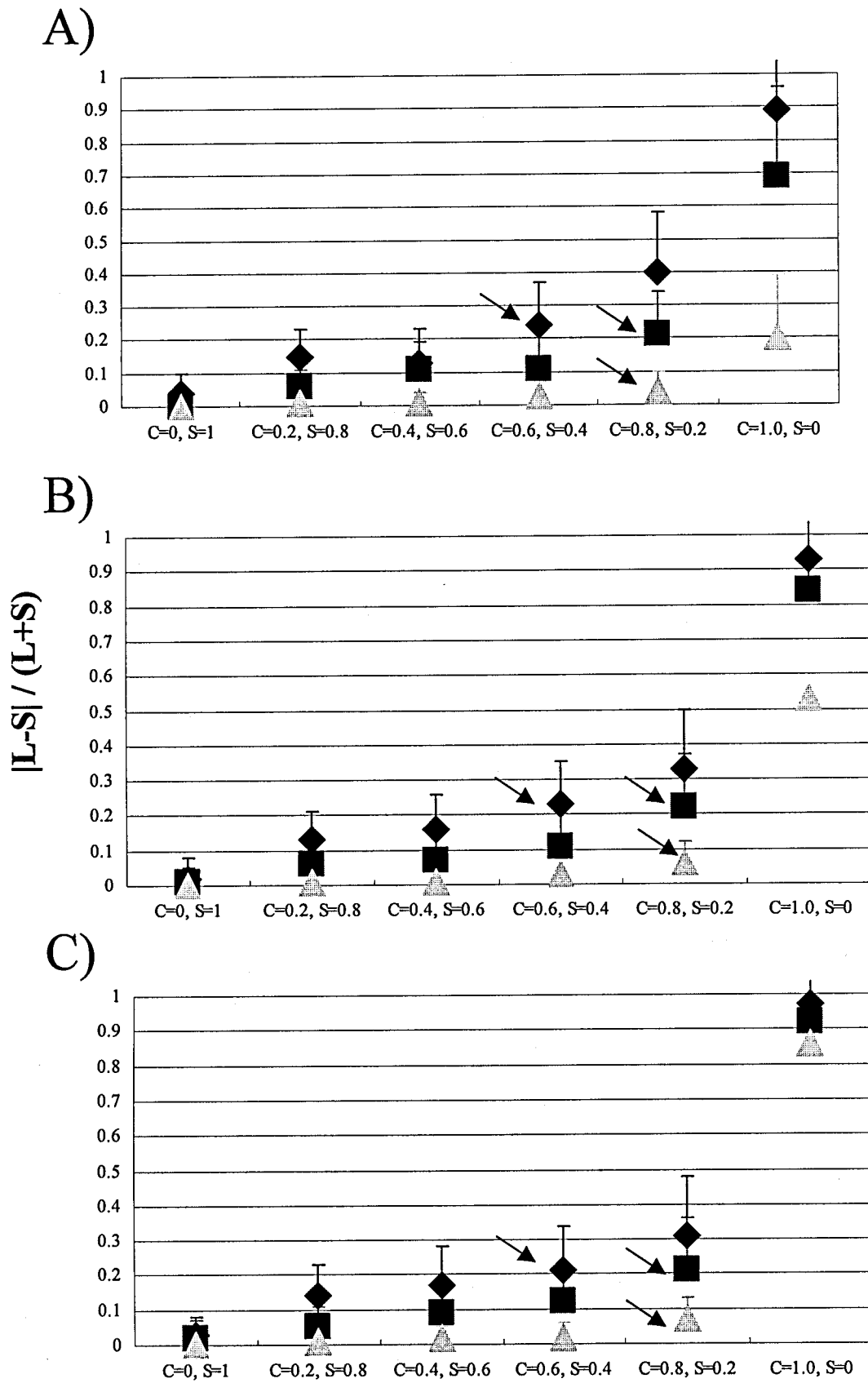


Fig. 5-8 (b). This case of simulation shows that the same reproductive mode as $I=0.8$ is replicated every year. The value was calculated by $|L-S| / (L+S)$. A): $|L-S| / (L+S)=0$; B): $|L-S| / (L+S)=0.4$; C): $|L-S| / (L+S)=0.8$. Arrows indicate the proximate value showing equal floral morph ratio based on G -test. ◆: $N=50$; ■: $N=100$; ▲: $N=500$.

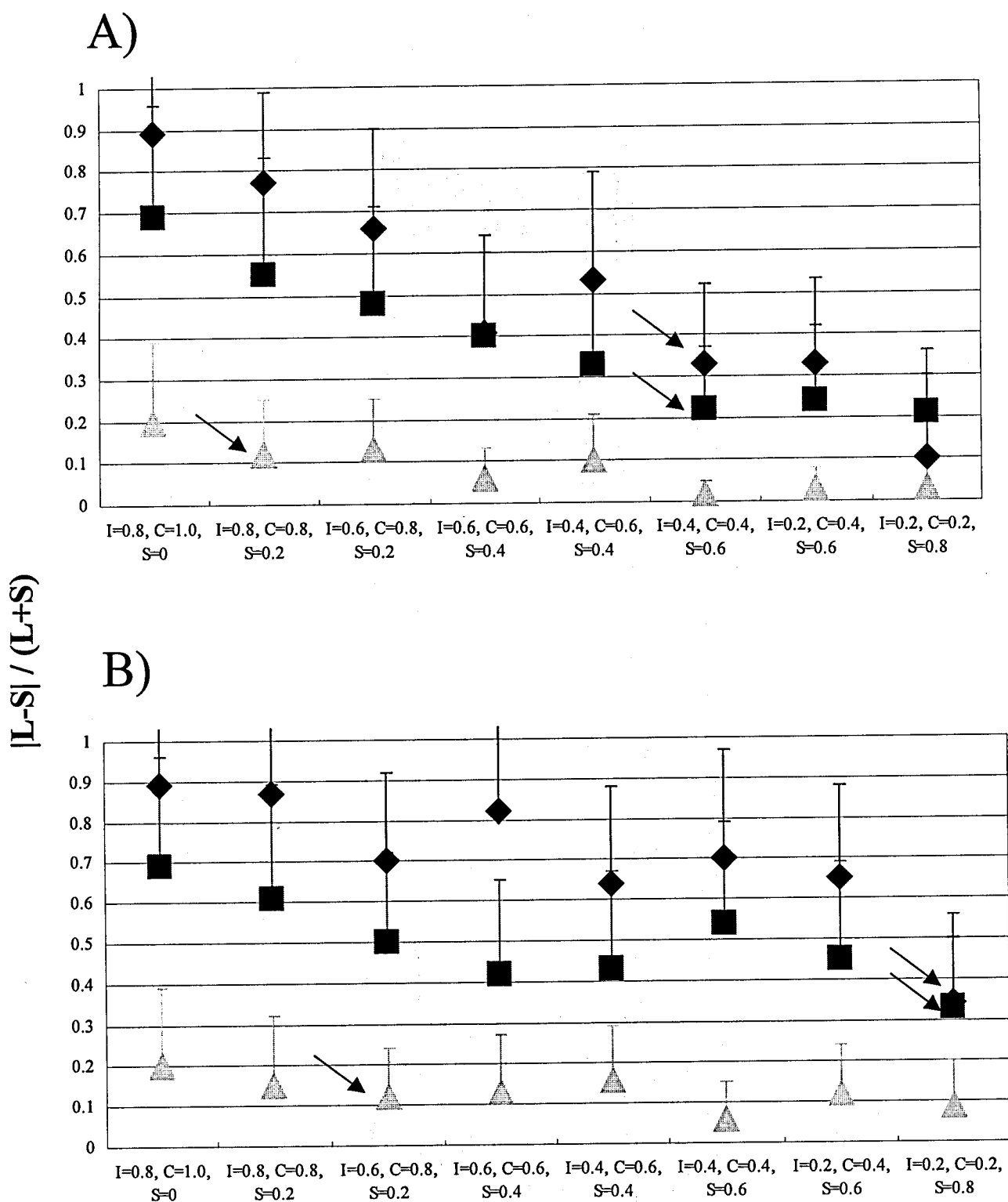


Fig. 5-9. This case of simulation shows that only a vegetative reproduction occurs when water level is high and sexual reproduction occurs simultaneously with vegetative reproduction in case of occasional water drawdown. The value was calculated by $|L-S| / (L+S)$. A): Occasional water drawdown occurs every ten years.; B): Occasional water drawdown occurs every thirty years. Arrows indicate the proximate value showing equal floral morph ratio based on G -test. $\blacklozenge$: $N=50$; $\blacksquare$: $N=100$; $\blacktriangle$: $N=500$.

Appendix 5-1. Effects of fruit setting on turion formation and growth of rootstocks.

Parameter	No-pollinated	Pollinated	Statistical analysis**
Number of turions per plant	6.83±0.52***	6.53±1.23	P=0.5127
Dry weight of individual turion (g)	0.32±0.16	0.25±0.16	P=0.0041
Dry weight of plant(g)	1.62±0.41	1.11±0.54	P=0.0169
Number of flowers / inflorescence	8.12±4.48	8.00±4.8	P=0.8780
Fruit set rate	-	0.56±0.25	-
Max of leaf area(cm ²)	56.61±11.03	52.35±12.49	P=0.3145
Turn over rate (day)	32.69±4.38	32.14±2.21	P=0.9008

*Parameters having significant difference($P < 0.05$).

**Mann-Whitney U-test

***Values of all parameters are Mean±SD in three containers.