Sex Organ Development And Interbreeding Mechanism of *Cyathea Spinulosa* (A Tree Fern)

Abstract The study is to investigate the out breed ing mechanisms of *Cyathea spinulosa*, a common Tai wan tree fern (Fig. 1). Fern gametophytes are called prothallia that are known to be bisexual with coexisting arche go nia and antheridia. From out door surveys, I found that matured pro th al lia of *C. spinulosa* can be either bisexual or unisexual (contain either archegonia or antheridia). The data also showed that the proportion of female prothallia is much higher in the "isolated" samples (prothallium that is at least 25 mm apart from the closest by neighbor) compared to that of the "clustered" prothallia. In the laboratory, all of the prothallia grown individually could only pro duce archegonia; however, the pro th al lia in multispore cultures could be bi sex u al or unisexual. Induction of an the rid i um development was studied by plac ing im ma ture pro th al lia next to a ma ture female prothallium. And the induction was both time- and dis tance-dependent. It takes ap prox i mate ly 8 days for the appearance of the antheridia and the optimal dis tance is 150-200 mm. Furthermore, pro longed multispore cul tures re sult ed in aborted pro th al lia (no zy gote formation) or sporophyte-car ry ing prothallia that were originally female or bisexual. These results indicate that: (1) arche go ni um de vel op ment is spontaneous, (2) an the rid ia development must be induced by ma tured arche go nia (from another prothallium), and (3) no antheridium can be induced in arche go nia-carrying prothallia; therefore the development of an the rid i um must pre cede that of arche go ni um in an individual prothallium. The above tem po ral and spatial regulation of sex organ de vel op ment in *C. spinulosa* gametophytes may rep re sent a mechanism in volved in the assurance of outbreeding in this fern.

1.Introduction

Cyathea spinulosa is a common tree fern found in the for ests in South east Asia. *C. spinulosa* is be lieved to be a homosporous fern and thus its ga me to phytes are believed to bisexual, i.e., they are her maph ro dites and con tain both arche go nia and antheridia on a sin gle prothallium (Pal, 1994). Based on the prox im i ty of antheridia and arche go nia on bi sex u al gametophytes, it was once thought that intragametophytic selfing would be the pre dom i nant mat ing system in homosporous ferns (Klekowski, 1979). In a preliminary outdoor survey, I was amazed to find some unisexual pro th al lia (females or males only) of *C. spinulosa.* The proportion of female prothallia is highly dif fer ent in sam ples grown un der different growing patterns. It is very high in

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isolated samples but very low in clus tered samples. After 2 weeks of further cul tur ing the uni sex u al pro th al lia in the laboratory, the male prothallia started to develop female organs (archegonia) but the fe male pro th al lia remained unchanged. Reviewing of the lit er a ture revealed that the mating system of *C. spinulosa* has not been published. I studied the sex ra tios of pro th al lia collected in outdoor sur veys and in lab o ra to ry cultures with re gard to their grow ing pat terns (in "clustered" or "iso lat ed" forms), the time- and distance-de pen dent induction of an the rid ia in immature prothallia by older fe male gametophytes, and the se quence of ga met an gia for ma tion in *C. spinulosa.* Based on the findings, I conclude that the asyn chro nous mat u ra tion of male and female ga met an gia can prevent intragametophytic selfing and that the distance-de pen dent induction of an the rid ia de vel op ment may help to promote intergametophytic crossing in this tree fern.

2.Materials and Methods

(Determination of sex expression of *C. Spinulosa* gametophytes in nat u ral habitats) *Cyathea Spinulosa*, a common tree fern in Taiwan, was the subject of this study. In three out door surveys, pro th al lia from 2 to 4 mm in radius were collected in two categories ac cord ing to their growing densities at the Kun-lun Herbal Drug Tour ing Park in Lungtan, Taoyuan County, Taiwan. The samples were respectively des ig nat ed as the "clus tered pro th al lia" when they grew as a group with the farthest in di vid u als less than 2.5 cm away from their neighbors, and the "iso lat ed pro th al lia" when the closest neighbor was at least 2.5 cm apart. The sex of the samples was determined under a mi cro scope according to the ex pres sion of sex organs as shown in Fig. 2. They were further classified into four categories, asex u al (no sex organ), male (contained only antheridia), female (contained only archegonia), and her maph ro dite (contained an the rid ia and archegonia). (Determination of sex expression of *C. Spinulosa* gametophytes in cultures) Segments of leaves bearing ma tured spo ran gia collected and stored dried from plants at the Kun-lun Herb al Drug Touring Park in Sep tem ber 1999 were dried and stored in the refrigerator. Spores were then col lect ed and kept at 4℃ until used. To establish aseptical gametophyte cultures, spores were sur face ster il ized with 2.5% NaOCl, rinsed in sterilized water, and sown on 1% so lid i fied agar medium with Bold's so lu tion (Bold, 1957) in 9-cm Petri dishes. The spores were al lowed to ger mi nate and grow in a growth chamber, under a 12 hr/day light cy cle (fluorescent light, ap prox i mate ly 2,500 lux) at 26 ｛? ℃. To simulate the ger mi na tion condition in the na ture habitat, the "clus tered ga me to phytes" cul tures were ini ti at ed from multispore cultures with the den si ty con trolled be tween 2 to 10 pro th al lia/cm. In addition, young pro th al lia (less than 2 mm in radius, 7 to 14 days after germination) were re plant ed and al lowed to grow individually in sep a rat ed con tain ers to simulate the "isolated gametophytes". For ty-five days after the spores were sown, the sex of the prothallia was de ter mined as de scribed above. For the de ter mi na tion of sex ratios of sporophyte-carrying gametophytes, multispore cultures were ini ti at ed as described above. The cul tures were allowed to pro 2

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ceed through the de vel op ments of sex organs (maturation), fertilization, and ger mi na tion of sporophytes. Sev en ty-five days after the spores were sown, the sporo phyte-car ry ing ga me to phytes were collected and ex am ined for the presence of still existing or de gen er at ed arche go nia and antheridia. The sam ples were recorded as fe males and her maph ro dites accordingly. Induction of antheridia in young pro th al lia Induction of antheridium de vel op ment was stud ied by using immature, i.e.,asexual pro th al lia as the re cip i ents and asexual, male as well as fe male pro th al lia as the inducers. The pro th al lia were re plant ed in con tain ers lined with 4 lay ers of gauze soaked in Bold's solution. In 9-cm Petri dishes, the inducers were placed at the center and 7 asexual prothallia were placed around the edge (i.e., 4.5 cm apart from the inducer) (Fig. 3A). Development of sex organs was monitored every day af ter replanting. In

Drug Touring Park, I col lect ed a total of 202 pro th al lia that grew in clustered con fig u ra tions (from 7 dif fer ent locations) and 8 prothallia that grew solitarily. The sex of these pro th al lia was de ter mined in the lab o ra to ry by viewing under a microscope. In addition to an ex pect ed great num ber of hermaphrodites, significant per cent ag es of unisexual prothallia were also found (Fig. 4). Moreover, the ratio of female pro th al lia in the iso lat ed sam ples was much higher than that in the clus tered samples (42% vs. 5%, as shown in the second column of Figs. 4A and 4B). Subsequent laboratory culture of the uni sex u al pro th al lia collected from the wild produced two very different results: the female prothallia re mained female but the male prothallia became bisexual. Sex order to study the distance-dependence of antheridia induction, inducers and recipients were replanted in a 60 x 8 cm glass trough in the following manner: An inducer was placed 5 cm from the edge (starting point) and 4 immature prothallia were placed every 5 cm apart from the inducer (Fig. 3B). Antheridia development in the recipient prothallia was determined 4 days later.3.Results Sex expression of *C. Spinulosa* gametophytes in natural habitats In three independent visits in the late fall (November to December) to the Kun-lun Herbal expression of *C.Spinulosa* gametophytes in cultures To rule out the environmental factors that would affect the sex expression in *C.spinulosa*, spores were allowed to germinate and grow under a controlled environment. In general, the spores germinated within 7 days and took 7 to 14 days to develop into young prothallia, approximately 2 mm in radius and remained asexual. To determine the sex ratios of prothallia grown in different configurations, the sex of the well-developed prothallia was determined 45 days after sowing. Of the 141 prothallia grown in the multispore cultures, 45 (31.9%) of them remained asexual (immature).Moreover, the mature prothallia could be classified as hermaphrodites, female, and male and their ratios were 27.0, 5.7, and 35.5% respectively (Fig. 5A). Therefore, the sex ratio of laboratory-grown prothallia resembles that found in the wild (compared Fig. 4A and Fig. 5A). In contrast, the laboratory-grown solitary prothallia were found to be either asexual or female (no hermaphrodite or male). At around 45 days after sowing, the asexual and female prothallia were 71% and 29% respectively (Fig. 5B). Induction of antheridiain young prothallia Although female, male, and asexual prothallia were used respectively as inducers in the induction experiments, only females were found to be effective (Fig. 6). When the distance of the recipient immature prothallia was kept at 4.5 cm as shown in Fig. 3A, the development of antheridia in the recipients took place in a time-dependent manner. Antheridia could be found in some of the recipients as early as 3 days later and all of them developed antheridia 8 days after they were placed in a Petri dish with a female prothallium at the center (Fig. 6A). The induction process was also found to be distance-dependent, the recipients at 15-20 cm away from the inducer were the first to develop antheridia and 100% induction was observed in these samples 4 days after replanting (Fig. 6B). The data showed that 15-20 cm is optimal for the induction process. Sex ratios of sporophyte-carrying gametophytes in prolonged multispore cultures It is conceivable that sporophyte-carrying gametophytes must arise from female or bisexual prothallia. The sex ratio of the sporophyte-carrying gametophytes was determined in the extended multispore cultures. It was found that the majority of the sporophyte-carrying gametophytes were originally hermaphrodites (93%). In other words, only 7% of the sporophyte-carrying gametophytes originated from female prothallia (Fig. 7). 4.Discussion Female prothallia have not yet been found to develop into hermaphrodites in all circumstances while all male prothallia would become hermaphrodites eventually.Therefore, archegonia development in *C. spinulosa* gametophytes is a spontaneous process. In contrast, the development of antheridia in young prothallia is precisely regulated. The process must be induced by nearby female prothallia that contain mature archegonia. Furthermore, the timing of this induction is critical.It must be initiated before the autonomous development of archegonia in the same prothallium. The developmental sequences of sexual expression of *C. spinulosa* gametophytes are summarized in Fig. 8. As it has been shown in other ferns, mature female *C. spinulosa* gametophytes may release chemical compounds that are collectively called antheridiogens, which act as the chemical inducer for antheridia development on nearby immature prothallia. For each prothallium, whether or not to develop antheridia depends on whether it receives the correct signal(s) at the right time with the right amount.In either case, development of archegonia in these prothallia would take place spontaneously. Moreover, in hermaphrodites, the maturation of archegonia occurred after the sperms were released from the antheridia of the same gametophyte. Finally, a successful fertilization (as indicated by the germination of the sporophytes) may or may not occur (Fig. 8). It is worth noting that only one sporophyte may geminate from one prothallium, indicating that sporophyte development on a gametophyte might be exclusive, it would suppress the development of other zygotes on the same plant.

Although the actions of antheridiogens have been reported extensively, the concentration effect(s) (reflected as the distance-dependent experiment in this study) was not well documented. Under the specified conditions, prothallia at 15-20 cm away from a female gametophyte responded best, development of antheridia in these plants occurred earliest. In nature, spores from the same sporangium are likely to be distributed very closely; therefore it is of interest not to stimulate the closest nearby gametophyte to decrease the chance of intergametophytic selfing.

5.Conclusion

In *C. spinulosa*, intragametophytic selfing is most likely prevented by asynchronous maturation of male and female gametangia on the same prothallium and intergametophytic selfing may be prevented by the dose-dependent response of antheridia development to the "not-so-close" female neighbor. Collectively, these mechanisms may evolve to promote intergametophytic crossing (interbreeding) in this tree fern.

6.Reference

1.Bold, H. C.(1957) Morphology of plants.1st ed. Haper and Row, New York.
2.Klekowski, E. J., Jr. (1979) The genetics and reproductive biology of ferns. In The experimental biology of ferns. Edited by A. F. Dyer. Academic Press, London U.K. pp. 133-170.
3.Masuyama, S. (1974) The sequence of the gametangium formation in homosporous fern gametophytes I. Sci. Rep. T.K.D. Sect. B. 16: 47- 69.
4.Pal, G. D.(1994) Fern and fern allies of Itanagar, Lower Subansiri District, Arunachal Pradesh. J. Econ. Taxon. Bot. 18: 153-163.

Fig. 1. The morphology of *C. spinulosa* sporophytes and parts. (A) mature *C. spinulosa*; (B) feather-like leave with leaflets; (C)(D) the ventral side of a leaflet, showing rows of sori; (E) sorum; (F) sorum; (G) spores.

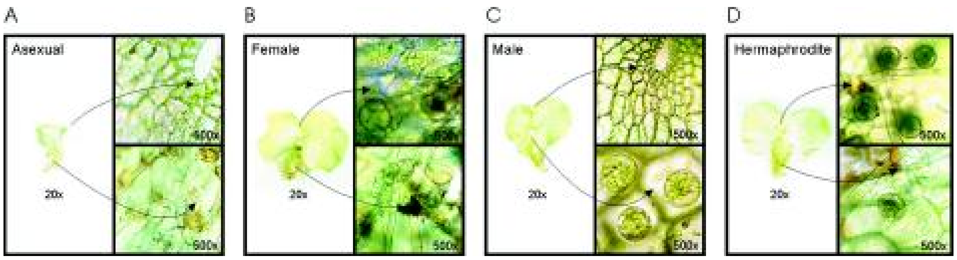
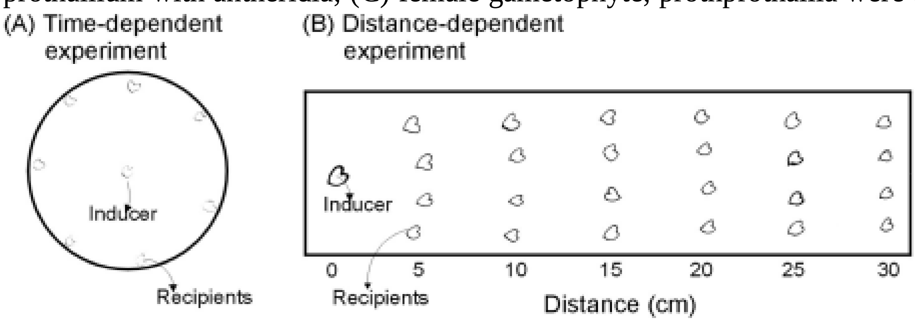
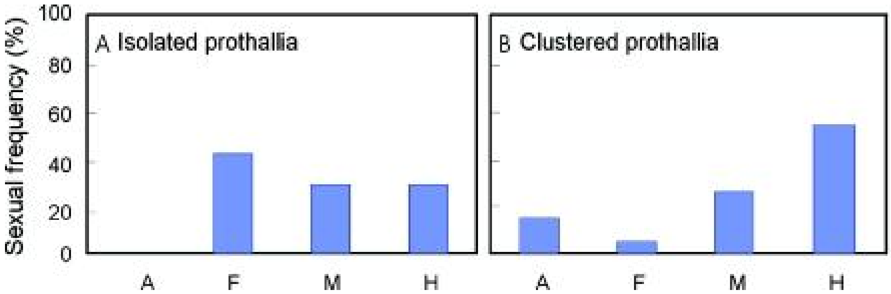


Fig. 2. Sex expression of *C. spinulosa* gametophytes. Gametophytes of *C. spinulosa* were classified by sexualexpressions: (A) asexual gametophyte, no sex organ was found; (B) malegametophyte, prothallium with antheridia; (C) female gametophyte, prothprothallia were used as recipients.



(A)Time-dependent experiments, the re cip i ent prothallia were placed 4.5 cm apart from the potential inducers (in 9-cm Petri dishes, the inducers were planted at the center while the re cip i ents were planted at the edge) and the deve lopment of antheridia on the re cip i ent prothallia was recorded on a daily basis. (B) Distance-dependent experiments, recipient prothallia were placed every 5 cm apart from the po ten tial inducers and the development of antheridianulosa gametophytes in natural habitats.



(A)Clustered gametophytes, ga me to phytes were found in-groups, with the farthest neighbor within 2.5 cm. (B) Isolated gametophytes; gametophytes with the closest-by neigh bor at least 2.5 cmapart. H: hermaphrodites; F: females; M: males; A: asexual.

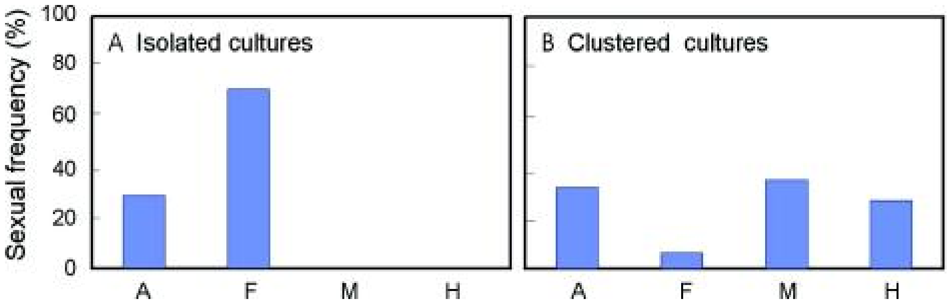


Fig.5. The sexual frequency (%) of *C. spinulosa* gametophytes in the laboratory. (A) Clustered gametophytes,gametophytes that were initiated from multispore cultures, 2-10 gameto phytes/c㎡ (B) Isolated gametophytes, gametophytes that were re plant ed after germination and allowed to grow individually, 1 plant per container. H: hermaphrodites; F: females; M: males; A: asexual.

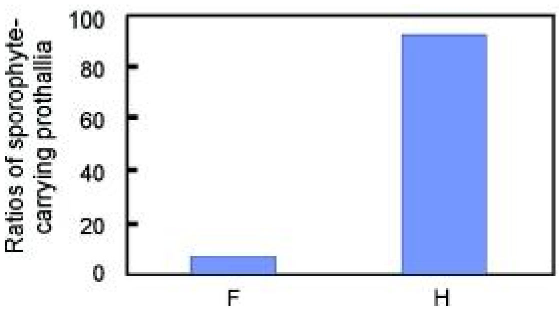


Fig.7 Ratio of female and bisexual ga me to phytes with developing sporophytes of *C. spinulosa.* Multispore cultures were initiated and the gametophytes were allowed to grow and develop for 75 days. At the end of this culturing period, young sporophyte-carrying prothallia were collected and observed with an aid of a microscope for the existence of the sex organs. Prothallia containing archegonia (or degenerated archegonia) were classified as female. Prothallia with both archegonia and an the rid ia (or degenerated organs) were classified as hermaphrodites. H: hermaphrodites; F: females.