Species-specific gating of gametangial dehiscence as a temporal reproductive isolating mechanism in *Coelomomyces*

(sympatric species/photoperiod/gamete release/fungal systematics)

BRIAN A. FEDERICI

Division of Biological Control, Department of Entomology, University of California, Riverside, California 92521

Communicated by John N. Couch, October 18, 1982

ABSTRACT Hybrid zygotes of *Coelomomyces dodgei* and *Coelomomyces punctatus* can be produced in the laboratory by making reciprocal crosses of their gametes. Although the F_1 hybrid sporophyte is viable, the F_1 gametophyte is inviable. Gametangial dehiscence (gamete release) in both species is relatively synchronous and follows a photoperiod-dependent diel periodicity, but even in populations grown under identical conditions, the gametes of each species are released during significantly different time frames. When the fungi are reared on an 8/16-hr dark/light regime, the gametes of *C. punctatus* are released during hours 7–11, whereas those of *C. dodgei* are released during hours 16–19. These results demonstrate that gametangial dehiscence is gated and, furthermore, indicate that this gating serves as a temporal reproductive isolating mechanism for these species of *Coelomomyces*. Additional preliminary studies with *C. dodgei* demonstrate that gametogenesis and gametangial dehiscence are photoperiod-dependent functions triggered by the onset of the dark period during the last day of gametophyte development, a dark period as short as 2 hr being sufficient to stimulate gametogenesis and gametangial dehiscence.

Coelomomyces dodgei and *Coelomomyces punctatus* (class Chytridiomycetes; order Blastocladales) are closely related invertebrate-parasitic fungi that occur sympatrically throughout the eastern United States (1, 2). The life cycle of both species involves an alternation of gametophytic and sporophytic generations, respectively, between the copepod *Acanthocyclops vernalis* and larvae of mosquitoes of the genus *Anopheles* (3, 4). A variety of evidence attests to the close relationship of *C. dodgei* and *C. punctatus*. They display marked similarities in size, shape, and surface structure of their sporangia (5), have overlapping host ranges (1, 2, 6, 7), and can be readily hybridized in the laboratory through reciprocal crosses of their gametes (8). The hybrids, however, are only partially viable. The F_1 zygote formed in reciprocal crosses is capable of infecting mosquito larvae and producing a viable sporophyte. This sporophyte develops normally and produces hybrid sporangia that exhibit surface features characteristic of both parental species and that cleave and dehisce normally. However, the F_1 meiospore produced by these sporangia, though capable of encysting on the copepod host, does not produce a functional gametophyte and, thus, is inviable (9).

Though *C. dodgei* and *C. punctatus* can be hybridized easily in the laboratory, naturally occurring F_1 hybrid sporophytes and sporangia are not known. In this report, data are presented which demonstrate that, although gametangial dehiscence (release of gametes) is gated in both species and follows a photoperiod-dependent diel periodicity, the gametes of each species are released during significantly different and relatively narrow,

nonoverlapping time frames. This interspecific, asynchronous dehiscence of gametangia apparently serves as a temporal reproductive isolating mechanism and may account for the apparent lack of naturally occurring hybrids. Additionally, preliminary studies with *C. dodgei* are reported that demonstrate that the time of gametangial dehiscence is determined primarily by the onset of the dark period during the last day of gametophyte development.

The following more detailed account of the life cycle of *Coelomomyces* is relevant to the experiments described herein.

In the life cycle, a biflagellate zygote infects a mosquito larva and develops into a sporophyte that produces resting sporangia in the larval hemocoel. Infection results in death, after which sporangia are liberated into the aquatic environment as the dead larva putrefies. Meiosis occurs in these sporangia (10), and, after cleavage, haploid uniflagellate spores (meiospores) are released. The meiospores infect the copepod and produce within its hemocoel a gametophytic thallus that reaches maturity in about 7 days, thereby becoming a gametangium. This gametangium cleaves into gametes during the 12-hr period preceding dehiscence, and, at a predetermined time, gametes begin swarming. The swarming results in rupture of the gametangium, followed within a few minutes by death of the copepod and escape of the gametes through fissures in the copepod body. Gametes liberated from the same or different copepods fuse to form biflagellate zygotes that complete the life cycle by infecting another mosquito larva.

In the gametophytic phase of *C. dodgei* and *C. punctatus*, male gametangia and gametes are orange due to the presence of β -carotene, whereas females are light amber (11). As the fungus matures, the color of the host indicates the sex of the infecting meiospore, except in cases where a copepod is infected by both types, wherein both colors occur. This pigmentation allows infected copepods to be distinguished from those that are uninfected and facilitates observations on patterns of gametangial dehiscence. In the present study, the time of dehiscence for each species was established by determining the time of death for infected copepods that were reared under identical conditions.

MATERIAL AND METHODS

The hosts used for *C. dodgei* and *C. punctatus* in these experiments were the copepod *A. vernalis* and larvae of *Anopheles quadrimaculatus*. Methods for infecting and rearing these hosts have been described (3, 12), and only procedures relevant to the present report are given here.

Copepods were infected by exposing populations of 1,000–2,000 48- to 72-hr-old copepod nauplii to approximately 2,000 sporangia in the process of releasing meiospores or to suspensions of meiospores only. Suspensions containing only meiospores were prepared by filtering suspensions of dehiscing sporangia through Whatman no. 5 filter paper, which retained the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

FIG. 1. Diel periodicity of gametangial dehiscence for *C. dodgei* and *C. punctatus* for 3 consecutive days in two separate populations of the intermediate copepod host, *A. vernalis*, reared on an 8/16-hr dark/light photoperiod. Copepods were infected in one population with *C. dodgei* and in the other with *C. punctatus* and were reared as described. Gametangial dehiscence times are expressed as the number of copepods that died as a result of dehiscence in each 15-min interval over the 3-day period. ■, Dark period.

sporangia. During and after exposure, copepods were reared at $26 \pm 2^\circ\text{C}$. Infected copepods were collected from the rearing trays as gametophyte pigmentation became obvious, indicating thereby that gametogenesis and dehiscence would occur within 24 hr. Each copepod was placed in a separate 35-mm Petri dish

in a weak salt solution (8). Randomly selected specimens were periodically examined microscopically for the initiation of gamete swarming, an indication that dehiscence would occur within 1 hr. Once this was detected in any copepod, all copepods were kept under surveillance, and the time of each individual death was recorded.

In the experiments designed to determine the time of gametangial dehiscence, infected copepods were reared under an 8/16-hr dark/light photoperiod from infection until death, which typically occurred 6–10 days later. In preliminary experiments with *C. dodgei* that were designed to determine the effect of different photoperiods on the time of gametangial dehiscence, the onset and length of the photoperiod were varied during the final 24-hr period of gametophyte development. Because these experiments demonstrated that the critical event in the initiation of gametogenesis and dehiscence in this species was the onset of the last dark period, all time data are expressed in terms of hours and minutes after the onset of darkness. In all experiments, light (ca. 850 lux) was provided by 125-W fluorescent bulbs.

RESULTS

In the first series of experiments, fungal meiospores were combined with copepod nauplii, and the diurnal periodicity of gametangial dehiscence for each fungal species was determined by recording the times of dehiscence on 3 consecutive days in two different populations of *A. vernalis*: one infected with *C. dodgei* and the other infected with *C. punctatus* (Fig. 1). On each day, dehiscence of *C. punctatus* occurred only from 7 to 11 hr after the onset of darkness, whereas under the same conditions, the gametangia of *C. dodgei* dehiscence from 16 to 19 hr after the beginning of the dark period. In *C. punctatus*, most gametangia dehiscence just prior to or within 2 hr of the onset of the light period, whereas in *C. dodgei* all dehiscence occurred during the light phase. The dehiscence periods for the two species were thus gated and separated by approximately 6 hr.

The mean time of dehiscence for both *C. dodgei* and *C. punctatus* was established by determining the dehiscence times for 1 day in three different populations of infected copepods (Table 1). Measuring again from the onset of the dark period, the mean time of dehiscence for *C. punctatus* was 8 hr and 8 min (SD, 29 min) and for *C. dodgei* was 17 hr and 25 min (SD, 30 min).

To ensure that the relatively synchronous release of gametes in each species was not due to similar rates of fungal development, meiospores of *C. dodgei*, at a meiospore/nauplius ratio of 1,000:1, were added once to separate copepod populations

Table 1. Dehiscence times for gametangia of *C. punctatus* and *C. dodgei* in infected copepods of *A. vernalis* reared under an 8/16-hr dark/light photoperiod*

Repli- cate	Dehiscence times for gametangia, hr:min														
	<i>C. punctatus</i>									<i>C. dodgei</i>					
	Orange			Amber			Orange and amber†			Orange			Amber		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
1	15	8:06	42	6	8:11	54	11	8:15	43	25	17:30	31	24	16:48	19
2	25	8:11	29	7	8:04	6	11	7:59	24	8	18:01	15	8	17:32	8
3	20	8:08	18	4	8:08	6	16	8:11	5	16	17:40	4	8	17:36	6
Total	60	8:09	30	17	8:07	31	38	8:08	26	49	17:38	26	40	17:06	28
Grand															
	n			mean			SD			n			mean		
	115			8:08			29			116			17:25		
													30		

* For each fungal species, values were obtained from copepods collected on a single day from three separate experiments. Values are expressed in terms of hours and minutes after the onset of the dark period; the standard deviations are in minutes. n, Number of infected copepods.

† Gametangia occurring in the same host typically cleave and dehiscence approximately at the same time.

Table 2. Dehiscence times for gametangia of *C. dodgei* obtained when two populations of the intermediate copepod host were infected once at two different times separated by 12 hr*

Time of exposure, [†] hr	n [‡]	Time of dehiscence, [§] mean hr:min ± SD in min
D + 5 hr		
Replicate		
1	11	D + 17:30 ± 17
2	10	D + 17:22 ± 20
3	21	D + 17:30 ± 21
Total	42	D + 17:28 ± 20
D + 17 hr		
Replicate		
1	10	D + 17:11 ± 15
2	6	D + 17:02 ± 7
3	18	D + 17:22 ± 20
Total	34	D + 17:15 ± 18

* Copepods of *A. vernalis* were infected as 72-hr-old nauplii by combining them with meiospores at a meiospore/nauplius ratio of 1,000:1. Dehiscence times are for gametangia in copepods collected 10 days later.

[†] Hours after the onset of the dark period (D).

[‡] Number of infected copepods. For convenience, times for different mating types are not separated here.

[§] Hours and minutes after the onset of D.

at two different times, the times being separated by an interval of 12 hr. The mean time of gametangial dehiscence for infected copepods in both populations was in the range of 17 hr and 10–30 min (Table 2). The times were not significantly different from one another or from the times obtained for *C. dodgei* in the experiments described above.

The results of the preliminary experiments on the effect of the onset and length of the last dark and light periods on gametogenesis in *C. dodgei* are shown in Fig. 2. For the conditions tested, the results of these experiments demonstrate that the most critical factor affecting the time of gametangial dehiscence is time of onset of the last dark period. In all groups, dehiscence occurred between 16 and 19 hr after the onset of the last dark

period. Delaying the onset of the final dark period, as was done in groups 2 and 6, delayed the time of dehiscence by a period similar to the length of the delay. In contrast to this, the length of the last dark or light period, or onset of the last light period, had comparatively little effect on the time of dehiscence (groups 3–5). The experiment with group 6 also demonstrated that the effect of the onset of the dark period could be nullified by insertion of a light period.

DISCUSSION

The results of these experiments indicate that the time of gametangial dehiscence in *C. dodgei* and *C. punctatus* is a function of a species-specific, photoperiod-dependent, diel periodicity rather than of the time of copepod infection or fungal developmental rates. More importantly, the results demonstrate that when reared under the same photoperiod, *C. dodgei* and *C. punctatus* release their gametes at significantly different times. Although the techniques used to determine the dehiscence times were not completely analogous to natural conditions in that dawn and dusk periods were not included in the dark/light regimens, it is nevertheless highly probable that the results reflect similar differences occurring in the field. Thus, the release of gametes of these species at different times of day functions as a temporal reproductive isolating mechanism.

The diel nature of the temporal isolation apparent in *C. dodgei* and *C. punctatus* has probably resulted from constraints imposed on these fungi by their relatively short life cycles and by their reproduction within the same or congeneric sympatric hosts during the same seasons (2), thereby precluding isolation through host specificity or a seasonal temporal mechanism. The brevity of the sexual phase imposes the most critical constraint. Gametophyte development requires an average of 6–8 days, with gamete maturation and release occurring during the last day. The gametes are phototactic and typically remain motile for less than 24 hr; yet upon encountering a gamete of opposite mating type, plasmogamy occurs immediately (8). Thus, under natural conditions, gametes released during any day will tend to congregate near the water surface and form zygotes quickly

FIG. 2. Effect of the onset and length of the last dark and light periods on gametangial dehiscence in *C. dodgei*. Copepods were infected and reared on an 8/16-hr dark/light photoperiod until the last day of gametophyte development, when the photoperiod was varied for groups 2–6 as indicated in the figure. Group 1 served as the control. Note that in all cases gametangial dehiscence occurred between 16 and 19 hr after the onset of the last dark period and that delaying the onset of the dark period delayed dehiscence by a period similar to the length of the delay (groups 2 and 6), whereas the length of the light or dark period or onset of the light period had comparatively little effect on the time of dehiscence (groups 3–5). The results obtained with groups 5 and 6 demonstrate that, under the conditions tested, the effect of the onset of darkness is reversible by insertion of a light period.

* Indicated as the absolute mean time ± SD (in minutes) after time 0.

† Expressed as the mean time ± SD (in minutes) relative to the onset of the last dark period.

upon encountering mates. The relatively synchronous release of gametes of the same species, rapid mating, and release of gametes of different species during significantly different periods of the day substantially increase the probability of the formation of intraspecific rather than interspecific zygotes. The formation of interspecific zygotes has no reproductive advantage for either species because, although such zygotes can infect mosquito larvae, the hybrid is ultimately inviable (9).

In summary, it appears that *C. dodgei* and *C. punctatus* are reproductively isolated in at least two ways: by a prezygotic mechanism in which the gametes of each species are released during different periods of the day and by a postzygotic mechanism that results in the inviability of any interspecific hybrids that do form. From the standpoint of evolution, it is likely that hybrid inviability provided the selection pressure that drove apart the mating periods of the two species.

Temporal reproductive isolation is well documented in animals, particularly insects, and higher plants (13, 14). However, although a diurnal rhythm has been found in the release of spores in many fungi (15), in no case has the periodicity been shown to function in sexual reproductive isolation. Thus, the allochronic release of gametes in *C. dodgei* and *C. punctatus*, species capable of forming interspecific zygotes, provides a unique example of temporal reproductive isolation in a group of lower eukaryotic organisms.

It will be of interest to determine how widespread species-specific gating of gametangial dehiscence is in *Coelomomyces*, a genus composed of several distinctive groups in which many species appear to be closely related. In addition to providing insights into the biology of these fungi, the time of gametangial dehiscence may prove to be a useful taxonomic characteristic.

In the case of *C. dodgei* and *C. punctatus*, the mean time of gametangial dehiscence is unique to each species, a factor supporting their credibility as species.

I thank P. W. Tsao and J. J. Johnson for their assistance with this study. The investigations were supported partially by Grant AI 12772 from the National Institutes of Health and a grant from the Biological Control of Vectors component of the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases.

1. Couch, J. N. & Bland, C. E. (1983) in *The Genus Coelomomyces*, eds. Couch, J. N. & Bland, C. E. (Academic, New York), Ch. 2, in press.
2. Chapman, H. C. & Glenn, F. E. (1972) *J. Invertebr. Pathol.* **19**, 256–261.
3. Federici, B. A. & Roberts, D. W. (1976) *J. Invertebr. Pathol.* **27**, 333–341.
4. Federici, B. A. & Chapman, H. C. (1977) *J. Invertebr. Pathol.* **30**, 288–297.
5. Bland, C. E. & Couch, J. N. (1973) *Can. J. Bot.* **51**, 1325–1330.
6. Federici, B. A., Smedley, G. & Van Leuken, W. (1975) *Ann. Entomol. Soc. Am.* **68**, 669–670.
7. Federici, B. A. (1977) *Proc. Pap. Annu. Conf. Calif. Mosq. Control Assoc.* **45**, 107–108.
8. Federici, B. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4425–4428.
9. Federici, B. A. (1982) *Mycologia* **74**, 555–562.
10. Whisler, H. C. (1979) in *Insect-Fungus Symbiosis*, ed. Batra, L. R. (Allanheld, Osmun, Montclair, NJ), pp. 1–32.
11. Federici, B. A. & Thompson, S. N. (1979) *Exp. Mycol.* **3**, 281–284.
12. Federici, B. A. (1980) *Entomophaga* **25**, 209–216.
13. Bush, G. L. (1975) *Annu. Rev. Ecol. Syst.* **6**, 339–364.
14. Levin, D. A. (1978) *Evol. Biol.* **11**, 185–317.
15. Ingold, C. T. (1971) in *Fungal Spores, Their Liberation and Dispersal* (Clarendon, Oxford), pp. 214–238.