PAPER

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Allorecognition behavior of slime mold plasmodium—*Physarum rigidum* slime sheath-mediated self-extension model

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Abstract

When allogeneic plasmodia of Myxomycetes encounter each other, they fuse or avoid each other depending on the combination, indicating that plasmodia can recognize self and nonself. The mechanisms of allorecognition are not fully understood from the perspective of behavior. In the present study design, *Physarum rigidum* geographical strains naturally encountered each other with the aim of formulating a model of allorecognition behavior. The plasmodia of *P. rigidum* can recognize each other by touching the cell membrane surface. However, contact is not necessary. Cases involving the absence of contact occur by the slime sheath of hyaline mucus that covers plasmodium. This so-called non-contact allorecognition has distinct characteristics regardless of distance and is faster compared to that involving contact. These facts suggest that the plasmodia of one *P. rigidum* can recognize others and can rapidly and safely decide whether to avoid or fuse with other plasmodia, using the non-contact allorecognition. Previous studies on *P. polycephalum* have regarded the slime sheath as a repellent or as an external memory for self. These studies advocated that the principle of the plasmodium is basically avoidance of other individuals. In this study, we propose the self-extension model based on data of the allorecognition behavior of *P. rigidum*. According to the model, the slime sheath functions as a signal that disperses information about self into the environment. Self-extension by the slime sheath enables non-contact allorecognition.

Keywords: myxomycetes, plasmodium, slime mold, *Physarum rigidum*, incompatibility, non-contact allorecognition, slime sheath

(Some figures may appear in colour only in the online journal)

Introduction

Slime mold plasmodium can separate into multiple individuals and these individuals can fuse back together. However, sometimes the plasmodium does not fuse with another individual even when that other individual is the same species. Previous genetic studies have demonstrated a specific fusion genetic locus for this plasmodial incompatibility separate from reproduction-related genetic loci, with fusion of two individuals occurring only when all these loci match [2, 4, 7]. *Didymium iridis* and *Physarum polycephalum* have multiple fusion gene loci, so it has been presumed that plasmodia allogeneic individuals do not fuse unless the strains are very closely related [3].

The main interest of plasmodial incompatibility research to date has concerned whether individuals will or will not fuse, and in the discovery of the genetic patterns involved in the incompatibility. The behavior of each individual when multiple individuals encounter each other is unclear. This lack of clarity has hampered the understanding of how plasmodium incompatibility actually manifests as allorecognition behavior and the significance of this behavior. Ashworth and Dee opined that the 'ecological significance of genes controlling plasmodial fusion is unknown' [1].

The response of fusion or non-fusion between individuals has been thought to manifest through contact of the cell membranes. The control mechanism of membrane fusion is not yet understood, but may involve protein or carbohydrate membrane components [2].

Plasmodium is wrapped in a hyaline mucus secreted by the plasmodium itself. This substance is known as a slime sheath (figure 1) [5]. The slime sheath is involved in movement of the plasmodium, and has a defensive role for the plasmodium, covering up the otherwise exposed cell [3]. The structural components have been studied [8, 11], but the findings have been inconsistent [3].

There is little knowledge about the allorecognition behavior of plasmodia. However, there are a number of theories concerning the response to other individuals and the slime sheath of other individuals, as well as behavior selection. It is thought that the slime sheath is involved in fusion of *P. polycephalum* plasmodium, but the mechanism is unclear [6]. *P. polycephalum* plasmodium may proactively avoid fusing with other individuals, and fusion is the final option only when avoidance space is unavailable [13]. However, verification method of this theory has not been shown. It was claimed that the slime sheath serves as a repellent in the fusion avoidance behavior of plasmodium [13], but this claim contradicts a published description in which the poly-galactose composition of the slime sheath may be chemoattractant for plasmodium [1]. Another theory hypothesizes that slime sheath shed by the plasmodium is used as external memory for plasmodium behavior selection, with the slime sheath functioning as a repellent [10].

These theories claim that the slime sheath is involved as a repellent in avoidance behavior of one plasmodium with other individuals. If this is correct, then the allorecognition behavior would be extremely simple, with avoidance of other individuals being the default behavior. However, is it possible that slime sheaths really are repellents? We have a decade of experimental data concerning the culture of plasmodium collected from the natural environment. We have frequently observed behavior that can be interpreted as plasmodium striving to fuse with other individuals. We have also found that the response to the slime sheath is not always consistent with the sheath being a repellant. For example, we have observed behavior where the plasmodia traced the slime sheath put on the media. Thus, it can be questioned whether plasmodium has repellent properties (i.e. dislike of fusion) separate from incompatibility.

The seemingly simple allorecognition is not fully understood from the perspective of behavior. The idea that
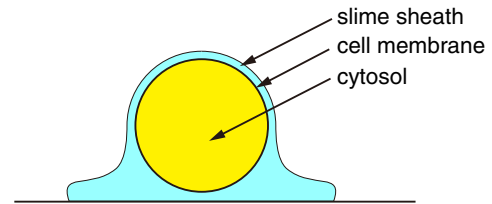

Figure 1. Cross-sectional view of slime mold plasmodium.

plasmodium secretes a repellent which other encountered individuals avoid [13] does not fully explain the plasmodium allorecognition behavior. In this study we aimed to develop a new model of allorecognition behavior based on the theory that plasmodia are able to assess the individuals they encounter and make a behavior selection concerning fusion or avoidance. We observed and quantitatively analyzed allorecognition behavior when plasmodia are allowed to behave freely with as little restriction as possible.

Materials

Five geographical strains (Tt, Ei, Tk, B1, B2) of *P. rigidum* plasmodium were collected through fieldwork in Japan, with the aim of sampling plasmodium strains with different genotypes of the gene involved in plasmodial incompatibility (figure 2). The strains differed in that only Ei was collected from a highland region at an elevation of approximately 1450 m and the other four strains were collected in plainland temperate forests. B2 was collected from a site 120 m away from the B1 collection site one year after the collection of B1. All five strains formed fruiting bodies and were identified based on the morphological characters of fruiting body, spores and calcareous.

In slime mold research, particularly in plasmodium research, *P. polycephalum* is often used as the model organism. The advantages of this species include its genetic homogeneity and the ease of culturing. However, *P. polycephalum* is not often found in the natural environment [12] and is even rarer in Japan [14]. Thus, it is difficult to collect the required geographical strains by fieldwork.

P. rigidum was used as the material in this study for three reasons. Firstly, this species is relatively prolific in Japan where the authors conducted their fieldwork, making it a suitable species for collection of geographical strains. Secondly, *P. rigidum* is positioned in the same clade as *P. polycephalum* on the phylogenetic tree [9], making it a closely related species. Thirdly, based on the authors' previous experience, *P. rigidum* is robust and impervious to environmental changes, which makes it relatively easy to culture as a wild strain in an artificial environment.

Methods

Two individual *P. rigidum* plasmodia on oat flakes were placed on 2% agar in a round polystyrene petri dish with a diameter of 50 mm. The individuals were allowed to behave freely (figure 3).
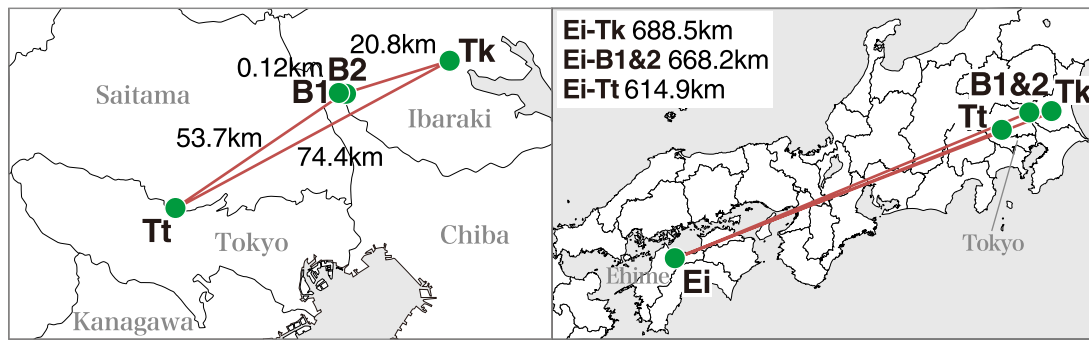
Figure 2. The collection sites of the five geographical strains of *Physarum rigidum*. Four strains were collected from plainlands in regions close to Tokyo on Honshu (largest of the main islands). Ei was collected from the highlands of Shikoku, the smallest of the main islands of Japan.

Using this method, the behavior of the ten combinations of the five geographical strains was observed concerning whether the individuals fused at encounter or avoided one another.

Furthermore, an encounter was arranged between the divided selves of a single individual. When this experiment was conducted, the Tk strain had died, so only four combinations of the remaining four strains could be assessed.

We prepared 3–28 replicates for each combination and performed 117 experiments in total. 205 encounter cases in total (1–8 encounter cases in each experiment) were observed.

To maintain a constant environment in these experiments, nutrients were not supplied, because nutrient application affects the plasmodia behavior. Drying of the medium was prevented by adding moisture drop-wise to the surface using a syringe. The samples were placed in a room with low light with the environmental temperature maintained at 25 °C to ensure active behavior of the *P. rigidum* plasmodium.

Whether the individuals avoided or fused was recorded for all encounter cases. Based on the observations of encounter cases, results of 117 experiments were assigned into two categories; 'fusible and compatible (the two individuals finally fused and began to behave as a single individual)' or 'infusible and incompatible'. The latter assignment was made in cases where the two individuals repeatedly avoided each other, even when the experiment was continued until the individuals weakened. Although it varies depending on cases, after about two or three days, they could not keep normal condition. In other words, they got thinner or scattered, and the movement became obviously dull.

The allorecognition behavior in each encounter case was quantified. The beginning of allorecognition was defined as the time when the plasmodium stopped moving or came into contact with the other individual. Completion of allorecognition was defined as a change in behavior (continuing straight, changing direction, or starting to fuse at the point of contact). The time from the beginning to completion of allorecognition was defined as the decision-making time (DMT). The point where the behavior change was observed and the distance to the other plasmodium was set as the decision-making distance (DMD). When an individual made contact without stopping immediately in front of the other individual, DMD was set as 0. DMT and DMD were recorded for all 205 encounter cases observed in 117 experiments.
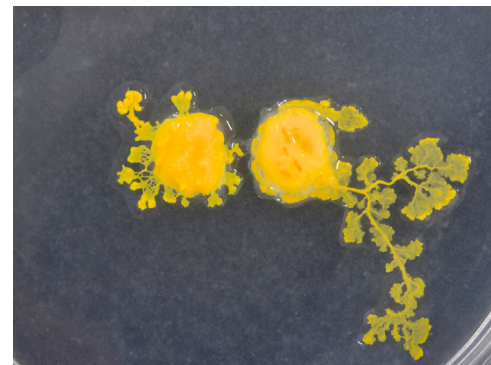

Figure 3. Two *P. rigidum* allogeneic individuals (geographical strains) on agar.

We quantitatively analyzed the distribution of DMT for each of the avoided and fused cases, the distributions of DMT and DMD for all cases, and any notable phenomena that occurred during observation. In these analyses, we conducted experiments until sufficient data are gathered, and then statistical tests (Kolmogorov–Smirnov test and Pearson's chi-square test) were performed.

Results

The five geographical strains recognized each other and the allorecognition behavior was distinct. Of the 10 combinations of five geographical strains (n = 104 encounter cases/38 experiments), only the experiments of a single combination of B1–B2 were assigned into 'fusible and compatible' (n = 4 encounter cases/3 experiments) (figure 4(a)). The encounter cases of this combination transitioned smoothly from encounter to fusion, became a single individual and continued to live. There was sufficient surrounding space, and in most cases the plasmodium developed in all the directions. Thus, although there would have been behavior options other than fusion, all individuals progressed through the stages of encounter, contact, and fusion.

Without exception, all experiments of the other nine combinations were assigned into 'infusible and incompatible' (n = 100 encounter cases/35 experiments) (figure 4(b)). In any encounter cases of these experiments, the individuals avoided each other through the behavioral changes by either
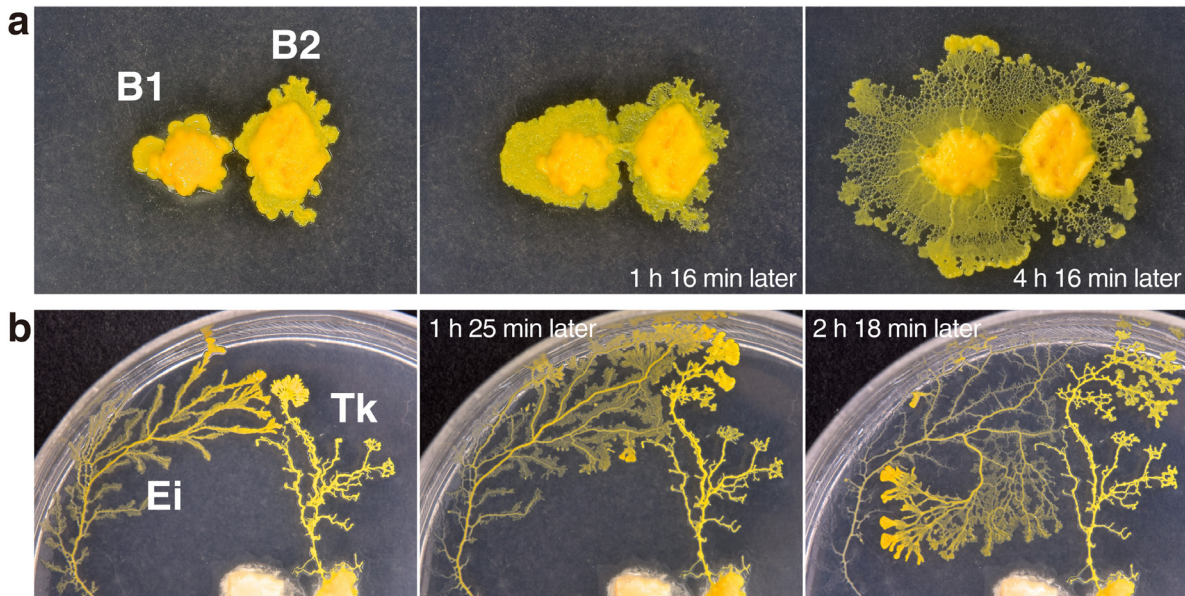
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Figure 4. Two typical encounter cases. (a) A fused case in which B1 and B2 completed fusion extremely smoothly to become a single individual. (b) An avoided case. As can be confirmed in this group of photographs, Ei and Tk encountered each other in at least five locations, recognized self and other, and chose to avoid the latter in all encounters. This avoidance behavior was very clearly observed.
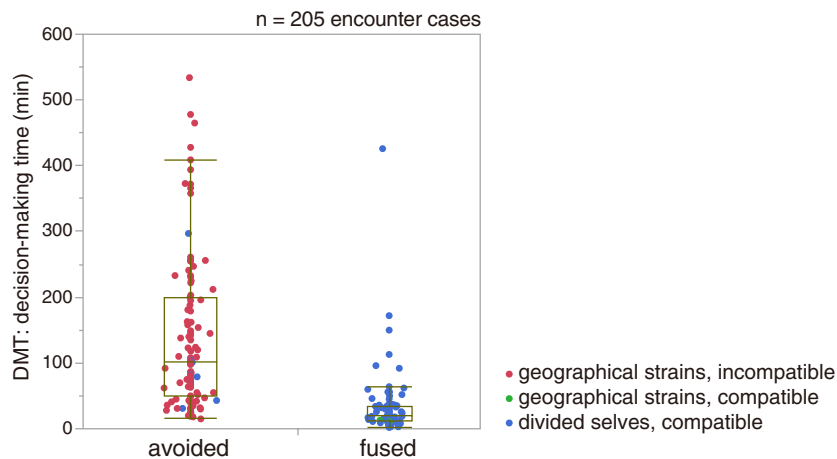

Figure 5. Comparison of DMT during the period when the two individuals encountered each other. The DMT was clearly longer with the avoided cases.

changing the direction of progression or retracting the part of their body. Even if two individuals came into contact, no change in the cell membrane was evident.

Within the combinations of divided selves (n = 101 encounter cases/79 experiments), the individuals failed to fuse in 10.9% of encounter cases (n = 11 encounter cases/8 experiments), but they all fused after one more or two encounters. Ultimately all 79 experiments were assigned into 'fusible and compatible'. These cases were the same as the B1–B2 cases, with a smooth transition through encounter, contact, and fusion.

When we compared fused and avoided encounter cases regarding the DMT, the avoided cases clearly tended to take much longer than the fused cases (figure 5). This tendency was significant based on the Kolmogorov–Smirnov test (D = 0.678 2634, p < .0001).

When two individuals encountered each other, in addition to the contact allorecognition cases where the cell membranes

of the two individuals came into direct contact (figure 6(a)), non-contact allorecognition cases were also observed, where the plasmodium recognized and maintained some distance from the other plasmodium (figure 6(b)). In these cases, the individuals stop the movement at a close distance from the other (in many cases, within a range of 0.1–1 mm). The deadlock state continues for from 13 min to approximately 7 h. Then, they resume moving forward and fuse each other or avoid by retracting the body or changing moving direction. From this, it can be seen that allorecognition is done without contact between cell membranes.

Non-contact allorecognition is thought to involve contact with a hyaline slime sheath that is secreted from the slime mold and subsequently spreads around the mold's exterior (figure 7). The spreading area of the slime sheath varies depending on the case, and the slime sheath of *P. rigidum* has high transparency, so in many cases its spread cannot be visually confirmed. In this experiment, it was confirmed that
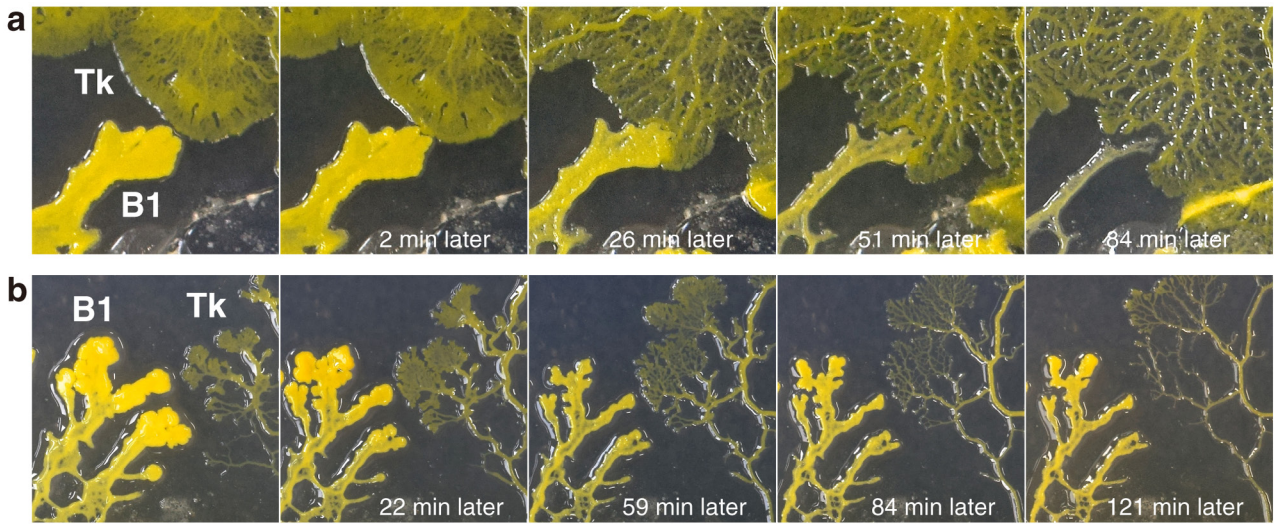
Figure 6. High magnification images of two typical avoided cases. (a) A case of contact allorecognition. After the cell membranes of the two plasmodia came into contact with the other, they then avoided each other. (b) A case of non-contact allorecognition. After the two individuals approached to approximately 0.2 mm, they avoided each other.
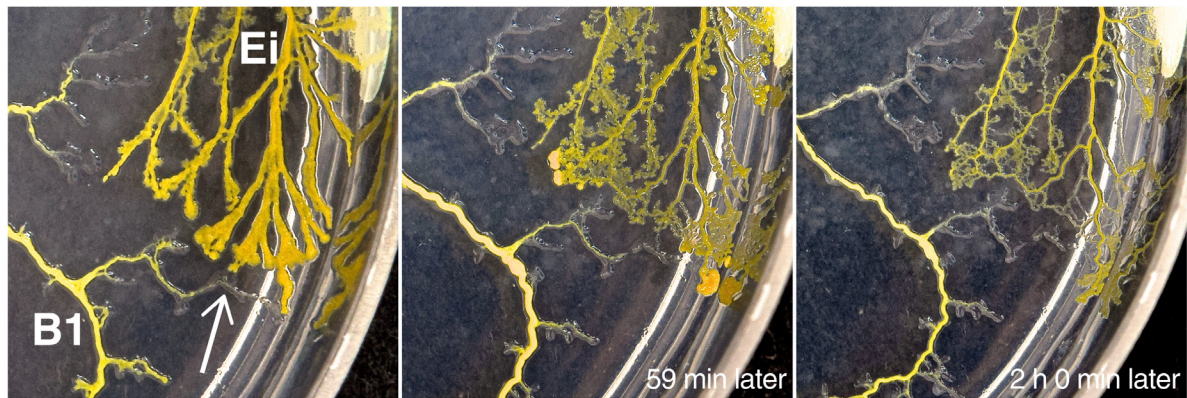

Figure 7. A case of non-contact allorecognition in avoided cases. Ei contact the B1 slime sheath, and then reversed the direction of progression to avoid B1. In the photograph, the hyaline slime sheath shed by B1 is clearly visible.

in at least 58.5% of the non-contact cases (n = 41 encounter cases), the deadlock states occurred within the spreading area of the slime sheath. It should be noted that this does not indicate that the remaining non-contact cases have no relationship with the slime sheath. Non-contact allorecognition was significantly more evident (p < .0001, Pearson's chi-square test) in avoided cases than in fused cases (figure 8). With the five non-contact, fused cases, after contact with the slime sheath and subsequent deadlock states continued for 13–60 min, the plasmodia processes of contact and fusion.

There was no correlation between DMT and DMD (figure 9). With contact allorecognition (DMD = 0) significant variation was seen in DMT, and quite many encounter cases were deadlocked for an extremely long time. Conversely, with non-contact allorecognition (DMD > 0), there was little variation in DMT, with most encounter cases concentrated within a short time.

Comparison of the DMT for the contact and non-contact types limited to the avoided cases revealed a longer time for the
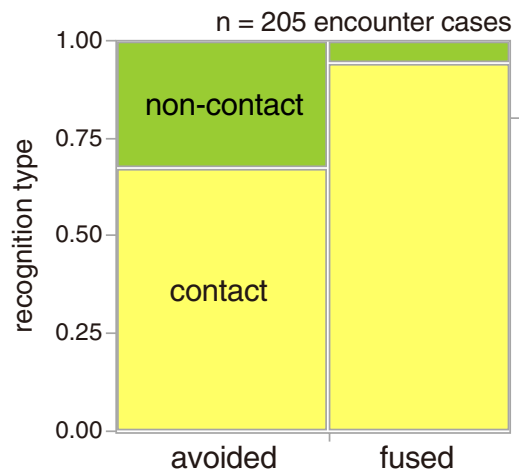

Figure 8. Comparison of the results when two allorecognition types encountered each other. Non-contact allorecognition was observed in 5.3% of fusion cases and 32.4% of avoided cases.
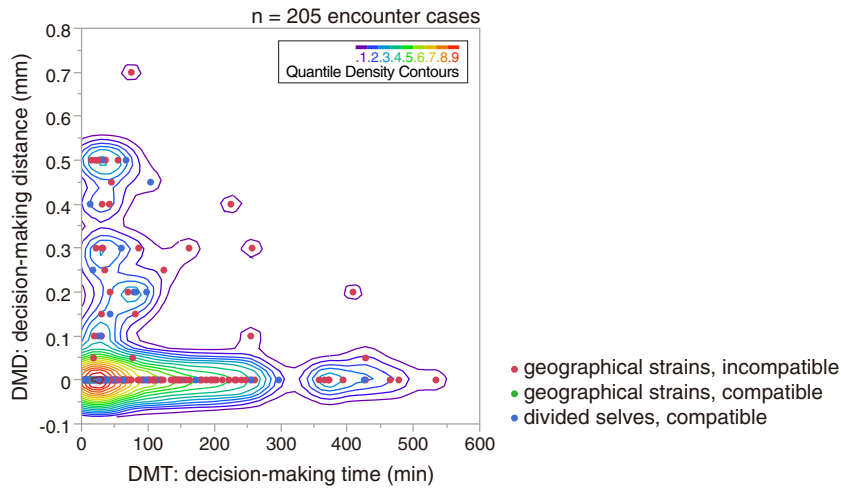
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Figure 9. Distributions of DMT and DMD during 205 encounter cases. While significant variation in DMT was seen with the contact type (DMD = 0), DMT for the non-contact type (DMD > 0) was concentrated in 150 min or less.
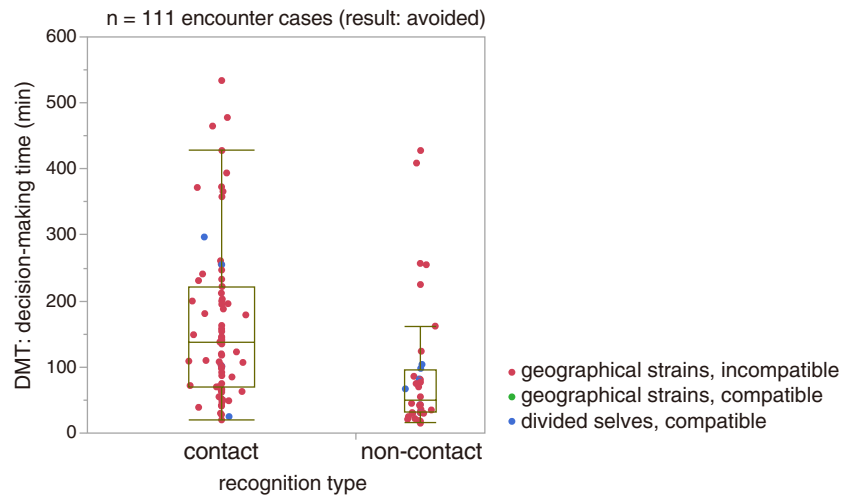

Figure 10. Comparison of DMT distribution for the contact and non-contact types for the avoided cases. The non-contact type tended to have shorter DMT than the contact type.

contact type, with a more rapid DMT for the non-contact type (figure 10). This behavior was significant (D = 0.443 3333, p = 0.0001, Kolmogorov–Smirnov test).

Discussion

Fusible and compatible combination of *P. rigidum* plasmodium geographical strains was rare (only one of the 10 combinations), consistent with the findings of previous genetic studies [3]. Plasmodium strictly identifies allogeneic individuals and adopts a fusion or avoidance behavior. This suggests that the plasmodium allorecognition system severely restricts fusion.

Whether plasmodium prioritizes the avoidance when encountering different individuals, as claimed in a previous study on *P. polycephalum* [13], was investigated. We found that even when *P. rigidum* plasmodium encountered an infusible and incompatible individual, it did not immediately avoid that individual. Instead, there was a period of assessment, during which the *P. rigidum* plasmodium drew closer or maintained
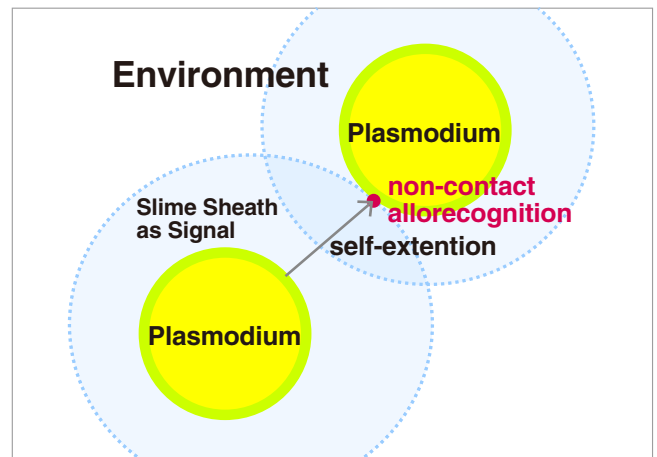

Figure 11. Self-extension model with non-contact allorecognition. Self-extension occurs using the slime sheath as a signal transmitted to the environment, which facilitates non-contact allorecognition.

contact until a final judgment was made. This behavior indicates that slime molds are searching for rare opportunities to

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fuse by taking some time to assess the encountered individual rather than prioritize the avoidance.

P. rigidum plasmodium recognizes self and other through contact with the secreted slime sheath as well as through direct contact between cell membranes. Non-contact allorecognition was seen not only in avoided encounter cases but also in fused ones, supporting the view that the slime sheath has functions other than evading other individuals. The results of this study suggest that in non-contact allorecognition, the slime sheath is a substance that functions to exchange signals with other individuals that helps determine recognition of self and other, rather than as a substance used to avoid other individuals [13] or as external memory of the plasmodium to streamline its' own actions [10]. Therefore, the signals could be regarded as a third role of the slime sheath, in addition to the known roles of movement and moisture retention.

The speed of decision-making in non-contact allorecognition (which exceeds contact type) and the ability to make decisions at a distance from the other individual without having to make a connection suggest that the non-contact type is an advantageous method of allorecognition for avoidance. In fact, the non-contact type occurs more frequently with avoidance than with fusion cases. It is thought that plasmodia expand their opportunities for decision-making by using the non-contact type of recognition, which enables early and safe allorecognition.

P. rigidum plasmodium seeks to fuse with the individuals it encounters. However, this creates a dilemma, since it wants to avoid all but a few select individuals. The mechanism of non-contact allorecognition enables the plasmodium to safely achieve both fusion and avoidance. To date, plasmodium allorecognition has been based on the premise of contact. The slime sheath has not been considered to be involved in allorecognition, instead has been regarded as just a simple repellent [10, 13]. However, the present observations indicate that the slime sheath is a substance that disperses allorecognition information about itself into the environment. This view led to the self-extension model, which posits that the plasmodium facilitates allorecognition through self-extension by transmitting information about itself (figure 11).

Outlook

Progress with verification of this self-extension model

Non-contact allorecognition using a slime sheath may be common to a wide range of myxomycete plasmodia. *P. rigidum* was used in this study. However, based on previous experience with wild strains, similar phenomena have been seen with *P. melleum* and *P. roseum*. It has also done with *P. polycephalum*. We would like to confirm the self-extension model in these various species.

We are currently conducting analysis of the slime sheath aiming to identify the signal substance involved in allorecognition in the self-extension model. Identification of signal substance and clarification of its function enable us to discover the shared mechanism among various species of slime molds and to conduct more precise tracking of allorecognition behavior, which has previously been limited to observation studies. Through the precise analysis of allorecognition behavior controlled by signal substance in slime sheath, it may be demonstrated that all contact type cases have aspects of the non-contact type during the allorecognition process. In other words, all plasmodium allorecognition can be explained by the self-extension model.

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