J. Parasitol., 98(5), 2012, pp. 946–950

EXCYSTATION SIGNALS DO NOT ISOLATE GREGARINE GENE POOLS: EXPERIMENTAL EXCYSTATION OF *BLABERICOLA MIGRATOR* AMONG 11 SPECIES OF COCKROACHES

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ABSTRACT: An experimental excystation assay was used to test the potential species isolating effects of excystation signaling among gregarines. Oocysts of a single gregarine species, *Blabericola migrator*, were tested for activation, excystation, and sporozoite motility by using intestinal extracts from 11 species of cockroaches representing a cohesive phylogeny of 7 genera, 3 subfamilies, and 2 families of Blattodea. Sporozoite activation, excystation, and motility were observed for all excystation assay replications using intestinal fluid from blaberid hosts, but delayed activation or excystation was observed for all assay replications using intestinal fluid from hosts in the family Blattidae. The results illustrate a trend toward a generalized excystation signal among gregarines that is conserved across the host clade at a subfamily or family level but that is unlikely to play a significant role as a species-isolating mechanism among sibling gregarine species.

Host specificity is a vague term that is not without conceptual merit, i.e., parasites have evolved in concert with their hosts and these evolutionary changes tend to increase compatibility, transmissibility, and parasite survival. The term is vague simply because it encompasses too many notional ideas and phenomena under a single rubric. Although often conceived as a compilation of known hosts, host specificity has intellectual utility only when it extends beyond a basic list of host species from which a parasite species occurs and begins to address patterns of evolutionary or ecological significance (see Barger, 2011 on the ecological significance of host specificity and host equivalency). Host specificity is a function of both physiological and ecological components. The physiological component is endogenous and evolutionary, reflecting basic signaling, recognition, and compatibility in a host–parasite relationship. The relevant question is, can this parasite recognize, infect, and complete its life cycle in this host? The ecological component is exogenous and contemporary, reflecting the encounter dynamics of hosts and parasite transmission stages. In this case, the relevant question is, does this parasite species have sufficient spatio-temporal overlap with this host species to establish a stable infective cycle?

Gregarine host specificity can be enforced during any 1 of 4 phases of the gregarine life cycle, i.e., gametogony and sporogony, excystation, establishment and growth, and sexual association and syzygy. Gametogony and sporogony are environmentally mediated processes correlated with abiotic conditions of the host niche, restricting encounter rates by influencing the distribution of oocysts in the environment and reflecting an ecological component of host specificity. Gregarines whose gametocysts cannot survive to produce infective oocysts under a host's preferred niche conditions will not have sufficient host species overlap and interaction to establish a stable infective cycle. In contrast, excystation is a signaling and recognition event, whereas establishment, growth, sexual association, and syzygy are compatibility-dependent processes. Together, they reflect evolved physiological components of host specificity and failure of a gregarine species to successfully passage any single phase in a suitable host species enforces stricter host specificity, ultimately leading to gene pool isolation and species divergence.

Received 23 February 2012; revised 24 April 2012; accepted 27 April 2012.
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DOI: 10.1645/GE-3122.1

Previous studies of gregarine host specificity are either empirical tests of excystation or infection (Corbel, 1968; Clopton et al., 1992; Clopton and Janovy, 1993; Clopton and Gold, 1995, 1996; Watwood et al., 1997; Wise et al., 2000; Smith and Cook, 2008) or depend upon conclusions drawn from observational and survey studies (Corbel, 1971; Clopton, 2004, 2006; Clopton et al., 2004; Cielocha et al., 2011). For any empirical test of host specificity, the level of perceived host specificity is a function of the relatedness of the experimental host taxa. Prior studies of host specificity reflect the ready availability of experimental host taxa rather than a cohesive host phylogenetic context. To accurately appraise host specificity across a host lineage, experimental host taxa must be chosen to reflect a series of more inclusive outgroups. The study presented here uses an experimental excystation technique to test the host specificity of *Blabericola migrator* across 11 species of cockroaches representing 7 genera, 3 subfamilies, and 2 families of Blattodea.

MATERIALS AND METHODS

An experimental excystation assay was used to evaluate the suitability of 11 cockroach species for oocyst activation and excystation of *B. migrator*, which is described from *Gromphadorhina portentosa*, but also normally infects *Princisia vanwaerebecki* (Clopton, 1996a, 1996b). For each cockroach species tested, 3 replicates of the excystation assay were conducted. Excystation assays using intestinal extracts of *P. vanwaerebecki* served as a positive control for the technique. Gregarine and cockroach culture, collection of infective oocysts, preparation of host intestinal extracts, and the excystation assay itself are described individually, as follows.

Gregarine and cockroach culture

Blabericola migrator maintained in vivo in breeding colonies of *P. vanwaerebecki* provided oocysts for experimental excystation studies. *Archimandrita tessellata*, *Blaberus giganteus*, *Blaberus craniifer*, *Blaberus discoidalis*, *Henschoutedenia flexivitta*, *G. portentosa*, *Gromphadorhina oblongonota*, *Elliptorhina chopardi*, *Periplaneta americana*, and *Periplaneta australasiae* maintained in breeding colonies provided host intestinal fluids for experimental excystation studies. Breeding cockroach colonies were maintained individually in 22-L polycarbonate containers with coir bedding and cardboard egg crate roosting habitat. Food (Complete & Balanced Purina® Dog Chow®, Nestle Purina Pet Care Company, St. Louis, Missouri) and water were provided ad libidum.

Collection of infective oocysts

Gregarine gametocysts were collected from *P. vanwaerebecki* by isolating 30–60 cockroaches in groups of 15–20 individuals in stacked 250-ml glass Carolina culture dishes (Carolina Biological Supply Company, Burlington, North Carolina). Groups were maintained in

isolation for 24 hr, and gametocysts were harvested from their accumulated feces as follows. Feces were collected and softened by soaking in half-strength Griffiths & Tauber saline (120 mM NaCl, 1.35 mM KCl, 1.8 mM $CaCl_2$, and 1.2 nM $NaHCO_3$; modified from Griffiths and Tauber [1943]). Gametocysts teased and collected from softened feces were triple rinsed in saline to reduce surface contamination by bacterial and fungal spores, transferred to 7-mm black cardstock disks saturated with a 0.1% aqueous methylparaben (methyl parahydroxybenzoate) solution, and placed in the well of a 60-mm center-well organ culture dish (BD Falcon™; BD Biosciences, Franklin Lakes, New Jersey). The outer trough of the dish was filled with a hydrating gel of finely milled cross-linked polyacrylamide (SoilMoist Granules®; JRM Chemical, Inc., Cleveland, Ohio) saturated with 0.1% aqueous methylparaben solution. The hydrating gel provides consistent high humidity for gametocyst development and dehiscence. Each dish was covered and placed inside a 100-mm glass Petri dish to reduce desiccation and maintained at 25 C for gametocyst development. Gametocysts matured and dehisced to produce infective oocysts for experimental studies within 3–4 days.

Preparation of cockroach intestinal extracts

Cockroach intestinal extracts were prepared by removing the intestinal tract from a single cockroach and extracting the fluid from the midgut lumen by using a 5-µl Drummond Wiretrol capillary tube and Microcap bulb with silicone tip (Drummond Scientific Company, Broomall, Pennsylvania). For each preparation, 2.5 µl of luminal fluid was collected, the microcap bulb was removed, and the end of the capillary tube was sealed with Critoseal® (Oxford Labware, St. Louis, Missouri). Prepared tubes were spun in a hematocrit centrifuge for 45 sec to separate bacteria and particulates from the intestinal fluid itself. Removal of bacteria and particulates enabled clear observation of changes in oocysts placed in the intestinal fluid and prevented bacterial overgrowth of experimental preparations.

Excystation assay

For each assay, a subsample of centrifugally cleared host intestinal fluid was placed on a 12-mm circular coverslip with several thousand *B. migrator* oocysts, and then the coverslip was inverted onto a glass slide to make a temporary wet mount. Each coverslip was sealed to its microscope slide with a meniscus of paraffin oil to prevent desiccation. Excystation preparations were observed at ×800 magnification by using a B-Max 50 compound microscope (Olympus, Tokyo, Japan) with 10× eyepieces, 40× universal planapochromatic objectives with phase contrast condensers, and an infinity-optics turret doubler. Observations were made at 5-min intervals for 1 hr, or until excystation was apparent in a majority of oocysts in the preparation. The excystation assay was replicated 3 times for each cockroach species in the study.

Host phylogeny

Cytochrome oxidase II sequence data was used to produce a maximum likelihood phylogeny for host taxa in the study. Groups, taxa, and corresponding GenBank accession labels were as follows: Blattodea, Blaberoidea, Blaberidae, Blaberinae: *Archimandrita tessellata* (DQ874263), *Blaberus craniifer* (DQ181518), *Blaberus discoidalis* (AB014063), *Blaberus giganteus* (EF363219); Blattodea, Blaberoidea, Blaberidae, Blaberinae: *Elliptorhina chopardi* (DQ874278), *Gromphadorhina oblongonota* (DQ874287), *Gromphadorhina portentosa* (EF363207), *Henschoutedenia flexivitta* (DQ874291), *Princisia vanwaerebecki* (DQ874318); and Blattodea, Blattoidea, Blattidae, Blattinae: *Periplaneta americana* (PELMTCOII), *Periplaneta australasiae* (DQ874310). Blattodea, Polyphagoidea, Polyphagidae, Polyphaginae: *Polyphaga aegyptiaca* (AB014069) served as an outgroup for rooting. The resulting topology corresponds to those of Kambhampati (1995) and Inward et al. (2007) but includes species relationships included in neither of these studies.

RESULTS

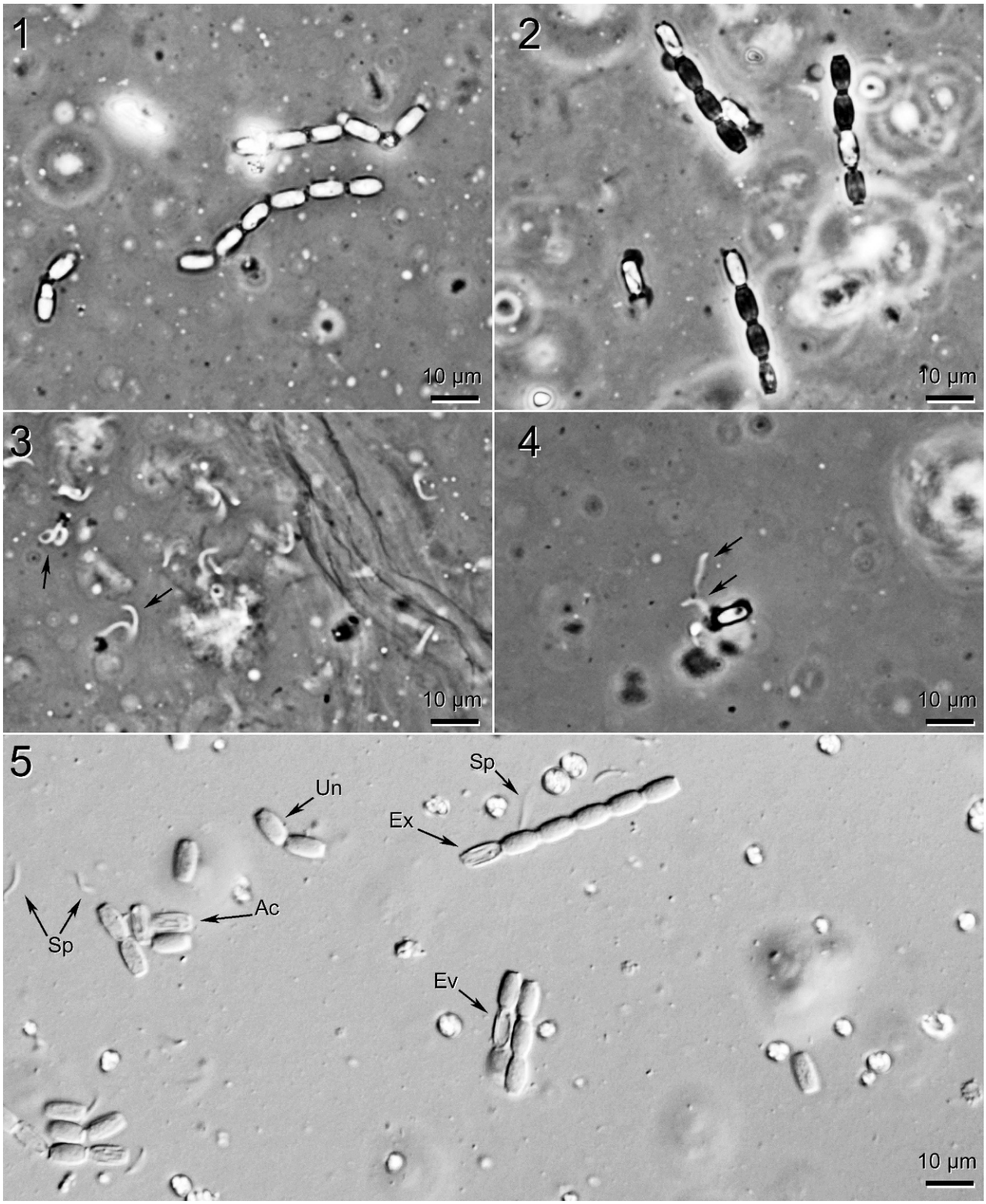
The normal response of oocysts to the excystation assay using *P. vanwaerebecki* intestinal extract is characterized by activation of sporozoites within the oocyst, sporozoite excystation, and sporozoite motility (Figs. 1–5). Initially oocysts are clear and glassine, with no visual evidence of internal dormant sporozoites (Figs. 1, 5). Activation of sporozoites is marked by darkening of individual sporozoites within the oocyst until both become opaque. Although activated, at this point sporozoites remain quiescent (Figs. 2, 5). Excystation is marked by emergence of sporozoites through polar channels in the oocyst wall. On contact with the surrounding host intestinal fluid, sporozoites become motile and begin to swim with a sinuate nematode-like motion (Figs. 3, 5). Once fully emerged, sporozoites remain motile and move away from the oocyst. Evacuated oocysts are visually distinct from unactivated oocysts. The oocyst wall in an unactivated oocyst appears as a boundary margin, whereas the thickness of the oocyst wall and polar channels is apparent in an evacuated oocyst (Figs. 4, 5).

Sporozoite activation, excystation, and motility were observed for all excystation assay replications using intestinal fluid from *P. vanwaerebecki*, *E. chopardi*, *G. portentosa*, *G. oblongonota*, *B. giganteus*, *B. craniifer*, *B. discoidalis*, and *H. flexivitta*. In these assays, sporozoite activation was complete within 15 min of exposure to intestinal fluid, excystation began 15–20 min post-exposure, and sporozoites remained motile well over 1 hr. Sporozoite activation, excystation, and motility were observed for all excystation assay replications using intestinal fluid from *A. tessellata*, but in all replications the excystation was delayed by 5–10 min and the overall rate of activation and excystation was notably lower (approximately an order of magnitude lower by visual estimate) than for the aforementioned host species. For all excystation assay replications using intestinal fluid from *P. australasiae*, sporozoite activation occurred normally (within 15 min), but excystation was delayed by 40–50 min. Post-excystation sporozoite motility was normal. For all excystation assay replications using intestinal fluid from *P. americana*, sporozoite activation occurred normally (within 15 min), but no sporozoite excystation or motility was observed, although observations were extended to 90 min post-exposure.

DISCUSSION

Results of experimental excystation studies are summarized in the context of the host phylogeny in Figure 6. Sporozoites of *B. migrator* activated, excysted, and demonstrated normal motility across all of the oxyhaloinid and most of the blaberinid cockroaches included in the study. Delayed excystation was observed only in *A. tessellata*, the most phylogenetically distant blaberinid cockroach in the study. Excystation was either delayed or not observed in both cockroach species representing Blattidae. *Blabericola migrator* was originally described from *G. portentosa*. Accordingly, successful excystation is expected in the internal *Gromphadorhina–Princisia* clade. The remaining pattern of successful excystation indicates that host signals eliciting activation, excystation, and motility in sporozoites of *B. migrator* are conserved across all blaberinids tested but that these signals are not fully conserved among blattids. These results support the conclusion that the host signals in the intestinal lumen eliciting activation, excystation, and motility in gregarine sporozoites are generalized signals rather than host specific signals, at least in the gregarines parasitizing blaberoideans.

Host specificity studies are more than questions of who infects whom. They are fundamental to understanding species boundar-



FIGURES 1–5. Excystation of *Blabericola migrator* oocysts in vitro. (**1**) Oocysts in host gut fluid before activation (phase contrast microscopy). Note the transparent nature of enclosed, quiescent sporozoites within each oocyst. (**2**) Activated oocysts in host gut fluid (phase contrast microscopy). Sporozoites become opaque within activated oocysts that appear dark under phase contrast microscopy. (**3**) Excysted sporozoites free in host gut fluid (phase contrast microscopy). Sporozoites are light colored and mobile, exhibiting a serpentine, nematode-like swimming motion. (**4**) Evacuated oocyst with free sporozoites in host gut fluid (phase contrast microscopy). (**5**) Oocysts and sporozoites in host gut fluid (differential contrast microscopy). Ac, unexcysted oocyst with activated sporozoites; Ev, evacuated oocyst; Ex, oocyst with excysting sporozoite; Sp, free sporozoite; Un, unexcysted oocyst with dormant sporozoites.

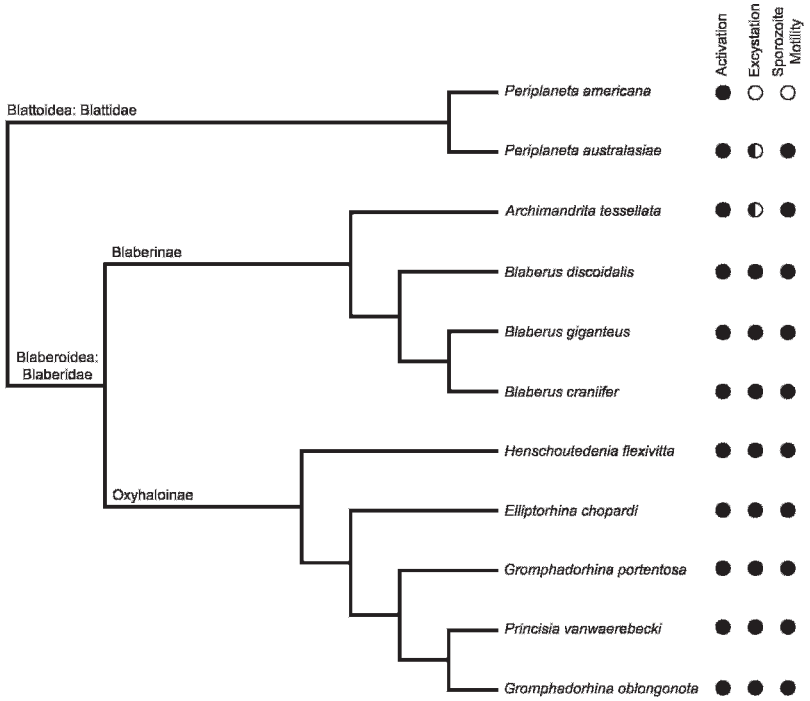


FIGURE 6. In vitro activation, excystation, and motility of *Blabericola migrator* sporozoites across the phylogeny of 11 species of cockroaches. (●, normal activation, excystation or motility; ◐, delayed excystation; ○, no excystation or motility).

ies and gene pool isolating mechanisms acting in parasitic lineages. Regardless of their specific form, species isolating mechanisms in parasitic lineages must prevent reproductive overlap in the definitive host. Several generalized gene pool isolating mechanisms have been proposed for gregarines parasitizing insects, postulating both exogenous and endogenous isolating effects.

Exogenous gregarine gene pool isolating mechanisms are the result of interactions between the host, the parasites' infective stages, and the environment. These mechanisms are ecological, depending upon overlap and interaction of host and parasite populations, given physiological compatibility of host and parasite species. Clopton and Janovy (1993) hypothesized that host specificity in the gregarine complex infecting *Tenebrio molitor* was reinforced by overlap of the host niche with the environmental requirements for gametocyst development. Similar examples of host niche fidelity aligned with gregarine gametocyst development requirements have been postulated for gregarines infecting tetrigid grasshoppers (Clopton et al., 2004). Host-mediated isolation is a variation of this phenomenon in which niche specialization and fidelity on the part of the host prevents cross-transmission of gregarines among related hosts in gregarines parasitizing calopterygid damselflies (Clopton, 2004) and tenebrionid beetles (Clopton, 2006).

Endogenous gregarine gene pool isolating mechanisms are the result of structural, metabolic, or physiological interactions between hosts and infective gregarine life cycle stages. These mechanisms are evolutionary or phylogenetic in nature, depending upon evolved compatibility among species. They may be simple physiological effects such as host intestinal pH (Clopton and Gold, 1995) or more complex physiological effects that seem to signal host suitability to an infective oocyst (Corbel, 1968; Patil et al., 1985; Clopton and Gold, 1996; Smith and Cook, 2008).

Corbel (1968) used experimental cross-infections to evaluate host specificity among 6 species of gregarines and 23 species of crickets and grasshoppers, experimentally testing 79 of 138 possible host–gregarine combinations and concluding that host specificity of gregarines infecting orthopteran hosts was a function of host superfamily rather than host genus or species. In contrast, Patil et al. (1985) conducted cross-excystation and cross-infection experiments using 9 gregarine species (3 genera, 1 family) and 8 species of tenbrionid beetles (7 genera, 4 tribes, 2 subfamilies, 1 family). In their experiments, all gregarine species excysted and established experimental infections in their natural hosts, but only 5 gregarine species excysted and no gregarine successfully established an infection in an experimental host species. It is worth noting that although most experimental excystations were unsuccessful, all successful experimental excystations were reciprocally successful; where experimental excystation was successful, host pairs were equally competent to elicit excystation in the normal gregarine parasites associated with each host species even though they were incapable of supporting establishment and infection.

Clopton and Gold (1996) used reciprocal cross-infections and reciprocal cross-excystation assays to test the host specificity of a single gregarine, *Gregarina blattarum*, across 5 species of domiciliary cockroaches. Although *G. blattarum* was able to excyst in all 5 cockroach taxa, infections were successfully established only in the natural host *Blatella germanica*. Similarly, Smith and Cook (2008) used reciprocal cross-infections and reciprocal cross-excystation assays to test the host specificity of 5 gregarine species across 6 cockroach host taxa. In contrast to Clopton and Gold (1996), Smith and Cook (2008) reported absolute host specificity mediated by excystation; gregarines could excyst or establish infections only in their natural hosts.

These and other studies of gregarine host specificity exist (Corbel, 1968, 1971; Clopton et al., 1992; Clopton and Gold, 1996; Watwood et al., 1997; Wise et al., 2000; Smith and Cook, 2008; Cielocha et al., 2011), but most lack a phylogenetic context. Thus, they often attempt to cross-infect distantly related hosts with distantly related gregarines and accept the failure to cross-infect as general evidence of strict host specificity among gregarines. But gregarine host specificity reflects a continuum of restrictivity. Although generally thought of as a ''1 host species for each gregarine species'' arrangement, host specificity can constrict to developmental stages of a single host species (Clopton et al., 1991, 1992) or expand to include several related host species (Corbel, 1968, 1971; Levine, 1988; Cielocha et al., 2011), so there is little evidence to suggest that the host specificity of gregarines justifies a maxim of ''different host, different gregarine.''

Our study illustrates a trend toward a generalized excystation signal among gregarines that is conserved across the host clade at a subfamily or family level. This trend corroborates the results of Corbel (1968), Patil et al. (1985), and Clopton and Gold (1996), but it stands in stark contrast to the results of Smith and Cook (2008). This discrepancy may be a methodological artifact. Smith and Cook (2008) used a buffer solution to dilute the host gut extracts used in their study, and this dilution may have reduced potential signaling or enzymatic effects to a sub-threshold level in experimental hosts. It should be noted that our study considers the response of a single gregarine species across an array of host taxa. Future, reciprocal studies using gregarine species that naturally infect other host taxa in the array can determine the degree to which this phenomenon is generalized. Nonetheless, the preponderance of available evidence supports the conclusion that excystation signaling is a relatively conserved, generalized phenomenon that reflects ancient associations between gregarines and their host lineages and that excystation signaling is unlikely to play a significant role as an endogenous species-isolating mechanism among gregarine sibling species.

ACKNOWLEDGMENTS

This material is based upon work supported in part by National Science Foundation grant NSF DEB-1019419. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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