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Discrimination, Crypticity, and Incipient Taxa in *Entamoeba*[1]

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ABSTRACT. Persistent difficulties in resolving clear lineages in diverging populations of prokaryotes or unicellular eukaryotes (protistan polyphyletic groups) are challenging the classical species concept. Although multiple integrated approaches would render holistic taxonomies, most phylogenetic studies are still based on single-gene or morphological traits. Such methodologies conceal natural lineages, which are considered "cryptic." The concept of species is considered artificial and inadequate to define natural populations. Social organisms display differential behaviors toward kin than to nonrelated individuals. In "social" microbes, kin discrimination has been used to help resolve crypticity. Aggregative behavior could be explored in a nonsocial protist to define phylogenetic varieties that are considered "cryptic." Two *Entamoeba invadens* strains, IP-1 and VK-1:NS are considered close populations of the same "species." This study demonstrates that IP-1 and VK-1:NS trophozoites aggregate only with alike members and discriminate members of different strains based on behavioral and chemical signals. Combined morphological, behavioral/chemical, and ecological studies could improve Archamoebae phylogenies and define cryptic varieties. Evolutionary processes in which selection acted continuously and cumulatively on ancestors of *Entamoeba* populations gave rise to chemical and behavioral signals that allowed individuals to discriminate nonpopulation members and, gradually, to the emergence of new lineages; alternative views that claim a "Designer" or "Creator" as responsible for protistan diversity are unfounded.

Key Words. Aggregation, behavior, chemical signals, cryptic species, design creationism.

AMOEBAE and amoeboid protists have transitioned from studies based on single morphological traits (pseudopodia; Levine et al. 1980), to single gene (subunit rRNA—SSU rRNA; Adl et al. 2005; Burki and Pawlowski 2006; Pawlowski and Burki 2009; Stensvold et al. 2010; Stensvold et al. 2011), and to multigene (Bapteste et al. 2002; Pawlowski and Burki 2009) phylogenies at the supertaxon, supergroup, class, genus, or species levels. Single-gene analyses of metabolic traits (e.g. alcohol dehydrogenase *adhe*) contribute to conflictive phylogenetic depictions, due to horizontal acquisition (horizontal gene transfer, HGT) of genes from prokaryotes and unicellular eukaryotes (Andersson 2005; Andersson et al. 2006; Bapteste and Boucher 2009; Espinosa et al. 2001, 2009; Paz-y-Miño-C. and Espinosa 2010).

In *On the Origin of Species* (1859), Charles Darwin questioned whether the species concept reflected natural groups, "we shall have to treat species in the same manner as those naturalists treat genera, who admit that genera are merely artificial combinations made for convenience ··· we shall at least be freed from the vain search for the undiscovered and undiscoverable essence of the term species." Persistent difficulties in resolving clear lineages have questioned the species concept to describe diverging populations of prokaryotes (Bacteria and Archaea, Doolittle and Zhaxybayeva 2009; Pace 2006) or unicellular eukaryotes (protistan polyphyletic groups, Finlay 2004). Although it is widely accepted that value of multiple integrated approaches would render "holistic taxonomies" (Finlay 2004; Pawlowski and Burki 2009), most phylogenetic studies are still based on single-gene or morphological traits. Such methodologies conceal natural lineages, which are considered "cryptic" (Mallet 2010). Stebbins (1950) defined cryptic species as " ··· population systems, which were believed to belong to the same species until genetic evidence showed the existence of isolating mechanisms separating them". In protists, unresolved phylogenies generate heated debates to unravel "cryptic" morphospecies (populations of unicellular eukaryotes with similar physiological properties and mating incompatibilities; Caron et al. 2009; Finlay 2004). Phylogenetic analyses would benefit from the integration of physiological, ecological, and behavioral studies. Most phylogenies of the *Entamoeba* lineage are based on SSU-rRNA analyses (Stensvold et al. 2010, 2011).

Kin discrimination has been suggested as a behavioral mechanism in social microbes to resolve "cryptic" varieties, given that individuals in a population signal to "like" individuals (Kalla et al. 2011; Pérez-Ponce de León and Nadler 2010; Sáez and Lozano 2005). In single or multicellular organisms, behavior toward kin differs from interactions with nonkin (Hamilton 1964; Queller et al. 2003; Wilson 2000). Altruistic behavior increases toward members of the same type, a phenomenon known as "the green beard effect" (Dawkins 1976). Discrimination at the unicellular level has been detected in bacteria (*Myxococcus xanthus*; Velicer and Vos 2009; Vos and Velicer 2009) and protists (*Dictyostelium, Polysphondylium violaceum;* Kalla et al. 2011; Li and Purugganan 2011; Mehdiabadi et al. 2006; Ostrowski et al. 2008; Queller et al. 2003). The *Entamoeba* lineage is an ideal model to combine discrimination in a nonsocial ameba with morphological, multigene, and ecological studies in attempting to resolve phylogenetic varieties considered "cryptic."

Here, we demonstrate that two strains of *Entamoeba invadens* (IP-1 and VK-1:NS) aggregate only with members of their own population suggesting they distinguish members of the same strain based on chemical and behavioral signals. Discrimination could help resolve conflictive branching and crypticity among *Entamoeba* varieties and demonstrate the evolutionary history of this lineage. We discuss how gradual genetic changes combined with selective pressures, that gave strain members the ability to discriminate between alike and non-alike individuals, drove the diversification of the *Entamoeba* lineage; we contrast this analysis with proposals invoking "common design" (independent major taxa emergence with no common ancestry; Nelson 1996) and "special *Entamoeba* creation" (Sherwin 2009), which are unfounded.

MATERIALS AND METHODS

Cells and reagents. All ameba cultures were obtained from Dan Eichinger (NYU School of Medicine) and Graham Clark (London School of Hygiene and Tropical Medicine).

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Entamoeba invadens strains IP-1 and VK-1:NS were grown at 25 °C under axenic conditions in flat-bottomed 48-well plates containing 1.4 ml of TYI-S-33 (Diamond 1968) containing 10% ABS (Sigma-Aldrich, St. Louis, MO) modified from Espinosa et al. (2001, 2009). Additional media components were purchased from Fisher Scientific (Agawam, MA), Sigma-Aldrich, and Atlanta Biologicals (Atlanta, GA). Growth counts were averaged from three replicate wells and three separate experiments.

Growth conditions and trophozoite growth rates. *Entamoeba invadens* strains were inoculated at 4×10^2 cells/ml in 1.4 ml of media in 48 well plates and incubated at 25 °C. Every 24 h aliquots of cells were harvested by chilling, and the cell density was determined using the Cellometer Vision HS RF-150 (Nexcelom BioScience LLC, Lawrence, MA). Trophozoites were subcultured every 72 h by transferring 4×10^2 cells/ml of culture into 1.4 ml of fresh medium and repeating the incubation and cell density determinations as described. Each time point cell density value was determined using triplicate cultures.

Morphological and aggregative measurements. Cell density, cell size, cell spatial distribution, number of aggregated trophozoites, number of aggregated clusters per surface area, and average distance between clusters were examined using a Zeiss Axiovert 40 CFL fluorescent microscope (10X or 32X; Micro-Tech Optical, New England Inc., Bloomfield, CT). Digital images of each well (1.4 ml) were analyzed to determine interactions among strain and nonstrain members. Images were processed with the Image-Pro Software (Micro-Tech Optical, New England Inc.).

Fluorescent labeling of *Entamoeba invadens* cells. CellTracker Red and Green CMFD (Invitrogen, Carlsbad, CA) were used to fluorescently label *E. invadens* VK-1:NS and IP-1 cells. Briefly, 1×10^5 trophozoites were harvested by chilling and centrifuged at 1,361 *g* for 20 min. Trophozoite pellets were resuspended gently in CellTracker Red (1:3 dilution in DMSO) and CellTracker Green CMFD (1:100 dilution in DMSO). Two incubation periods (30 min) followed by a 5-min PBS plus formaldehyde fixing period and a final resuspension in 1.4 ml media were performed following the manufacturer's protocol. Cells were then incubated at 25 °C for 36 h and analyzed at 12, 14, 18, and 36 h following the dyeing procedure. All experiments were performed in triplicate. To eliminate potential toxicity of both dyes, nondyed trophozoites were analyzed at the same time points. Table 1 shows the six combined sets. *Entamoeba invadens* IP-1 and VK-1:NS strains were dyed with both fluorescent tags, alone and together.

Table **1.** Experimental combinations of *Entamoeba invadens* IP-1 and VK-1:NS labeled with CellTracker Red and/or Green CMFD fluorescent tags (Invitrogen).

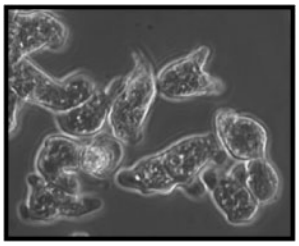
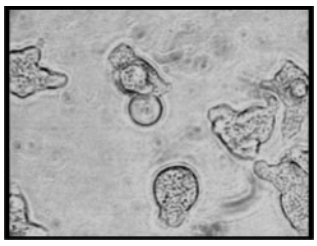
Unlabeled	Labeled (green or red)
IP-1/VK-1:NS	IP-1 (green)/VK-1:NS (red)
	VK-1:NS (green)/IP-1 (red)
IP-1 alone	IP-1 (green)/IP-1 (red)
VK-1:NS alone	VK-1:NS (green)/VK-1:NS (red)

RESULTS

Entamoeba invadens IP-1 and VK-1:NS aggregate with members of their own strain and maintain separation from clusters of nonalike ameba. Quantitative data of individual trophozoites (average length, width, and surface area) show that both strains are morphologically distinguishable when combined traits are examined. As seen in Table 2, IP-1 is larger, wider, and elongated (length 28.77 ± 3.52 μm; width 23.04 ± 1.81 μm) and VK-1:NS is smaller, narrower, and rounded (length 21.20 ± 2.04 μm; width 17.50 ± 1.45 μm). The average distance between IP-1 clusters is smaller (17.10 μm) than between VK-1:NS clusters (69.71 μm) when examined at the three time points (12, 18, and 36 h).

Pair combinations of IP-1/VK-1:NS labeled with green/red or, in the reciprocal, red/green dyes (Table 1) were placed together and grown in the same well. IP-1 trophozoites formed distinct and separate color clusters, which expanded in 12, 18, and 36 h without mixing with members of the other strain; a similar pattern of fluorescent single color clusters was observed for VK-1:NS trophozoites. For example, IP-1 aggregated in green clusters; VK-1:NS in red clusters; or IP-1 aggregated in red clusters; VK-1:NS in green clusters (Fig. 3–8). In contrast, when *E. invadens* VK-1:NS trophozoites were labeled with green and red dye and placed together in the same well, aggregation occurred between all trophozoites, showing strong strain association behavior. Large fluorescent yellow clusters (green + red) increased after 12, 18, and 36 h (Fig. 9–11). Similar behavior was shown by pair combinations of *E. invadens* IP-1 trophozoites that were labeled with green/red dyes (Fig.

Table **2.** Phenotypic characterization of *Entamoeba invadens* IP-1 and VK-1:NS.

Characteristics	*Entamoeba invadens* IP-1	*Entamoeba invadens* VK-1:NS
Average length (μm)	28.77 ± 3.52	21.20 ± 2.04
Average width (μm)	23.04 ± 1.81	17.50 ± 1.45
Number of ameba per cluster (μm)	> 20	> 20
Average distance between clusters (μm)	17.10	69.71
Unlabeled trophozoites		

12–14). Histograms showing fluorescent yellow surface area at 12, 18, and 36 h for aggregating strain members (IP-1/IP-1 or VK-1:NS/VK-1:NS two-color overlapping clusters) are depicted in Fig. 15. IP-1/VK-1:NS growing together showed little or no mixed aggregation, no yellow overlapping clusters (Fig. 15). There was no detectable toxicity in the trophozoites with either dye for the length of the experiments (36 h, control data not shown).

DISCUSSION

Entamoeba invadens IP-1 and VK-1:NS have been studied as molecular models of encystation (Byers et al. 2005; Mitra et al. 2010), or as part of single-gene phylogenetic analyses of the *Entamoeba* lineage (Stensvold et al. 2010, 2011). Little is known about their morphological, ecological, and behavioral properties or their evolutionary history inside their reptilian hosts' gut. Based on combined measurements of individual trophozoites (average length, width, Table 2), we established clear differences: IP-1 is larger, wider, and elongated (length 28.77 ± 3.52 μm; width 23.04 ± 1.81 μm, Fig. 1) and VK-1:NS is smaller, narrower, and rounded (length 21.20 ± 2.04 μm; width 17.50 ± 1.45 μm, Fig. 2). The average distance between aggregated clusters of IP-1 (17.10 μm) is greater than between aggregated clusters of VK-1:NS (69.71 μm) (Table 2).

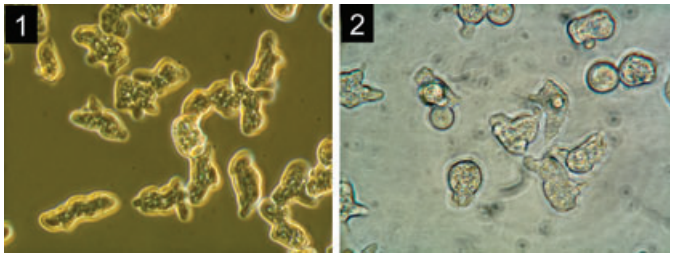


Fig. **1, 2.** Phenotypic characterization of *Entamoeba invadens,* see figures within Table 2.

Our findings provide evidence that nonsocial protists have evolved discrimination skills previously attributed mainly to social organisms. *Entamoeba invadens* IP-1 and VK-1:NS are able to discriminate between and interact preferentially with strain members. Distinct green or red clusters of combined sets (e.g. IP-1 red/VK-1:NS green or IP-1 green/VK-1:NS red; Table 1) suggest discrimination and nonaggregative behaviors with nonstrain members (Fig. 3–8). Fluorescent yellow clusters formed by *E. invadens* trophozoites increased in size with time if placed with members of the same strain (IP-1 green/IP-1 red; VK-1:NS green/IP-1 red; Fig. 9–14).

The number of studies on cooperative behaviors of social microbes have increased in the last decade: discrimination-dependent resource production (e.g. siderophores; Griffin et al. 2004), quorum sensing (Keller and Surette 2006; Parsek and Greenberg 2005), biofilms (Nadell 2009), aggregative motility (Chaine et al. 2010; Kraemer and Velicer 2011; Vos and Velicer 2009), and formation of fruiting bodies (Kalla et al.

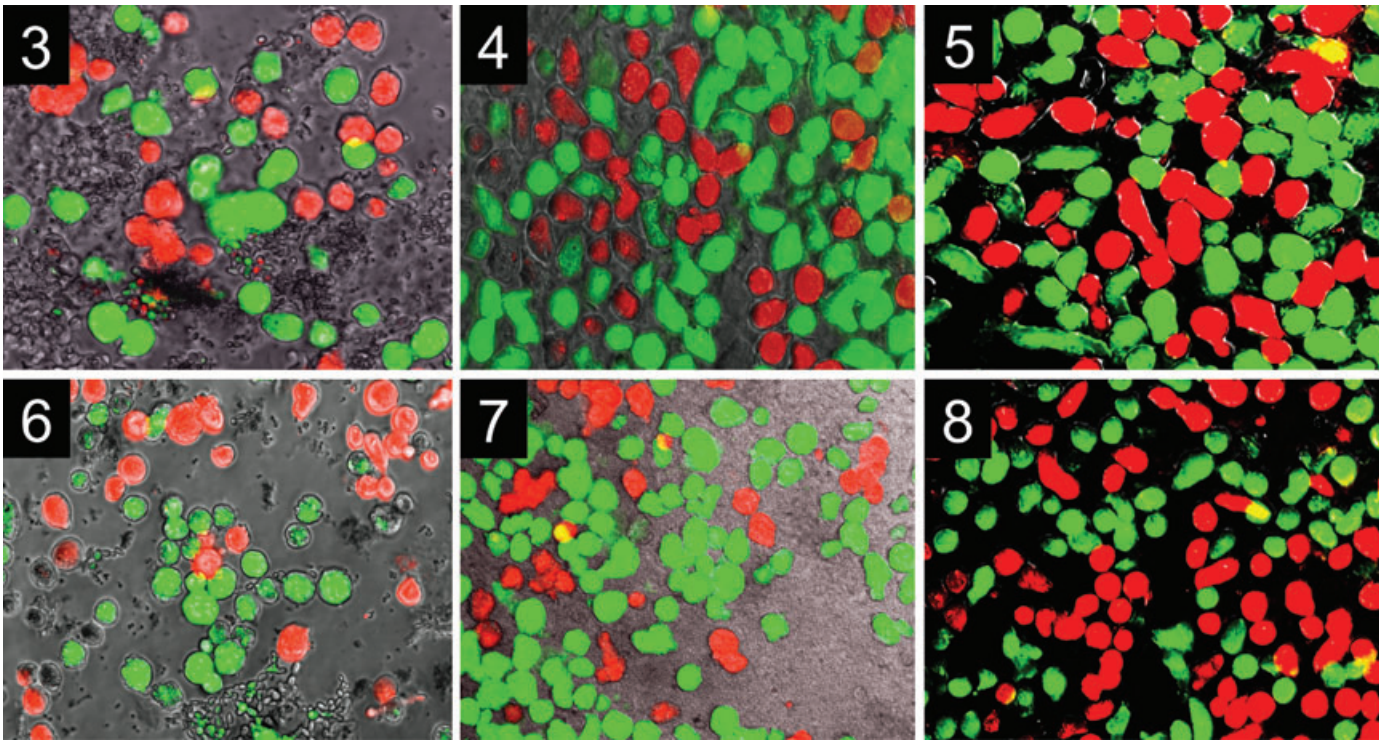


Fig. **3–8.** Discrimination shown by two strains of *Entamoeba invadens*, IP-1 and VK-1:NS; Fig. 3–5. Fluorescent micrographs of IP-1, labeled green, and VK-1:NS, labeled red, were taken with the same field of view at three different times. **3.** Initial aggregates show distinct clusters formed by two strains at 12 h. **4.** Intermediate aggregates show distinct clusters of two strains at 18 h. **5.** Large aggregates show distinct clusters formed by two strains at 36 h. Fig. **6–8**. Fluorescent micrographs of inversely labeled strains, IP-1, labeled red; VK-1:NS, labeled green, were taken with the same field of view at three different times. **6.** Initial aggregates show distinct clusters at 12 h. **7.** Intermediate aggregates show distinct clusters at 18 h. **8.** Large aggregates show distinct clusters formed by two strains at 36 h. The surface area of individual ameba, aggregates, and overlapping areas were determined by Image-Pro software (Micro-Tech Optical New England Inc.). Scale bar, 1 mm.

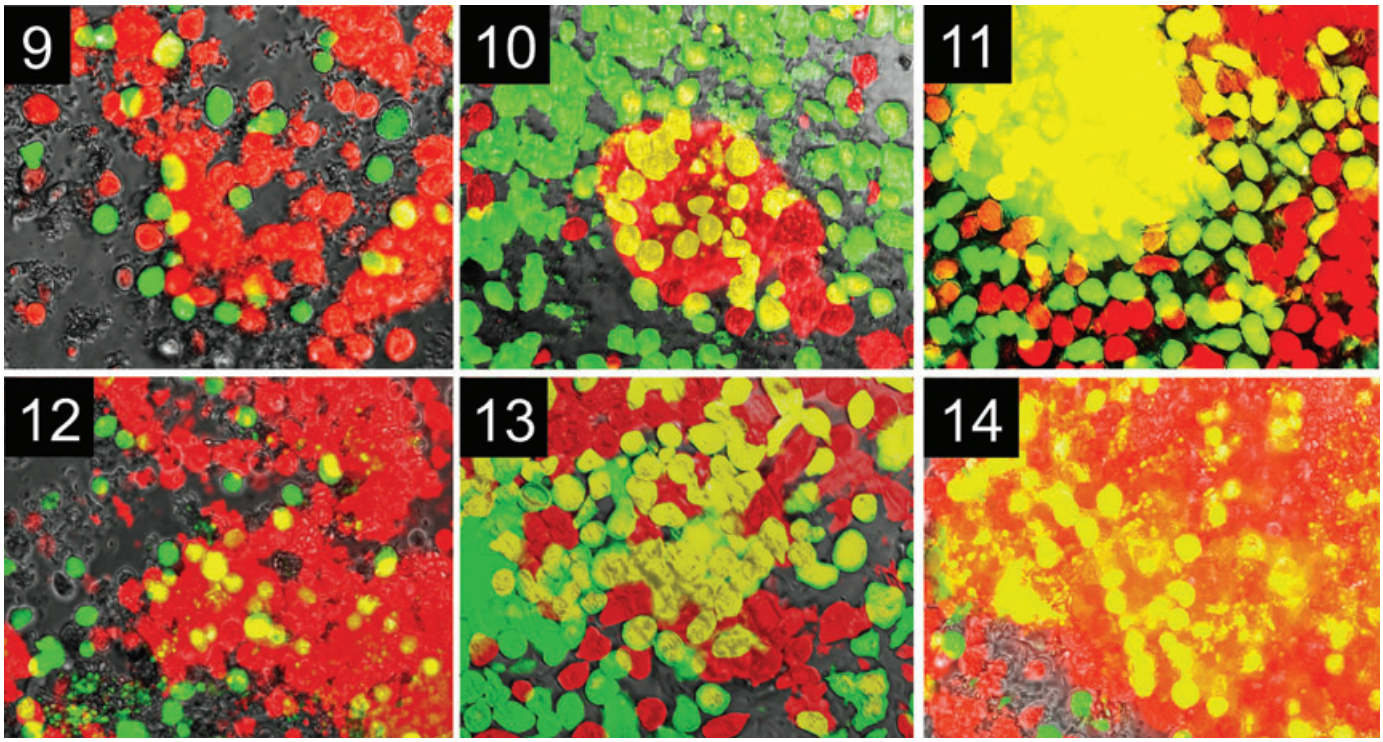


Fig. **9–14.** Aggregative behavior shown by strains of pure *Entamoeba invadens* cultures with either IP-1 or VK-1:NS cultures. Fluorescent micrographs were taken with the same field of view at three different times. **9–11.** *E. invadens* IP-1 (half of the cells are fluorescently labeled green, and the other half fluorescently labeled red) trophozoites mix equally creating an overlapping fluorescent yellow area that increases with time. **9.** Yellow surface area at 12 h. **10.** Yellow surface area at 18 h. **11.** Yellow surface area at 36 h. **12–14.** *E. invadens* VK-1:NS (half of the cells are fluorescently labeled green, and the other half fluorescently labeled red) trophozoites mix equally creating an overlapping fluorescent yellow area that increases with time. **12.** Yellow surface area at 12 h. **13.** Yellow surface area at 18 h. **14.** Yellow surface area at 36 h. The surface area of individual ameba, aggregates, and overlapping areas were determined by Image-Pro software (Micro-Tech Optical New England Inc.). Scale bar, 1 mm.

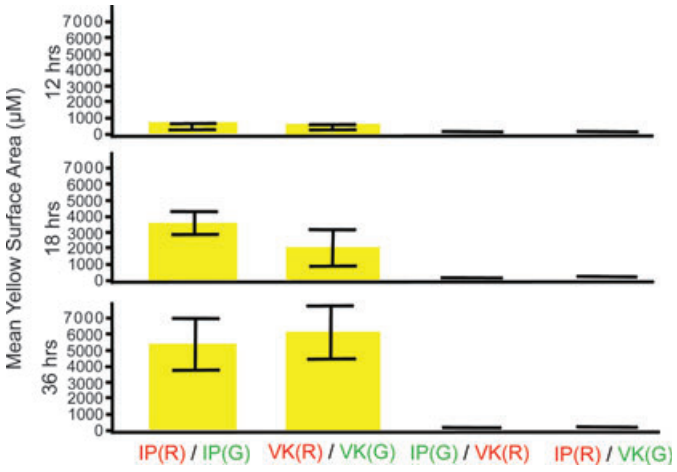


Fig. **15.** Yellow surface area (µm) histograms that quantitate the aggregative behavior of each combination of *Entamoeba invadens* IP-1 and/or VK-1:NS cultures (pure and mixed). The increasing yellow surface area is shown at 12, 18, and 36 h with error bars. The surface area of individual ameba, aggregates, and overlapping areas were determined by Image-Pro software (Micro-Tech Optical New England Inc.). Results were averaged from three replicate wells and three separate experiments (error bars shown).

2011; Mehdiabadi et al. 2006; Strassmann and Queller 2011). Most research in chemical signaling in nonsocial protists has focused in feeding, defense, invasiveness, or reproductive behaviors (e.g., marine eukaryotes; dinoflagellates; algae, parasitic amoeba; Brodsky 2009; Paul et al. 2007; Strom et al. 2007; Zaki et al. 2006). Upon sensing the human pro-inflammatory cytokine tumor necrosis factor, *E. histolytica* trophozoites migrate directionally and initiate infection (Blazquez et al. 2008; Tavares et al. 2000). This is the first study to show strain association as a trait in nonsocial ameba, suggesting that discrimination might have evolved as an adaptation not limited to sociality.

In protists, "cryptic" varieties are defined as morphospecies composed of strains with different physiological abilities or mating incompatibilities (Caron et al. 2009). Morphological phylogenies ignore the importance of physiological, ecological, and behavioral traits (Finlay 2004; Pawlowski and Burki 2009). The anaerobic Archamoebae (Entamoebae and pelobionts) were historically placed at the base of the eukaryotic tree, as "primitive eukaryotes" that lacked mitochondria (Embley and Martin 2006). Recent phylogenomic analyses of 100 genes support the grouping of three highly divergent amoebae *Dictyostelium, Entamoeba*, and *Mastigamoeba* within the class Conosea (Bapteste et al. 2002). Single-gene analyses of metabolic traits (e.g. alcohol dehydrogenase *adhe*) contribute to conflictive phylogenetic depictions, due to horizontal acquisition (HGT) of genes from prokaryotes and unicellular eukaryotes (Andersson et al. 2006; Bapteste and Boucher 2009; Espinosa et al. 2001, 2009; Paz-y-Miño-C. and Espinosa

2010). If single-celled protists display aggregative behavior only with alike-members, we can use measurable traits to reveal traditionally "cryptic" varieties in the *Entamoeba* lineage and improve the Archamoebae phylogenies and, possibly, of other nonsocial unicellular eukaryotes.

This study demonstrates that trophozoites aggregate only with members of their strain suggesting that they may also distinguish among close and distant relatives based on chemical and behavioral signals. Adaptations to different ecological environments (37 °C vs. 23 °C hosts; intestinal pH, oxygen sensitivity) and horizontal gene exchange could have influenced diversification.

CONCLUSIONS

Here, we demonstrate that two strains of *E. invadens* (IP-1 and VK-1:NS) aggregate only with members of their own population. Chemical discrimination could help resolve conflictive branching and crypticity among *Entamoeba* varieties and clarify the evolutionary history of this lineage. But the generation of new lineages via classical evolutionary trajectories has been challenged by proponents of "common design" or "separate ancestry" of complex molecular structures (Luskin and Gage 2008) and major taxonomic lineages (Nelson 1996) and by advocates of a "creation model" for *Entamoeba* parasitic origin (Sherwin 2009). Design creationists claim that "common ancestry is merely an assumption that governs interpretation of the data, not an undeniable conclusion" (Luskin and Gage 2008). The creation model proposes that created Entamoeba "progressed from originally free-living single-celled eukaryotes (neutral or beneficial) toward a pathogenic condition after 'The Fall'" (Sherwin 2009). Both alternatives hypothesize supernatural causation to life's essential evolutionary processes. Our case study suggests behavioral traits can be used to address the origin and evolution of the *Entamoeba* lineage, strengthening the Darwinian perspective. It is possible to envision an evolutionary process in which selection acted continuously and cumulatively on intermediates and ancestors of *Entamoeba* populations, which evolved chemical and behavioral cues to discriminate nonstrain members and gradually diversified into new lineages; sudden emergence of newly "designed" or "created" extant *Entamoeba* is improbable.

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