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REVIEW ARTICLE

Discrimination Experiments in *Entamoeba* and Evidence from Other Protists Suggest Pathogenic Amebas Cooperate with Kin to Colonize Hosts and Deter Rivals

Avelina Espinosa[a,b] & Guillermo Paz-y-Miño-C[b]

a Department of Biology, Roger Williams University, Bristol, Rhode Island
b New England Center for the Public Understanding of Science, Roger Williams University, Bristol, Rhode Island

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Correspondence
A. Espinosa, Department of Biology, Roger Williams University, One Old Ferry Road, Bristol, Rhode Island 02809
Telephone number: +1 401-254-3137;
FAX number: +1 401-254-3310;
e-mail: aespinosa@rwu.edu

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ABSTRACT

Entamoeba histolytica is one of the least understood protists in terms of taxa, clone, and kin discrimination/recognition ability. However, the capacity to tell apart same or self (clone/kin) from different or nonself (nonclone/nonkin) has long been demonstrated in pathogenic eukaryotes like *Trypanosoma* and *Plasmodium*, free-living social amebas (*Dictyostelium*, *Polysphondylium*), budding yeast (*Saccharomyces*), and in numerous bacteria and archaea (prokaryotes). Kin discrimination/recognition is explained under inclusive fitness theory; that is, the reproductive advantage that genetically closely related organisms (kin) can gain by cooperating preferably with one another (rather than with distantly related or unrelated individuals), minimizing antagonism and competition with kin, and excluding genetic strangers (or cheaters = noncooperators that benefit from others' investments in altruistic cooperation). In this review, we rely on the outcomes of in vitro pairwise discrimination/recognition encounters between seven *Entamoeba* lineages to discuss the biological significance of taxa, clone, and kin discrimination/recognition in a range of generalist and specialist species (close or distantly related phylogenetically). We then focus our discussion on the importance of these laboratory observations for *E. histolytica*'s life cycle, host infestation, and implications of these features of the amebas' natural history for human health (including mitigation of amebiasis).

SCIENTIFIC observations indicate that a large variety of unicellular eukaryotes (protists), members of major phylogenetic lineages, can tell apart same or self (clone/kin) from different or nonself (nonclone/nonkin). Indeed, protists have taxa, clone, and kin discrimination/recognition ability (Paz-y-Miño-C and Espinosa 2016, 2018). One of such protists is *Entamoeba histolytica*, an iconic Amoebozoa due to its relevance to human health (Fig. 1).

The capacity to discriminate/recognize kin, which is also common in invertebrates, vertebrates, and plants (reviews in Biedrzycki and Bais 2010; Blaustein et al. 1988; Dudley et al. 2013; Fletcher and Michener 1987; Hepper 1991; Holmes and Sherman 1983; Penn and Frommen 2010; Sherman et al. 1997; Starks 2004; Tang-Martínez 2001), has been explained historically under inclusive fitness theory (Hamilton 1963, 1964); that is, the reproductive advantage that genetically closely related organisms (kin) can gain by cooperating preferably with one another (rather than with unrelated ones), minimizing antagonism and competition with kin, and excluding genetic strangers (or cheaters = noncooperators that benefit from others' cooperation). The term inclusive fitness refers to the total genetic contribution an individual makes to future generations via its/his/her own reproduction (direct fitness) or via helping others (kin) to reproduce (indirect fitness; Gardner et al. 2016). For comprehensive reviews of kin recognition in unicellular eukaryotes, as well as prokaryotes (bacteria and some archaea), see Espinosa and Paz-y-Miño-C (2014a) and Paz-y-Miño-C and Espinosa (2016, 2018).

In recent clone recognition experiments, we demonstrated that amebas of diverse species could discriminate self from different (Espinosa et al. 2016). We first showed that seven *Entamoeba* cell lines kept in our laboratory could be characterized (by human observers) morphometrically as

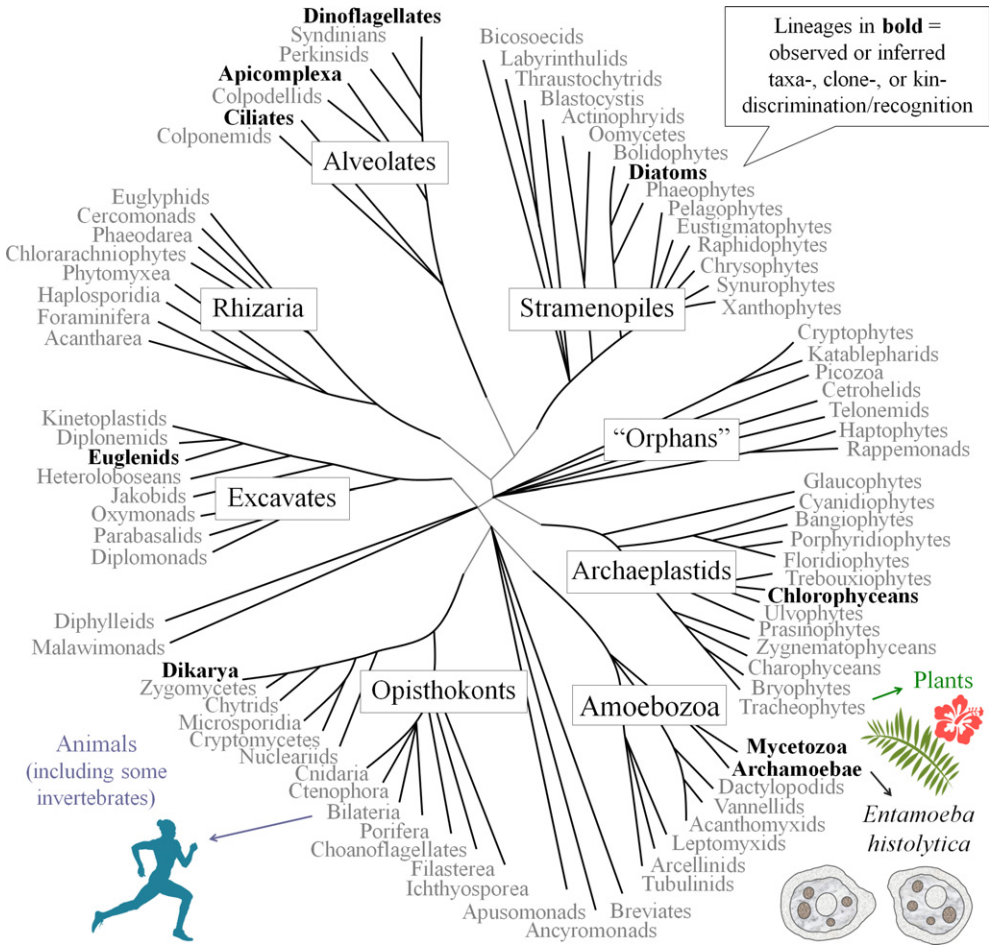

Figure 1 *Entamoeba histolytica*, a member of the Amoebozoa/Archamoebae (bottom right), belongs to one, among many, eukaryotic unicellular lineages (highlighted in bold) in which taxa, clone, or kin discrimination/recognition ability has been documented in the laboratory or inferred from observations in the wild. The academic field of kin recognition can be traced back to the 1960s and experiments with eukaryotic invertebrates and vertebrates (Opisthokonts/Bilateria, bottom left) and some plants (Archaeplastids/Tracheophytes, right). The advent of protists models into this field is new, early 2000s onwards, and *Entamoeba* species are among the least studied (redrawn and modified from Paz-y-Miño-C and Espinosa 2018; after Worden et al. 2015).

per the amebas' length, width, and cell surface area (*E. histolytica* HM-1:IMSS, *E. dispar*, *E. moshkovskii* Laredo, *E. moshkovskii* cf. Snake, *E. terrapinae*, *E. invadens* IP-1, and *E. invadens* VK-1:NS); we documented that the lineages differed statistically from one another based on these measurements (i.e., single-variable analyses of length, width, and cell surface area; or canonical discriminant analyses involving length × width; Fig. 2; Espinosa et al. 2016). More importantly, we also demonstrated that in mix-cell-line cultures between closely related (*E. invadens* IP-1 vs. *E. invadens* VK-1:NS) or distant-phylogenetic clone lines (*E. terrapinae* vs. *E. moshkovskii* Laredo), amebas consistently aggregated with clone members and avoided unrelated clones (which happen to be heterospecifics). Our studies included the identification of putative cell signals secreted by the amebas (RasGap/Ankyrin, coronin-WD40, actin, protein kinases, heat shock 70, and ubiquitin) and which known functions in *Entamoeba* spp. included cell proliferation, cell adhesion, cell movement, and stress-induced encystation (Espinosa et al. 2016).

In this review, we center our analysis on *E. histolytica* HM-1:IMSS and discuss the significance of its taxa, clone, and kin discrimination/recognition potential for the life cycle, host infestation, and implications of these aspects of the amebas' biology for human health (i.e., mitigation of amebiasis). Based on our in vitro discrimination/recognition experiments and analogous evidence reported in the literature about other pathogenic or free-living protists (i.e., *Trypanosoma*, *Plasmodium*, *Dictyostelium*, *Polysphondylium*, and *Saccharomyces*), we propose that *E. histolytica* can cooperate with kin to colonize hosts and deter genetically unrelated, or distantly related rivals. Even though kin recognition alleles have not yet been identified in *E. histolytica*, we think that such alleles do exist and that silencing them, or chemically altering the proteins they encode for, the amebas' capacity to aggregate, and,

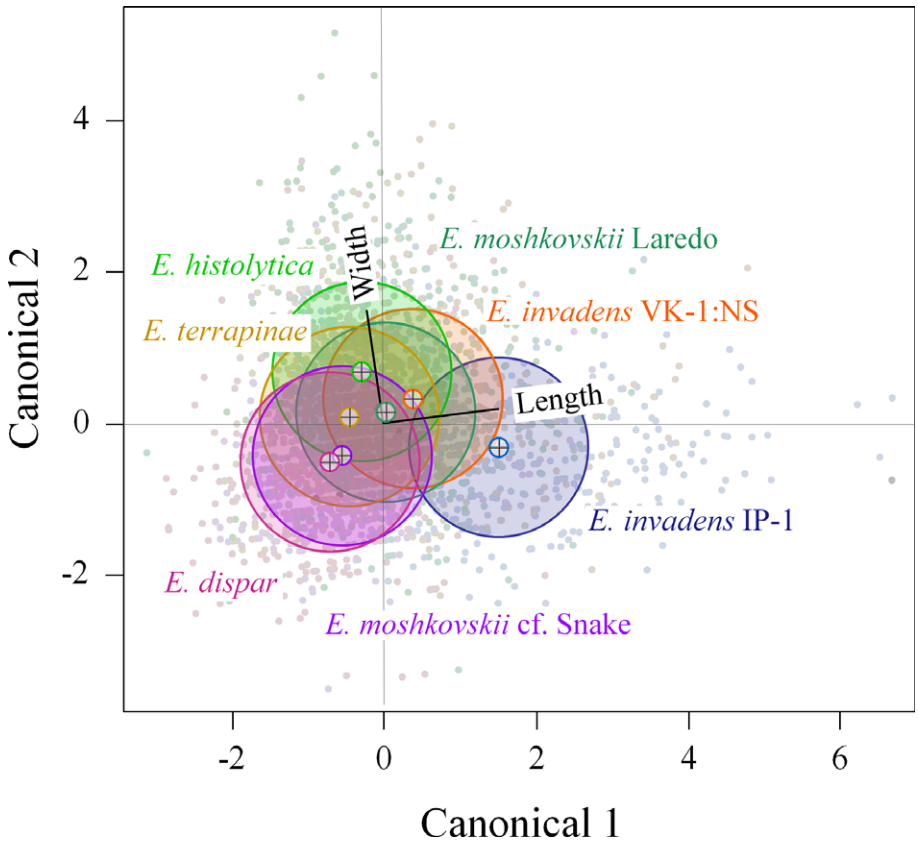

Figure 2 The seven *Entamoeba* clones kept in our laboratory were morphometrically different from one another as per cell length, width, and soma surface area. Of these three measurements, cell length was the most informative for clone-morphometric discrimination purposes. Length allowed us to distinguish statistically among most clones, except for *E. terrapinae* that was statistically indistinguishable from *E. moshkovskii* cf. Snake, which, in turn, was statistically indistinguishable from *E. dispar*. Cell width and soma surface area were less informative than length for clone-morphometric discrimination purposes, but both allowed us to roughly group amebas of distinctive widths and soma areas; as follows: according to width, *E. histolytica* was the widest-bodied ameba; *E. invadens* VK-1:NS, *E. terrapinae*, and *E. moshkovskii* Laredo were intermediate-width-bodied amebas; and *E. moshkovskii* cf. Snake, *E. dispar* and *E. invadens* IP-1 were thin-bodied amebas. According to soma area, *E. invadens* IP-1 and *E. invadens* VK-1:NS were the largest-bodied-area amebas; *E. moshkovskii* Laredo, *E. histolytica*, and *E. terrapinae* were the intermediate-area-bodied amebas; *E. moshkovskii* cf. Snake was a small-area-bodied ameba; and *E. dispar* was the smallest-area-bodied ameba. When we combined length and width in a canonical discriminant analysis (the plot shown in this figure), we could tell each ameba clone apart with 95% confidence, except for *E. moshkovskii* cf. Snake and *E. dispar*, which mean lengths/widths intercepts overlapped partially. Therefore, morphometrics alone (i.e., either single variables or combinations of variables) allowed us to tell apart each ameba clone. The canonical plot depicts the spatial dimensions for maximum morphometric separation among the *Entamoeba* cell lines. The labeled rays (length and width) show the directions of the covariates in the canonical space; the rays branch out from the point 0,0, which corresponds to the grand mean (N = 2,777 amebas); each multivariate mean is denoted by a + symbol, inside a small circle, as per *Entamoeba* clone (the small circle corresponds to the 95% confidence level area for each mean); the large circles depict the regions that contain 50% of the data points per clone. Note how *E. invadens* IP-1 (right-bottom circle) is the most elongated ameba with respect to the other taxa; *E. dispar* is the smallest (short and thin; left-bottom circle), and *E. histolytica* is the widest (top-left circle). Wilks $\lambda = 0.572$, $F_{12,\ 5{,}538} = 148.51$, $P < 0.0001$; the full model explains 43% of the variance shared between the variables length and width, with length alone capturing most of the association (34%) between extent-of-elongation and ameba clone identity; width alone captured 14% of the association. Redrawn and modified after Espinosa et al. (2016) and Paz-y-Miño-C and Espinosa (2018).

therefore, colonize hosts, encyst, or form abscesses inside them, can potentially be disrupted (treatment).

INTRA- AND INTERLINEAGE DISCRIMINATION ABILITY

Although evidence of taxa and kin discrimination/recognition in invertebrates, vertebrates, and some plants has been gathered since the 1960s (references above), the advent of protists models into this field of research is new, early 2000s onwards (Espinosa and Paz-y-Miño-C 2014a; Paz-y-Miño-C and Espinosa 2016, 2018). *Entamoeba* species are among the least understood protists in terms of taxa, clone, and kin discrimination/recognition potential (Espinosa and Paz-y-Miño-C 2012, 2014a,b). Here, we often use the terms discrimination/recognition

together in reference to the academic field of *kin recognition* and for that practical reason (i.e., discrimination/recognition = ability to distinguish one cell line, or clone, from another, or between "same" and "different" in the context of genetic relatedness), but there is a historical debate about the proper use of both terms that goes beyond the scope of this review (Blaustein et al. 1988; Paz-y-Miño-C and Espinosa 2018; Penn and Frommen 2010; Sherman et al. 1997; Starks 2004; Tang-Martínez 2001).

The seven cell lines kept in our laboratory are representative of major lineages within the genus *Entamoeba* (Fig. 3; for a detailed phylogeny of the *Entamoeba* genus see Elsheikha et al. 2018). Some, like *E. invadens* IP-1 and *E. invadens* VK-1:NS, differ only in a single nucleotide in the ssrRNA sequence (Espinosa and Paz-y-Miño-C 2012, 2014a,b), yet they discriminate one another as much as any other pair of phylogenetically more distant taxa (we infer that *Ei*-IP-1 and *Ei*-VK-1:NS differ substantially in their nuclear genomes, as distinctive species, but only the *Ei*-IP-1 genome has been sequenced; Ehrenkaufer et al. 2013). In fact, all cell lines segregate into single-clone clusters

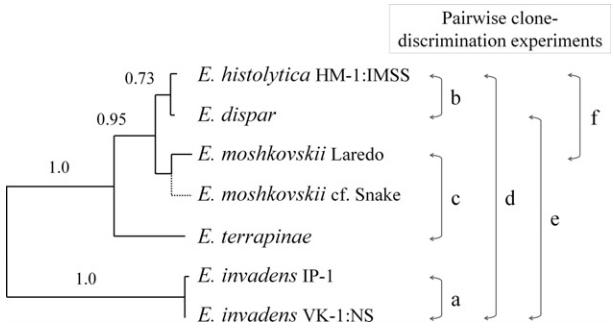

Figure 3 Phylogeny based on ssrRNA sequences of the *Entamoeba* clones discussed in this review. The seven clones depicted are representative of major lineages within the genus *Entamoeba* (for a detailed phylogenetic reconstruction of the genus see Elsheikha et al. 2018). Arrows indicate pairwise clone recognition experiments, as conducted in our laboratory, between: most closely related sister taxa *E. invadens* IP-1 vs. *E. invadens* VK-1:NS (marked as a); more distantly related sister taxa *E. histolytica* HM-1:IMSS vs. *E. dispar* (marked as b); between a representative of a known multispecies clade (e.g., *E. moshkovskii* Laredo) vs. the most distant member of that clade (*E. terrapinae*; marked as c); and between members of two distinct clades (*E. histolytica* HM-1:IMSS vs. *E. invadens* VK-1:NS, marked as d; or *E. dispar* vs. *E. invadens* VK-1:NS, marked as e). We have obtained comparable results (i.e., discrimination between same/self/clone and different/nonself/nonclone) with the pair *E. histolytica* HM-1:IMSS vs. *E. moshkovskii* Laredo (marked as f). Accession numbers NCBI: *E. histolytica* HM-1:IMSS X56991; *E. dispar* Z49256.1; *E. moshkovskii* Laredo AF149906.1; *E. terrapinae* AF149910.1; and *E. invadens* IP-1 AF149905.1. The ssrRNA sequence of *E. invadens* VK-1:NS was provided by C. Graham Clark. The placement of *E. moshkovskii* cf. Snake in the phylogeny (dashed-line branching) is tentative because its ssrRNA sequence is under review. Numbers indicate statistical branch support based on Approximate Likelihood-Ratio Test.

when grown in diverse combinations of mixed cultures (Espinosa and Paz-y-Miño-C 2012, 2014a,b; Espinosa et al. 2016), as noted in the phylogeny of Fig. 3: most closely related sister taxa *E. invadens* IP-1 vs. *E. invadens* VK-1:NS (marked as a in connecting arrows in Fig. 3); more distantly related sister taxa *E. histolytica* HM-1:IMSS vs. *E. dispar* (marked as b in connecting arrows in Fig. 3); between a representative of a known multispecies clade (e.g., *E. moshkovskii* Laredo) vs. the most distant member of that clade (*E. terrapinae*; marked as c in connecting arrows in Fig. 3); as well as between members of two distinct clades (*E. histolytica* HM-1:IMSS vs. *E. invadens* VK-1:NS, marked as d in connecting arrows in Fig. 3; or *E. dispar* vs. *E. invadens* VK-1:NS, marked as e in connecting arrows in Fig. 3). Note that we have obtained comparable results with the pair *E. histolytica* HM-1:IMSS vs. *E. moshkovskii* Laredo (marked as f in connecting arrows in Fig. 3). In Fig. 4 we only show the microscopy images corresponding to the exemplar pair *E. histolytica* HM-1:IMSS vs. *E. dispar* (laboratory protocols and images of other pairings are provided in Espinosa and Paz-y-Miño-C 2012, 2014a,b; Espinosa et al. 2016).

The morphometric data (Fig. 2; Espinosa et al. 2016) combined with the mix-cell-line culture outcomes between phylogenetically close or distant lineages (above and Figs. 3 and 4) demonstrate that *Entamoeba* spp. are capable of in vitro taxa and clone discrimination/recognition. Equal abilities have been reported previously in other Amoebozoa/Mycetozoa (Fig. 1), including the free-living social amebas in the genera *Dictyostelium* and *Polysphondylium* (Benabentos et al. 2009; Hirose et al. 2011; Kaushik et al. 2006; Li and Purugganan 2011; Mehdiabadi et al. 2006; Noh et al. 2018; Ostrowski et al. 2008; Queller et al. 2003; Smith et al. 2016). Note that the cell lines in our laboratory correspond to niche generalists/specialists: commensal (*E. dispar* and *E. terrapinae*) and pathogenic (*E. histolytica* HM-1:IMSS, *E. moshkovskii* Laredo, *E. moshkovskii* cf. Snake, *E. invadens* IP-1, and *E. invadens* VK-1:NS; Clark and Diamond 1991; Eichinger 2001; Espinosa and Paz-y-Miño-C 2012, 2014a,b; Faust and Guillen 2012; Weedall and Hall 2015). *Entamoeba moshkovskii* can be found in riverine sediments to brackish coastal pools (Clark and Diamond 1997) or, like *E. dispar*, it can also be pathogenic (Ali et al. 2003; Ngobeni et al. 2017; Oliveira et al. 2015; Shimokawa et al. 2012).

IMPLICATIONS OF CLONE DISCRIMINATION ABILITY FOR *E. HISTOLYTICA*'S LIFE CYCLE

Host-biont colonization in various pathogenic protists (e.g., *Trypanosoma*, *Plasmodium*) includes sequential population crashes (bottlenecks), from the moment of arrival at the host biont (either insect or vertebrate in the cases of *Trypanosoma* and *Plasmodium*) to the colonization of tissues and organs, proliferation within them, and subsequent dispersal to new host bionts (Caljon et al. 2016; Dyer et al. 2013; Koffi et al. 2015; Neal and Taylor 2014; Peacock et al. 2014; Sinden et al. 2007). The host-biont immune system, the gut digestive enzymes (e.g., when

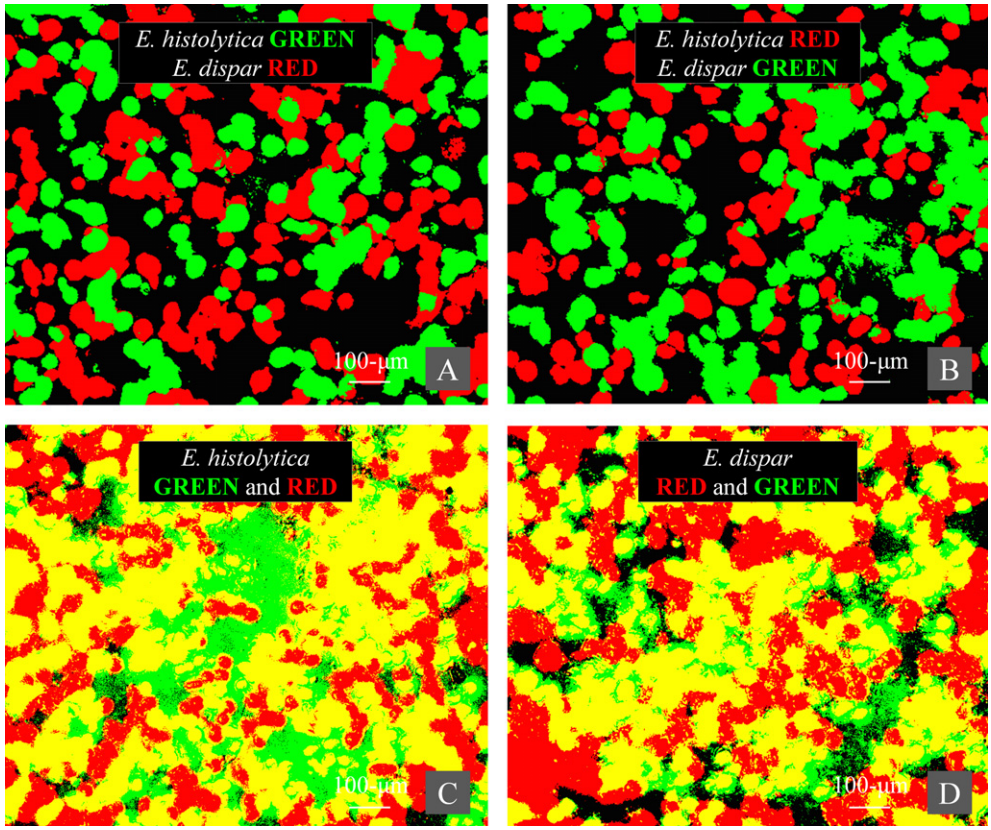

Figure 4 Clone discrimination/recognition experiments in which clone aggregation preference is shown by *Entamoeba histolytica* HM-1:IMSS and *E. dispar* in mixed- (top) or single-cell-line (bottom) cultures. **A.** Fluorescent micrograph of *E. histolytica* HM-1:IMSS labeled green and *E. dispar* labeled red, each clone aggregates in distinct clusters. **B.** Reverse-color labeling of trophozoites of *E. histolytica* HM-1:IMSS (red) and *E. dispar* (green), the clones aggregate in distinct clusters. **C.** *Entamoeba histolytica* HM-1:IMSS labeled with both green and red dyes; trophozoites mix equally and look yellow under the microscope. **D.** *Entamoeba dispar* labeled with both green and red dyes. In all trials, cells were labeled with CellTracker Green CMFD and Red (Invitrogen, Carlsbad, CA). All images taken at 36-h, scale bar = 100-µm, 10× magnification (laboratory protocols and images of other pairings, as pointed at in Fig. 3, are provided in Espinosa and Paz-y-Miño-C 2012, 2014a,b; Espinosa et al. 2016).

Trypanosoma or *Plasmodium* cross the insect's gut to invade the body cavity or arrive at the salivary glands), or competition with con- and heterospecifics that had settled previously in the host-biont anatomy (i.e., priority effects that favor first colonizers) contribute to crash the populations of invaders (Kraemer et al. 2016; Rendueles et al. 2015). Repetitive population bottlenecks combined with serial founder effects lead to clonality in surviving invaders, which ultimately benefit from cooperating with kin (e.g., Nkhoma et al. 2012; Trevino et al. 2017). In instances in which *Trypanosoma* or *Plasmodium* detect the presence and proliferation of competitor clones inside the host (by means of cell-signaling mechanisms of quorum sensing), the cells can respond via fast clonal expansion, active inhibition of competitors (e.g., excretion of hormones that block competitors' growth; Portugal et al. 2011), habitat/niche partitioning within the host by colonizing distinctive host environments (Ramiro et al. 2016), or cooperative behaviors with kin, like social motility in *Trypanosoma* (SoMo, below), which exclude unrelated cells (Roditi 2016; Roditi et al. 2016). Comparably, *Plasmodium* can reduce the investment in sexual reproduction (i.e., the production of male or female gametocytes, which *Plasmodium* can fine regulate depending on crowding; Reece et al. 2008) and instead continue in blood stages, replicate clonally, achieve "safety in numbers," and postpone the terminal maturation into gametocytes (Bechtsi and Waters 2017; Guttery et al. 2015; Pollitt et al. 2011; Portugal et al. 2011). These strategies help explain why *Trypanosoma* and *Plasmodium* monoclonal infections (or coinfections of genetically related strains) tend to be more frequent than polyclonal infections (but see dos Santos Lima et al. 2014; Gaunt et al. 2003; Messenger et al. 2015), although ecological contingencies inside and outside the host-biont environments (e.g., antipathogen drugs, drug resistance, or host population dynamics, spatial distribution, nutrition, and health) also play a role in inducing clonality (Duffy et al. 2009, 2013; Falk et al. 2015; Paz-y-Miño-C and Espinosa 2018; Tibayrenc and Ayala 2017; Weir et al. 2016).

We think that *E. histolytica* experiences analogous population dynamics during host-biont colonization, from the instant of arrival at the host's oral cavity (an enzymatic environment), to the exposure to digestive secretions in the gut (high acidity), colonization of the intestinal lumen

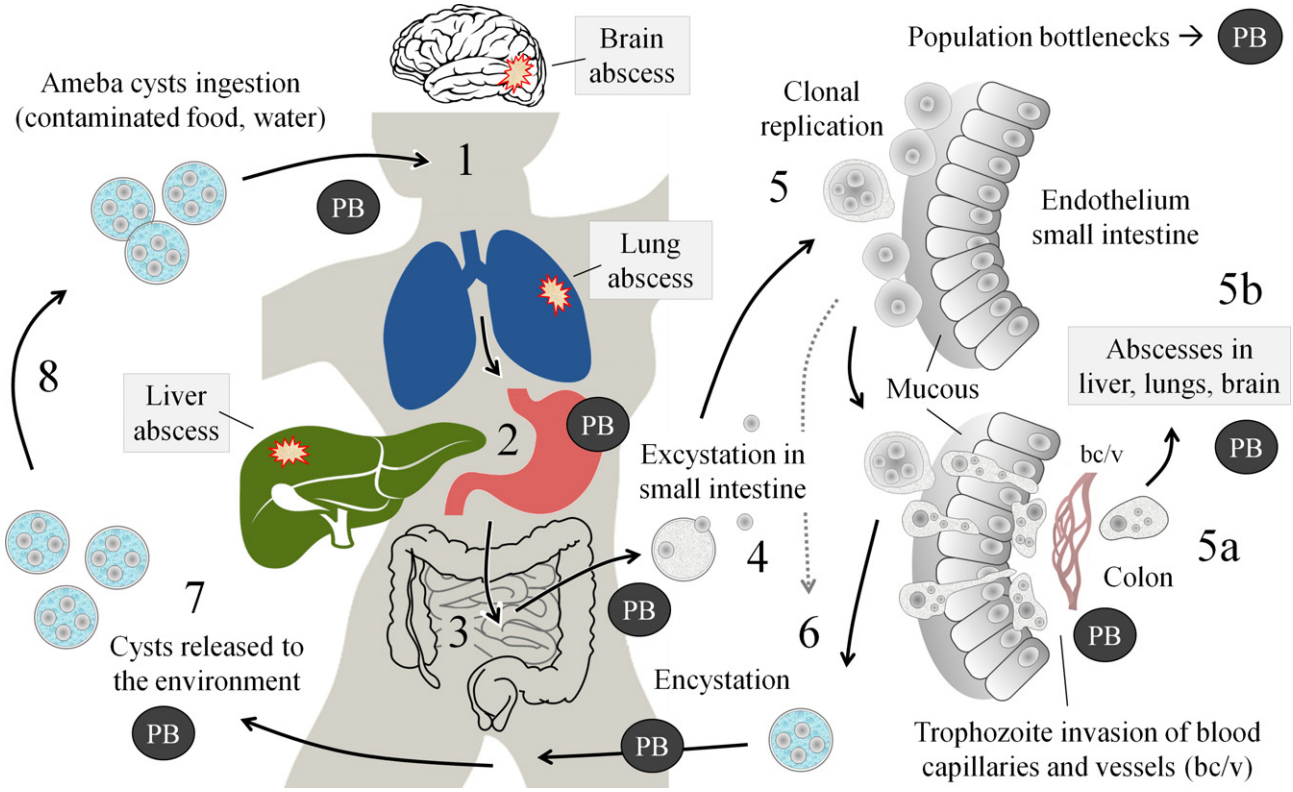

Figure 5 Population bottlenecks (PB) in the life cycle of *Entamoeba histolytica* (as suggested in this review). (1) Upon host's ingestion of contaminated food and water, ameba cysts (dormant stage of the organism) will face the enzymatic milieu of the oral cavity (the mouth environment can be highly variable in temperature, concentration, and mix of chemicals originated from diverse foods and the host's own microbiota); (2) once reaching the stomach, the cysts will be exposed to high acidity, an inducer of severe ameba cysts–population crashes; (3) cysts arrival at the small intestine, a more favorable environment for excystation; (4) not all amebas released during excystation will survive (simply due to intrinsic differential survival); (5) the colonization of the mucous layer on the small intestine endothelium (nutrient-rich) will induce fast ameba clonal proliferation, but the successful population expansion will depend on variable conditions inside the host and be limited by the presence of different clone competitors (priority effects, see text); (5a) a potential trophozoite invasion of the colon in the large intestine (if it occurs) will be countered by the host's immune responses (i.e., endothelium guarded by white-blood cells) and also by other ameba clones already established in the colon; (5b) in the uncommon cases of systemic infection, the liver, lungs, and other organs (rarely the brain) can be colonized by amebas, which form abscesses, but abscess formation involves high mortality (both caused by host immunity and amebas' own programmed cell death, PCD, imposed by abscess development); (from 5 to 6) prior to being eliminated from the body, the amebas must encyst, but not all cells will successfully form cysts; (7) and (8) cysts released into the external environment will face additional population crashes, although not directly associated with the fate of the host. To overcome the challenges of population demise (PBs) and stochastic opportunities to recover inside the host, amebas will need to associate and cooperate with clone members (kin); single amebas will not survive and associations or cooperation with genetic strangers will be maladaptive (prone to cheating). Dotted line indicates cases of direct elimination of cysts from the small intestine.

(excystation in the small intestine) and the mucous layer in the small/large intestines, and encystation prior to dispersal out, to other host bionts (Fig. 5). Host colonization infrequently includes the perforation of the colon endothelium (a tissue guarded by white blood cells), invasion of the blood capillaries and vessels (also protected by the immune system), and migration to the host-biont organs (i.e., the occasional abscesses in the liver, lungs, or rarely in the brain; Fig. 5). In fact, just like in other pathogenic or free-living protists (*Trypanosoma*, *Plasmodium*, or the social amebas *Dictyostelium* or *Polysphondylium*), each successful stage of *E. histolytica*'s life cycle is probably preceded by a population bottleneck (PB as suggested in Fig. 5).

Why are the in vitro taxa and clone discrimination experiments (Fig. 3 and 4) informative of *E. histolytica*'s life history? To address this question, we would like to take a look at *Trypanosoma*'s social motility (SoMo), which was discovered in the laboratory fairly recently (Oberholzer et al. 2010, 2015). When *T. brucei* cells were inoculated in the center of an agar surface (on a petri dish), the trypanosomes formed migration swarms, star-shaped, and with multiple spreading fronts or "spokes." The collective in vitro motility depended on each individual cell's flagellum for propulsion (cells with abnormal flagellum could not swarm). It was later proposed that swarming was central to the trypanosomes journey from the vector-biont midgut (the tsetse fly) to the salivary glands, where the cells

completed development into mammalian-infective morphs (trypomastigotes; Oberholzer et al. 2010, 2015; Imhof and Roditi 2015). Imhof et al. (2015) documented that intact social motility gave a virulence advantage to wild-type *T. brucei* carriers of the *Rft1* allele over null mutants *Rft1*$^{-/-}$ whose ability to migrate was compromised and, therefore, could not perforate the insect's midgut, mobilize into the fly's body cavity (i.e., the ectoperitrophic space) and, therefore, aim for the salivary glands (i.e., for transmission into new hosts). A parallel scenario of arduous migration, population crashes (bottlenecks), and repetitive founder effects is experienced by *Plasmodium* spp. once inside a mosquito biont (Neal and Taylor 2014; Pollitt et al. 2010; Reece et al. 2011; Sinden et al. 2007). Cell death is such that in *P. berghei* (agent of the African rodent malaria) 80–90% of the infective cells (ookinetes) perish inside the insect vector. Part of this mortality is attributable to the vector's immunity and the toxicity inside the mosquito's midgut (review on the molecular aspects of *Plasmodium* spp. development inside the vector in Bennink et al. 2016).

Thus, our in vitro clone discrimination/recognition experiments with *Entamoeba* spp. (Fig. 3 and 4) are, possibly, an indication that, to maximize fitness and deter rivals, amebas associate and cooperate with clone members (kin) precisely to overcome the predictable challenges of population demise and stochastic opportunities to recover inside the host (Fig. 5). Single amebas cannot survive, and associations or cooperation with genetic strangers will be maladaptive in terms of fitness and prone to cheating (Gilbert et al. 2007; Ho and Shaulsky 2015; Levin et al. 2015; Paz-y-Miño-C and Espinosa 2018; Santorelli et al. 2008). Consequently, clonal proliferation of kin, or cooperation with the closest genetic relatives that are still compatible for cell–cell alliances, shall be most advantageous (Espinosa et al. 2016; Paz-y-Miño-C and Espinosa 2018). In cases of coinfections by diverse clones or ameba species (as reported in human, great ape, and rhesus-macaque hosts; Guan et al. 2016; Ngobeni et al. 2017; Vlčková et al. 2018), the ameba ability to discriminate/recognize taxa, clone, or kin will also facilitate fine-scale (millimeter, perhaps microscopic) niche partitioning when invading the host and colonizing its tissues/organs, or forming abscesses (the latter as multitaxa communities of bacteria and ameba species and their clones; Reyna-Fabián et al. 2016). Reyna-Fabián et al. (2016) have reported bispecies (*E. histolytica* HM1:IMSS and *E. dispar* SAW-760) infection in a liver abscess from a single human host in Mexico (genotyped as per tRNA short-tandem repeats, STRs, 6DA, or DA(1), respectively; see Ali et al. 2005 for genotyping approach). Ali et al. (2008) have documented genetically distinctive *E. histolytica* infections in 16 patients from Bangladesh (where amebiasis is endemic), one from the United States, and one from Italy (both countries with no endemic amebiasis), when contrasting the ameba genotypes in the intestine (i.e., stool samples) vs. the liver (i.e., abscess samples; also via tRNA-linked STRs loci analysis, corroborated by PCR of the SREHP gene). Because liver abscesses developed during months after the initial amebas arrived in the gut (and once the early infection had cleared), it was likely that the distinctive genotype in the intestine, which differed from the liver-abscess genotype, originated in secondary infections among the Bangladeshi patients (i.e., intestine vs. liver-abscess genotypes varied at six loci assessed). By contrast, the United States and Italy patients carried closely related genotypes in the intestine and liver (i.e., five of the six loci were identical in each case, and one locus was different in each case), possibly derived from a single ancestor (Ali et al. 2008). In the total 18 cases examined, the liver abscesses were monoclonal. We are confident to suggest that in these scenarios of mixed-species (or potentially mixed clones) presence in liver abscesses, amebas use their ability to tell apart same (clone) from different (other clone or species) to actively segregate and associate preferentially with kin or with the closest genetic relative available. Fine-scale clone segregation (at the millimeter scale) has been widely documented in both free-living and laboratory experiments with social amebas (*D. discoideum*, *D. purpureum*, and *D. giganteum*; Fortunato et al. 2003; Sathe et al. 2010; Smith et al. 2016).

Although chimeric clusters of unrelated cells have been observed in free-living social amebas when forming fruiting bodies (e.g., *D. discoideum*, *D. giganteum*, or *D. purpureum*; Fortunato et al. 2003; Kaushik et al. 2006; Sathe et al. 2010), clonal associations for fruiting body formation and spore production often surpass reproductive success (i.e., spore production) in respect to mixed cell aggregations (Mehdiabadi et al. 2006, 2009). Mating types have also been reported in *D. purpureum* and successful crossings (i.e., during the sexual part of the amebas' life cycle in which cells merge) tend to occur preferentially between genetically closely related, compatible cell lines (Gilbert et al. 2012; Mehdiabadi et al. 2009). In dictyostelids, kin discrimination/recognition ability is crucial because fruiting body formation and spore production involve inevitable cell death (20–30% of the cells can die while forming the stalk that supports the fruiting body; Ostrowski et al. 2008). Likewise, mortality in *E. histolytica* during host invasion has been linked to the interaction among host immunity, virulence of the ameba strain, and species composition of the gut biome (Burgess and Petri 2016; Gilchrist et al. 2012; Morgado et al. 2016; Morton et al. 2015; Nakada-Tsukui and Nozaki 2016; Watanabe and Petri 2015). Thus, in our view, altruistic cooperation between close *E. histolytica* relatives shall maximize survival and reproduction of self, self-like, or kin (Paz-y-Miño-C and Espinosa 2018).

Moreover, in cyst-forming taxa like *E. histolytica* as well as in other protists (e.g., *Balantidium coli*, *Cryptosporidium parvum*, *Giardia lamblia,* and *Toxoplasma gondii*), encysting allows groups of cells to survive the harshness of the host environment (as well as outside the host; see molecular mechanisms of encystation in Schaap and Schilde 2018), but because both cyst formation and programmed cell death (PCD) co-occur as integral parts of these organisms' life cycles (including during abscess formation in *E. histolytica*), the need for a synchronous balance between encysting/abscessing and PCD has been proposed (Khan

et al. 2015). Because abscesses are communities of organisms living in close proximity, sometimes formed by multiple clones prone to cheat on one another (i.e., genetically unrelated noncooperators taking advantage of the PCD benefits intrinsic to kin, as per inclusive fitness), the occurrence of adaptive PCD requires the ability to discriminate between same/clone and different/nonclone members (Paz-y-Miño-C and Espinosa 2018).

Keep in mind that like *Trypanosoma* and *Plasmodium*, pathogenic or social amebas do undergo instances of sexual reproduction in their life cycles (i.e., genetic recombination and its benefits; dos Santos Lima et al. 2014; Koffi et al. 2015; Manske et al. 2012; Nkhoma et al. 2012; Peacock et al. 2014, 2016; Trevino et al. 2017; Weedall and Hall 2015), but the predominant clonal expansion of populations and subpopulations has long been associated with opportunistic fitness advantage during host invasion (to cope with host immunities) and when facing stochastic environmental change inside the host or in the wild (Duffy et al. 2013; Koffi et al. 2015; Suriptiastuti 2010; Tibayrenc and Ayala 2017). Recombination, therefore, does not occur with enough frequency to alter the prevalent pattern of clonal population structure (Tibayrenc 1993; Tibayrenc and Ayala 2017; Tibayrenc et al. 1990). The role of clonality as a maximizing fitness strategy (correlated with density-dependent factors linked to pathogen–host ecological interactions) has also been documented in *Giardia*, *Leishmania*, *Naegleria*, *Toxoplasma*, and *Trichomonas* (Tibayrenc and Ayala 2017; Tibayrenc et al. 1990).

POSSIBLE RELEVANCE TO HUMAN HEALTH

As encystation (Coppi et al. 2002; Schaap and Schilde 2018) and/or abscess formation require cell aggregation (abscesses depend on cells coming together), we think that disruption of the amebas' capacity to group (via gene silencing or drugs) might be a viable approach to mitigate amebiasis. Blocking encystation could reduce the dissemination of infection because transmission relies on cyst dormancy (Herman et al. 2017; Mi-ichi et al. 2016), but it shall not cure amebiasis considering that most hosts are asymptomatic and release cysts undetected (Mi-ichi et al. 2016). However, we know little, if anything, about the genes involved in *E. histolytica* taxa, clone, or kin discrimination/recognition. We speculate that such potential genes might operate analogously to the "green-beard" alleles *csA* or *tgrB1* and *tgrC1* in *D. discoideum* (green beards = tag genes for recognition; Dawkins 1976), but homologs to these genes in *Entamoeba* spp. have not been identified (we have searched for them in public databases: AmoebaDB, *Entamoeba* Welcome Sanger Institute, EnsemblProtists, NCBI, and UniProt; see links in references). To remain in intimate proximity, *D. discoideum* amebas rely on cell-membrane adhesion proteins encoded by *csA*; when wild-type csA^{+} cells are grown in mixed cultures with csA^{-} knockouts, the amebas cluster preferentially with those equipped with fully functional adhesion polypeptides (Paz-y-Miño-C and Espinosa 2016, 2018; Queller et al. 2003). Interestingly, the *tgrB1* and *tgrC1* alleles work as complementary pairs; *tgrB1* and *tgrC1* are necessary and sufficient for amebas to discriminate between same (self or clone) and different (other clones), and to adhere to each other during the social phase of the life cycle (Hirose et al. 2011). Just like *csA*, the *tgr* genes are polymorphic and encode for plasma membrane glycoproteins (Hirose et al. 2011). Hirose et al. (2015) have demonstrated that the *tgrB1-tgrC1* genes not only work in reciprocal recognition between cells (=allorecognition) but also that these alleles are required for two major functions during the amebas' social cycle. First, discrimination/recognition between genetically compatible cell lines (clone/kin) during active aggregation for social cooperation and fruiting body formation (i.e., the amebas actively pursue such aggregations); this includes prevention of exploitation by unrelated cells (nonclone/nonkin) or cheaters (i.e., the active exclusion of the latter). And, second, coordinated and cooperative cell motility, cell polarization for collective movement (i.e., correct positioning of each cell in respect to the neighboring cells, something mediated by the *tgrB1-tgrC1*-encoded plasma membrane proteins), and later cell differentiation during fruiting body formation (i.e., the allocation of specific cells to the specialized structures of the fruiting body; this involves PCD; Paz-y-Miño-C and Espinosa 2018). These allorecognition-mediated events, without which the multicellular aggregation cannot occur, are likely coupled by cell signaling codependent on the compatibility and matching between cells carrying the right copies of the *tgrB1-tgrC1* alleles (some of these molecular pathways can be explored in Du et al. 2015; Glöckner et al. 2016; Heidel et al. 2011; Kawabe et al. 2015; and Paz-y-Miño-C and Espinosa 2018). Note that green-beard gene-mediated cellular aggregations for cooperation have also been documented in budding yeast (*FLO1* in *Saccharomyces cerevisiae*, a member of the Dikarya in Fig. 1; Gardner and West 2010; Goossens et al. 2015; Smukalla et al. 2008), the bacteria *Myxococcus xanthus* (*trA*; Cao and Wall 2017; Cao et al. 2015; Pathak et al. 2012, 2013; Wall 2016), *Burkholderia thailandensis* (operon *bcpAIOB* and CDS system; Garcia et al. 2016), *Escherichia coli* (operon *cdiBAI*, *bamA*, and CDI system; Aoki et al. 2005; Danka et al. 2017; Ruhe et al. 2013; Willett et al. 2015), as well as in *Agrobacterium*, *Bacillus*, *Citrobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Neisseria*, *Paenibacillus*, *Pectobacterium*, *Photorhabdus*, *Proteus*, *Rhizobium*, *Salmonella*, *Pseudomonas*, *Xenorhabdus*, *Vibrio*, and *Yersinia* (i.e., CDI systems; KDLs gene array; *idsABCDEF* operon; *idrABCDE* cluster and *tss* cluster—Type VI Secretion System; Ti plasmid including *virB1-11* cluster which encodes the Type IV Secretion System; *rhsA*, *rhsB*, *rhsI*, *mosABC*, and *mocABRC* clusters; and *dfsB*; review in Paz-y-Miño-C and Espinosa 2018). In essence, green-beard genes are quite common in microbes and it is reasonable to suspect their existence in *Entamoeba* spp.

As noted earlier, we have identified putative cell signals secreted by the amebas during cogrowth (*E. invadens* IP-1 vs. *E. invadens* VK-1:NS; Fig. 3) in clone discrimination/recognition experiments (Espinosa et al. 2016; Paz-y-Miño-

C and Espinosa 2018). Briefly, we found six proteins common to *Ei*-IP-1 and *Ei*-VK-1:NS, as follows: RasGap/Ankyrin, coronin-WD40, actin, protein kinases, heat shock 70, and ubiquitin. Some of their known functions in *Entamoeba* spp. included: cell proliferation, cell adhesion, cell movement (i.e., pseudopod formation assisted by actin polymerization), migration, and stress-induced encystation (which are all relevant in the context of cell–cell recognition for aggregation; Bosch and Siderovski 2013a,b; Eckert et al. 2011; Espinosa et al. 2016; Herrera-Martínez et al. 2013; Paz-y-Miño-C and Espinosa 2018; Weber et al. 2006). Specifically, in *E. histolytica*, heat shock 70 (*Eh*Hsp70) helps amebas cope with oxidative stress and it is expressed during liver-abscess formation (Akbar et al. 2004; Nagaraja and Ankri 2018; Santos et al. 2015; Weber et al. 2006). Heat-shock proteins are also synthesized during thermal stress in *S. cerevisiae* and encystation in *G. lamblia* (Luján et al. 1996; Morano et al. 1998). And in *E. invadens*, a variety of analogous polypeptides (to the ones found by us, above) are expressed during stress-induced encystation (G proteins, protein kinase phospho-EieIF2α, heat-shock proteins Hsp70/90, ubiquitins, Gal/GalNAc lectin; Ehrenkaufer et al. 2013; Field et al. 2006; Hendrick et al. 2016; Mi-ichi et al. 2016; Nagaraja and Ankri 2018). Moreover, comparable protein functions to those listed here have been identified in homologs in *Dictyostelium* and *Plasmodium* (Loomis 2015; Olshina et al. 2015; Dictybase, see link in references). In *P. falciparum*, coronin coordinates the alignment of actin filaments that seem to guide directional motility in the cells (Olshina et al. 2015).

Other researchers have proposed that during encystation, *Entamoeba* cells (observations based on the reptilian pathogen *E. invadens*) attach to one another via molecular bindings of galactose (Gal)-terminated ligands to specific receptors on neighboring cells (Eichinger 2001; Mi-ichi et al. 2016). Although the identity of the hypothetical (Gal)-terminate ligand is unknown, another protein, the Gal/GalNAc lectin, is suspected to be "the receptor." The latter inference is based on experiments with *E. histolytica* in which Gal/Gal-NAc lectin mediates cell–cell adherence (between trophozoites), binding of trophozoites to the mucous layer of the host's intestinal endothelia, or to bacteria and red-blood cells during colonization of the gut (Eichinger 2001). Thus, the hypothetical galactose (Gal)-terminated ligand and its suspected Gal/Gal-NAc lectin receptor might mediate trophozoite aggregation for encystation (in our view of kin trophozoites), but this needs extensive research (Mi-ichi et al. 2016).

In sum, we have suggested that these extra- and intracellular signals likely interact in the overall physiology of *Entamoeba* spp., inducing cells to respond adaptively to clone–clone associations (same vs. different) for proliferation, locomotion, or encystation. In this respect, there is evidence that secretion of coronin to the milieu, by *E. histolytica*, and of RasGap, actin, protein kinases, heat shock, and ubiquitin, by *D. discoideum*, is directly involved in cell–cell adhesion, regulation of proliferation, or cell aggregation (proteomic analyses; Bakthavatsalam and Gomer 2010; Biller et al. 2014; Espinosa et al. 2016; Paz-y-Miño-C and Espinosa 2018). This implies that the proteins we have identified have similar functions in other *Entamoeba* clones. Realize that this area of work requires the identification of the genes involved in these cell–cell interactions (above), but it is evident that *E. invadens* IP-1 and *E. invadens* VK-1:NS (as per our observations; Espinosa et al. 2016), and likely *E. histolytica*, excrete to their surroundings (and secrete intracellularly) chemical cues for recognition and aggregation (Espinosa et al. 2016; Paz-y-Miño-C and Espinosa 2018). Thus, by interfering with the production of these secretions and/or altering their active chemical properties once present in the milieu, we think that amebas' aggregations (i.e., *E. histolytica*'s) could be disrupted and, therefore, their capacity to encyst and be transmitted from infected to healthy individuals. Current antiamebic drugs seem to work either at the level of the intestinal lumen, where trophozoites proliferate via cell divisions (Fig. 5, life-cycle stage number 5; e.g., drugs paromomycin, iodoquinol, and diloxanide furoate) or at the level of the intestine's endothelium or tissue abscesses, on the invasive trophozoites that have already penetrated the gut or colonized internal organs (Fig. 5, life-cycle stages numbers 5a and 5b; e.g., drugs metronidazole, tinidazole, and ornidazole, generically called nitroimidazoles), but not at both levels (Ali and Nozaki 2007; Anesi and Gluckman 2015; El-Dib 2017). Note that the localized efficacy of these drugs is linked to their differential absorption in the lumen vs. the intestinal endothelium/tissue abscesses, rather than to the amebas' sensitivity to the compounds; in fact, some nitroimidazoles can work well in the lumen if they are chemically prevented from being absorbed (through the gut lining) and allowed to remain in the lumen where they can act upon the trophozoites (Mirelman et al. 1989). The nitroimidazole drugs (as well as auranofin, which still is in clinical trials) apparently bind to amebas' metabolic enzymes (i.e., thioredoxin dismutase, thioredoxin, and superoxide dismutase) and block the trophozoites' capacity to cope with oxidative stress (Andrade and Reed 2015; Leitsch et al. 2007). However, these drugs, particularly the nitroimidazoles that are most commonly prescribed, are highly toxic to patients (causing nausea, vomiting, headaches, dizziness, vertigo, and neuronal damage; Ali and Nozaki 2007), have generated resistance (*E. histolytica* to metronidazole; Wassmann et al. 1999), or can be mutagenic and carcinogenic (as reported in bacterial DNA, rodents, and humans after long exposures to the drugs; Bendesky et al. 2002; Cavaliere et al. 1983; Löfmark et al. 2010). For these reasons, we think that exploring the possibility of managing amebiasis from an alternative perspective (i.e., disrupting the amebas capacity to form aggregations, as suggested above) may supplement current therapeutics. But these research programs need further development.

CONCLUSIONS

The *Entamoeba* spp. ability to discriminate/recognize taxa, clone, or kin, as observed in laboratory aggregation/

segregation experiments (Espinosa and Paz-y-Miño-C 2012, 2014a,b; Espinosa et al. 2016; Paz-y-Miño-C and Espinosa 2016, 2018), suggests that amebas use it during their life cycles to associate and cooperate preferentially with kin, and to deter genetically distant or unrelated rivals (i.e., potential cheaters). Because taxa, clone, and kin discrimination/recognition has been documented in a variety of unicellular eukaryotic lineages (Fig. 1), bacteria, and some archaea (review in Paz-y-Miño-C and Espinosa 2018), as well as in animals and plants (references above), we infer that in *Entamoeba* spp. (Fig. 3), and specifically in *E. histolytica* (Fig. 3 and 4), taxa, clone, and kin discrimination/recognition are—like in other organisms—crucial for survival and reproduction in terms of direct and indirect fitness (inclusive fitness; Hamilton 1963, 1964).

Analogously to the in vitro social migration (SoMo) observations in *Trypanosoma* (Oberholzer et al. 2010, 2015), which were later linked to swarm coordinated movements (kin) inside the tsetse fly gut and body cavity (Imhof and Roditi 2015), our in vitro observations of *Entamoeba* spp. aggregation behavior (particularly *E. histolytica*), in which cells segregate with kin and avoid unrelated clones (Fig. 4), are an indication that group forming with kin helps amebas to survive population bottlenecks awaiting them inside the host (Fig. 5). Population recovery via fast clonal propagation ("safety in numbers") and subsequent association and cooperation with close genetic relatives will secure highest fitness gain (as documented in *Trypanosoma* and *Plasmodium*; Bechtsi and Waters 2017; Guttery et al. 2015; Pollitt et al. 2011; Portugal et al. 2011; Reece et al. 2008; Roditi 2016; Roditi et al. 2016).

In cases in which *Entamoeba* spp. (e.g., *E. histolytica* or *E. dispar*) form abscesses in the host's tissues and/or organs (i.e., the Reyna-Fabián et al.'s 2016 study, above), it is possible that amebas segregate spatially according to kinship and clone identity, similar to the segregation observed in social amebas in the field or laboratory (*D. discoideum*, *D. purpureum*, and *D. giganteum*; Fortunato et al. 2003; Sathe et al. 2010; Smith et al. 2016). But this needs to be investigated by carefully sampling the abscesses at increasing microscopic (μm)-to-macroscopic (mm) distances in order to assess clone–clone spatial distribution in multiclone or multispecies infections (as it has been done with social amebas and myxobacteria; Kraemer et al. 2016; Smith et al. 2016). This is relevant as abscesses are communities of microbes and studies suggest that they can be composed of genotype mixes (e.g., Reyna-Fabián et al. 2016), although the prevalence of clonality in protistan infections is often assumed (Tibayrenc 1993; Tibayrenc and Ayala 2017; Tibayrenc et al. 1990); thus, clone segregation in abscesses needs to be confirmed.

Even though we do not know the genes encoding for *E. histolytica* preferential aggregation with kin, we think that such alleles exist and operate comparably to the *csA* or *tgrB1-trgC1* green beards in *D. discoideum* (i.e., plasma membrane glycoproteins; Hirose et al. 2011, 2015; Queller et al. 2003) or perhaps *FLO1* in *S. cerevisiae* (i.e., Flo-adhesin plasma membrane proteins encoded by the gene, which form lectin-like bonds with mannan oligosaccharide chains in the surface of the cell walls; the proteins stick cells to each other; Smukalla et al. 2008; Verstrepen and Klis 2006). But we do know that aggregations of *E. invadens* IP-1 and *E. invadens* VK-1:NS (and likely *E. histolytica*) secrete into the milieu signals associated with cell proliferation, cell adhesion, cell movement, and stress-induced encystation (RasGap/Ankyrin, coronin-WD40, actin, protein kinases, heat shock 70, and ubiquitin; Espinosa et al. 2016). Thus, because encystation (Coppi et al. 2002; Schaap and Schilde 2018) and abscess formation require cell aggregation (i.e., abscesses are bound to cell recruitment), we postulate that disruption of *E. histolytica* capacity to group, via silencing of alleles yet to be discovered, or drugs that alter the chemistry of the recruitment signals secreted into the milieu, might be approaches to mitigating amebiasis.

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