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ORIGINAL PAPER

Evidence of taxa-, clone-, and kin-discrimination in protists: ecological and evolutionary implications

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Abstract Unicellular eukaryotes, or protists, are among the most ancient organisms on Earth. Protists belong to multiple taxonomic groups; they are widely distributed geographically and in all environments. Their ability to discriminate among con- and heterospecifics has been documented during the past decade. Here we discuss exemplar cases of taxa-, clone-, and possible kin-discrimination in five major lineages: Mycetozoa (*Dictyostelium*, *Polysphondylium*), Dikarya (*Saccharomyces*), Ciliophora (*Tetrahymena*), Apicomplexa (*Plasmodium*) and Archamoebae (*Entamoeba*). We summarize the proposed genetic mechanisms involved in discrimination-mediated aggregation (self vs. different), including the *csA*, *FLO* and *trg* (formerly *lag*) genes, and the Proliferation Activation Factors, which facilitate clustering in some protistan taxa. We caution about the experimental challenges intrinsic to studying recognition in protists, and highlight the opportunities for exploring the ecology and evolution of complex forms of cell–cell communication, including social behavior, in a polyphyletic, still superficially understood group of organisms. Because unicellular eukaryotes are the evolutionary precursors of multicellular life, we infer that their mechanisms of taxa-, clone-, and possible kin-discrimination gave origin to the complex diversification and sophistication of traits associated with species and kin recognition in plants, fungi, invertebrates and vertebrates.

Keywords Altruism · Green-beard effect · Kin selection · Local mate competition · Recognition alleles · Sex ratio

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Abbreviations

HGT Horizontal gene transfer
EPAFs *Entamoeba* Proliferation Activating Factors
TPAFs *Tetrahymena* Proliferation Activating Factors

Introduction

Organisms that discriminate between distant and close genetic relatives can use that information to maximize survival and reproductive success (Hamilton 1964; Maynard-Smith 1964). Closely related individuals should be more likely than distantly related or non-related individuals to engage in altruistic cooperation and pass on the shared genes; competition between kin should be minimized via the ability to discriminate and/or recognize conspecifics' distinct levels of genetic proximity (Hamilton 1964; Maynard-Smith 1964; Herbers 2013). In the past decade, unicellular eukaryotes have been tested for discrimination/recognition ability (Mehdiabadi et al. 2006; Ostrowski et al. 2008; Reece et al. 2008; Chaine et al. 2010; Kalla et al. 2011; Nkhoma et al. 2012; Espinosa and Paz-y-Miño-C 2012) and the genetic mechanism of detection of conspecifics, and possible recognition of kin, has been proposed for some taxa (Queller et al. 2003; Smukalla et al. 2008; Benabentos et al. 2009; Hirose et al. 2011). Here we discuss studies conducted with protists in which the ecological and evolutionary significance of a potential capacity to distinguish between same (clone/kin members) versus different (distantly related con- or heterospecifics) is highlighted. We draw attention to experimental challenges intrinsic to culturing cell-lines in the laboratory, which can lead to confounding interpretations of results in discrimination or recognition tests. Finally, we highlight the opportunities that the studies with protists offer to hypothesize about the origin and evolution of ancient cell-to-cell mechanisms of discrimination in a diverse group of organisms, observe their behavior and ecology in the field and laboratory.

How do protists discriminate between same versus different?

Despite their vast lineage diversity, studies on clone-to-clone discrimination, con- and heterospecifics, or kin recognition in protists are scarce. In Table 1, we summarize chronologically (2003-present) exemplar work conducted in five major lineages: Mycetozoa (*Dictyostelium*, *Polysphondylium*), Dikarya (*Saccharomyces*), Apicomplexa (*Plasmodium*), Ciliophora (*Tetrahymena*), and Archamoebae (*Entamoeba*). Note that the *Entamoeba* examples are discussed in the section Laboratory Challenges in Protists' Discrimination Trials, below.

Mycetozoa: *Dictyostelium*, *Polysphondylium*

The social amoeba *Dictyostelium discoideum* is particularly well understood. Upon environmental stress, like starvation, thousands of soil free-living individuals aggregate in "mounds" which turn into "slugs" that move synchronously; slugs anchor on a substrate to form a "fruiting body" by allocating dying cells to a stalk that supports a spore-encasing structure (inside of which, cells differentiate into spores); once released, under favorable

Table 1 Exemplar studies in which taxa-, clone-, and possible kin-discrimination in protists is documented

Organism	Behavioral trait	Experimental observation	Proposed mechanism	References
Dictyostelium discoideum	Fruiting-body formation	*csA*+ cells form fruiting bodies with same	*csA* gene	Queller et al. (2003)
Dictyostelium purpureum	Fruiting-body formation	Highly related (r) fruiting-body formation	Unknown	Mehdiabadi et al. (2006)
Dictyostelium giganteum	Fruiting-body formation	Clonal/non-clonal fruiting body formation	Unknown	Kaushik et al. (2006)
Dictyostelium discoideum	Fruiting-body formation	Highly related (r) fruiting-body formation	Unknown	Ostrowski et al. (2008)
Saccharomyces cerevisiae	Flocculation biofilm-like clusters	*FLO1*+ cells cluster with carries of gene	*FLO1* gene	Smukalla et al. (2008)
Plasmodium chabaudi	Among-clone competition	Selfing to outcompete unrelated	Unknown	Reece et al. (2008)
Dictyostelium discoideum	Mound formation, slug migration	Clonal aggregation/ migration in cultures	*lagB1 lagC1* genes[a]	Benabentos et al. (2009)
Tetrahymena thermophila	Aggregation in clusters	Motility toward and aggregation with clones	TPAF[b] molecules	Chaine et al. (2010)
Polysphondylium violaceum	Fruiting-body formation	Clonal fruiting bodies form in mixed cultures	Unknown	Kalla et al. (2011)
Dictyostelium discoideum	Fruiting-body formation	Clonal fruiting bodies form in mixed cultures	*tgrB1 tgrC1* genes[a]	Hirose et al. (2011)
Plasmodium falciparum	Within-clone competition	Kinship patterns of infection in host	Unknown	Nkhoma et al. (2012)
Entamoeba invadens	Aggregation in clusters	Clonal aggregation in mixed cultures	Unknown	Espinosa and Paz-y-Miño-C (2012)
Entamoeba moshkovskii	Aggregation in clusters	Clonal aggregation in mixed cultures	EPAF[c] molecules	Espinosa and Paz-y-Miño-C (in press)

[a] *lag* and *tgr* are synonymous for the genes *lagB1*/*lagC1* and *trgB1*/*tgrC1*

[b] *Tetrahymena* Proliferation Activating Factors

[c] *Entamoeba* Proliferation Activating Factors

conditions, the spores mature into free-living, propagating amoebae (Romeralo et al. 2012). To remain in intimate proximity, amoebas rely on cell-membrane adhesion proteins like those encoded by the *csA* gene; when wild-type *csA*+ cells are grown in mixed soil cultures with *csA*− knockouts, the amoebas cluster preferentially with those equipped with fully functional adhesion polypeptides (Queller et al. 2003). Analogous experiments (wild-type vs. knockout effects in binary cultures) have been conducted with the *tgrB1* and *tgrC1* genes (formerly *lagB1* and *lagC1*), which also encode for cell adhesion transmembrane proteins (Benabentos et al. 2009). However, distinctive from *csA*+, which function is primarily adhesive (although clone specific), the *trg* genes work in complementary pairs directly involved in cell–cell discrimination and possible recognition (Benabentos et al. 2009; Hirose et al. 2011; Strassmann and Queller 2011). When fully functional pairs of *tgrB1*+ and *tgrC1*+ are extracted from the wild (genetically different clones), expressed in

identical cells which *tgr* genes have been previously knocked out in the laboratory (i.e. *tgrB1*− and *tgrC1*−), and grown in mixed cultures, the descendants proliferate and segregate into distinctive mounds and fruiting bodies resembling the strains from which the wild-type genes originated (Hirose et al. 2011). Moreover, a positive relation has been documented between the degree of genetic distance and the degree of recruitment of cells for the formation of fruiting bodies among clone-isolates from three social Mycetozoans (*D. discoideum*, *D. purpureum*, *P. violaceum*) collected in diverse localities (Mehdiabadi et al. 2006; Ostrowski et al. 2008; Kalla et al. 2011). This raises the question if social amoebae can or need to discriminate kin from non-kin, but this possibility needs unequivocal confirmation that kin discrimination has an adaptive value for the population structure of social *Dictyostelium* or *Polysphondylium* considering that their fruiting bodies in the wild are often composed of clonal clusters (Gilbert et al. 2012). Furthermore, because studies with another social amoebae, *D. giganteum*, have generated inconclusive results, in which cell-lineages collected from the wild and grown together cluster in fruiting bodies of variable degree of chimerism, aggregation could result from epigenetic phenomena (i.e. interaction of environmental factors that influence gene expression and the trajectory of cell development toward cluster formation) rather than from kinship (Kaushik et al. 2006).

Dikarya: *Saccharomyces*

Free living yeast, *S. cerevisiae*, also depend on clumping behavior (flocculation), or biofilm-like formation, to survive under stressful environmental conditions. Cells equipped with *FLO* genes can express cell-surface proteins that allow cell–cell adhesion during flocculation. Yeast carriers of *FLO1* aggregate with thousands of other *FLO1*+ cells regardless of their close or distant genetic relatedness in the rest of the genome (Smukalla et al. 2008). This single-gene-mediated example of behavioral modulation, among only those carrying *FLO1*, is consistent with the recognition alleles model or "green-beard effect" (Hamilton 1964; Dawkins 1976) in which a single gene promotes cooperation toward other carriers of the gene even if they are non-kin.

Apicomplexa: *Plasmodium*

Malaria-causing protists, *Plasmodium*, seem to discriminate between closely and distantly related conspecifics and use that information to maximize reproduction and host infestation (Schall 2008). *Plasmodium* replicates asexually within a host and also via production of male and female cells (gametocytes) that are carried by mosquito-vectors; gametocytes develop into gametes, which later combine during sexual reproduction inside the insect (Reece et al. 2008). When single *P. chabaudi* clones (sporozoites) infest a laboratory rodent, the sex ratio of the later emerging gametocytes is conspicuously female-biased (i.e. a few males suffice to fertilize the available same-clone females since equal sex-ratio would lead to non-adaptive excess of male gametes); but when multiple-clone infections occur within the same host, *P. chabaudi* increases the representation of male gametocytes in the population (Reece et al. 2008). This is explained by Hamilton's (1967) model of local mate competition which predicts that female-biased sex allocation will be favored when closely related males compete for mates, as in the same-clone environment. In mixed-clone infections, however, the optimal sex ratio for each genotype depends on the probability of selfing, thus it is expected to shift toward males, i.e. more males are needed to probabilistically encounter and fertilize same-clone females in a multiple-clone milieu

(Hamilton 1967; Reece et al. 2008; Schall 2008). *Plasmodium chabaudi* increases the proportion of male gametocytes as function of two factors: the differential degree of genetic distance *between* clones and the relative abundance of different-clone-cells versus self (Reece et al. 2008). For such adjustment to occur, *P. chabaudi* might rely on a mechanism (possibly plasma-membrane mediated, as in *Dictyostelium*, above) for the discrimination between self and different (Reece et al. 2008); but this is yet to be genetically characterized after excluding environmental factors, like host-immune responses (coming from the wild-type-rodent host rather than from the experimental laboratory mice, which are often immuno-compromised) or cellular-toxin production during clone–clone competition, which could still influence sex-ratio allocation independently from cell–cell discrimination ability.

Evidence that malaria pathogens can discriminate self versus different as function of genetic distance *within* clones, and relative to the presence of multiple clone competitors, comes from *P. falciparum*, which infects humans (Nkhoma et al. 2012). After male–female gamete fertilization, which occurs inside the mosquito vector, the resulting diploid zygote undergoes meiosis to generate recombinant sporozoites, each with differential potential to survive and reproduce, as a merozoite clone, in the host. The proportion of genetic relatedness among infecting merozoites *within* hosts (i.e. half-sibling or greater) is much higher than between hosts (i.e. 60 vs. 3 %); thus suggesting an intrinsic ability of *P. falciparum* to colonize hosts via transmission of multiple-closely-related sporozoites. But the selective pressure imposed by the host-immune system can also contribute to the observed patterns of kinship infestation by excluding other competing clones in multiple infections (Nkhoma et al. 2012). In this context, it is plausible that the reciprocal antagonistic and co-evolutionary interaction between *P. falciparum* and its host's immune system leads to a "matched" compatibility; in it, the infection does not depend solely on the human susceptibility or resistance to *P. falciparum*, but also on the "matched status" of pathogen and host phenotypes (matching phenotype model; Théron and Coustau 2005; Mitta et al. 2012; Medeiros et al. 2013). This opens an important area of investigation where the molecular determinants of compatibility need to be identified: the pathogen's antigens (which could be both polymorphic and clone specific) and the cellular immune receptors in the host (Mitta et al. 2012; Medeiros et al. 2013).

Ciliophora: *Tetrahymena*

Tetrahymena thermophila is a ciliate which genetic polymorphisms encode for differential levels of cell clustering and dispersal. Aggregation can be costly since it decelerates growth rate and reduces cell size, although it improves survival by both increasing tolerance to crowding and gaining access to patchy, ephemeral resources (Chaine et al. 2010). Cells exude *Tetrahymena* Proliferation Activating Factors (TPAFs) that are used by con- or heterospecifics to detect each other in the environment. When cells of genetically distinctive levels of aggregation (i.e. high, medium or low) are given the choice in the laboratory to disperse toward either the TPAFs previously exuded by an unrelated clone (relatedness $r = 0$) or by themselves ($r = 1$), they migrate toward their own cell-line TPAFs if they belong to the high-aggregation genotype; in contrast, medium- or low-aggregation genotypes have no preference or avoid their own cell-line TPAFs, respectively (Chaine et al. 2010). Thus, *T. thermophila* seems capable of discriminating between self and different, and also of modulating dispersal behavior as function of aggregative genotype.

Laboratory challenges in protists' discrimination trials

In recent studies on discrimination in protists, a potential methodological problem has been highlighted. Laboratory strains customarily classified within single taxonomic lineages might belong to distinctive taxa and, therefore, generate confounding interpretations of results in discrimination or recognition tests (Espinosa and Paz-y-Miño-C 2012, in press). The strains of *E. invadens*, IP-1 and VK-1:NS, illustrate this scenario. Both differ in a single nucleotide of the small subunit ribosomal RNA (ssrRNA) and are considered strains of the same species (Clark et al. 2006; Stensvold et al. 2010), even though they have been isolated from phylogenetically distant hosts: IP-1 from the turtle *Chrysemys picta* and the snake *Natrix cyclopion* (Meerovitch 1958), and VK-1:NS from the Komodo Dragon, *Varanus comodoensis* (Gray et al. 1966). When grown in mixed cultures, each strain aggregates only with self and maintains separation from clusters of the non-alike amoebae; moreover, each strain can be characterized by its distinctive morphology and pattern of aggregation (i.e. cell size within cluster, number of amoebas per cluster, size of and distance between clusters; Espinosa and Paz-y-Miño-C 2012, in press). These observations, together with the fact that cell lines have been isolated from multiple hosts, suggest that *E. invadens* IP-1 and VK-1:NS belong to separate taxa, possibly distinct biological species, capable of discriminating between one another. Therefore, kin bias or kin discrimination is yet to be documented in *E. invadens*. (i.e. measured as function of close vs. distant genetic relatedness among clones).

Two other *Entamoeba* varieties represent a similar challenge; *E. moshkovskii* Laredo, isolated from humans (Dreyer 1961), and *E. moshkovskii* Snake, isolated from Ophidia (Clark and Diamond 1997). In Fig. 1, we show the aggregation preference of *E. moshkovskii* Laredo and Snake in self versus mixed cultures; for comparison, we also show in Fig. 2 the *E. invadens* clones IP-1 and VK-1:NS under same laboratory conditions. Each of the *E. moshkovskii* and *E. invadens* clones aggregates only with self, regardless of cell-color labeling, and when grown in single clone cultures with equal ratios of cells tagged with a different color, the amoebas intermingle evenly. *Entamoeba* spp. probably exude *Entamoeba* Proliferation Activating Factors (EPAFs, analogous to the TPAFs above) that are used to detect each other in the environment.

It is, therefore, crucial that studies on species–species, clone–clone, or potential kin discrimination ability in protists confirm, prior to experimentation, the degree of *genomic* distance (i.e. based on multiple genetic markers across the entire genome, rather than on low-resolution taxonomic classifications relying on ssrRNA, as in the *E. invadens* and *E. moshkovskii* cases, above) between and within the cell lines to be used in laboratory trials. This is a challenge considering that protists' taxonomy is still superficially understood (Caron 2013; Pawlowski 2013).

If kin discrimination/recognition is indeed essential for survival advantage and reproduction of unicellular eukaryotes, the studies on protistan behavior, behavioral ecology and evolution need to explore the spatio-temporal effect of kin discrimination/recognition on fitness (i.e. the kin population structure resulting from the mechanisms of discrimination/recognition; Kamel and Grosberg 2013). The relationship between pathogens and hosts seem illustrative of these investigations, thus far conducted mostly in the laboratory, but from which future research on free-living lineages might benefit. For example, *Plasmodium* responds to both the genetic diversity and density of self versus co-infecting conspecifics by maximizing transmission of male/female gametocytes when the host environment is either favorable (i.e. high density of matching-to-parasite blood cells) or diminished (i.e. hosts carrying anti-malaria drugs or undergoing an anemic drop in red-blood-cells; Pollitt et al.

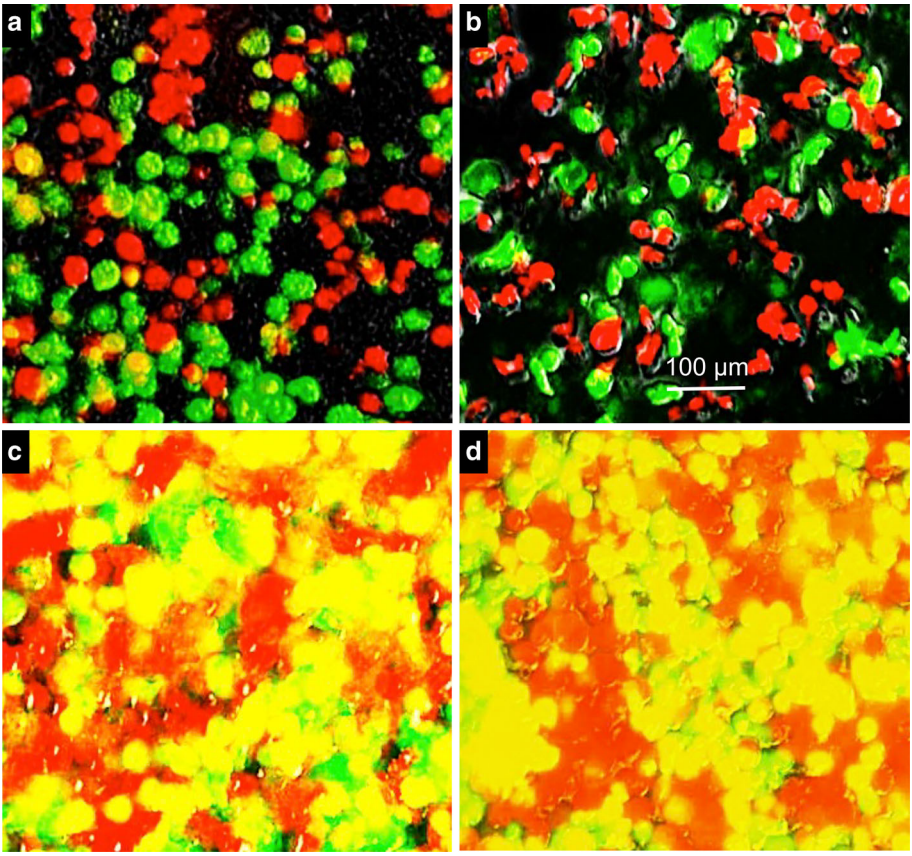

Fig. 1 Clone-aggregation preference shown by *Entamoeba moshkovskii* Laredo and *E. moshkovskii* Snake in mixed- or single-cell-line cultures. **a** Fluorescent micrograph of *E. moshkovskii* Laredo labeled *green* and *E. moshkovskii* Snake labeled *red*, each clone aggregates in distinct clusters. **b** Reverse-color labeling of trophozoites of *E. moshkovskii* Laredo (*red*) and *E. moshkovskii* Snake (*green*), the clones aggregate in distinct clusters. **c** *E. moshkovskii* Laredo labeled with both *green* and *red* dyes; trophozoites mix equally and look *yellow* under the microscope. **d** *E. moshkovskii* Snake labeled with both *green* and *red* dyes. In all trials, cells were labeled with CellTracker *Green* and *Red* CMFD (Invitrogen, Carlsbad, CA). All images taken at 36 h, scale bar = 100 µm, ×10 magnification. (Color figure online)

2011). In contrast, when *Plasmodium* experiences intermediate host-stress levels (i.e. low host-immune factors, relaxed competition among genetically unrelated co-infecting strains, or low levels of anti-malaria drugs), it reduces production of gametocytes and relies chiefly on asexual proliferation of sporozoites (Pollitt et al. 2011). This strategy of resource allocation trade-off between maintaining the infection (survival) and investment in transmission (reproduction) has been linked to *Plasmodium* capacity to discriminate between kin and non-kin and its direct association with fitness (Pollitt et al. 2011).

Ecological and evolutionary implications

Due to their vast phylogenetic diversity and geographic distribution in all Earth's environments (Stoeck and Stock 2010; Caron 2013; Pawlowski 2013), the patterns of taxa-,

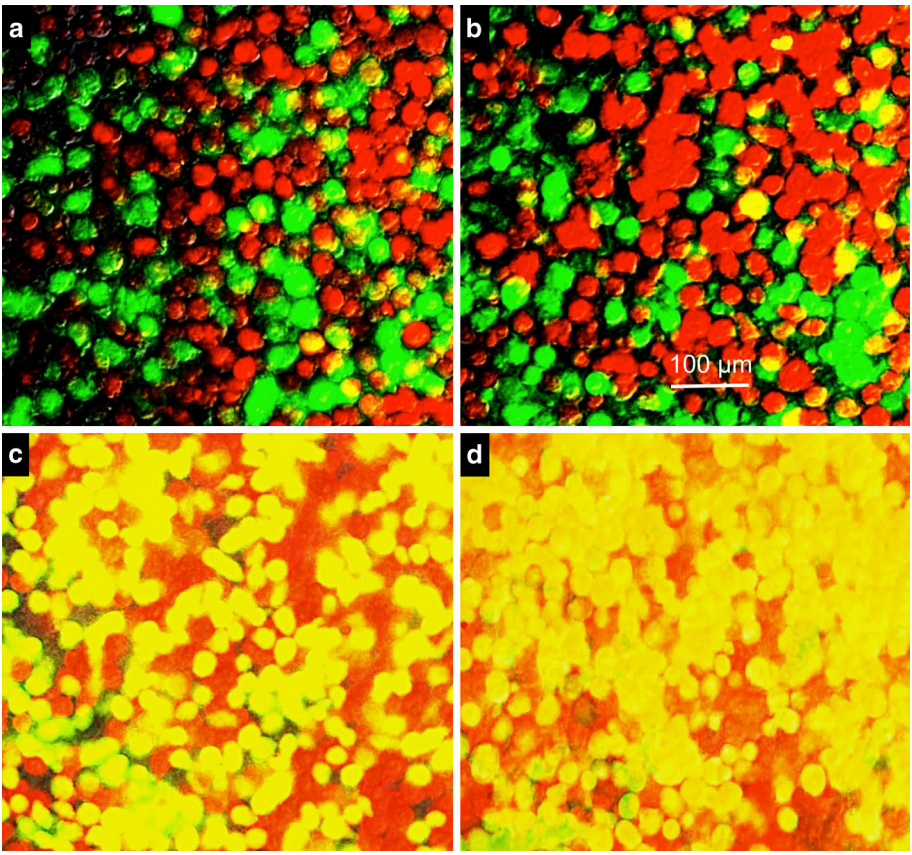

Fig. 2 Clone-aggregation preference shown by *Entamoeba invadens* IP-1 and *E. invadens* VK-1:NS in mixed- or single-cell-line cultures. **a** Fluorescent micrograph of *E. invadens* IP-1 labeled *green* and *E. invadens* VK-1:NS labeled *red*, *each clone* aggregates in distinct clusters. **b** Reverse-color labeling of trophozoites of *E. invadens* IP-1 (*red*) and *E. invadens* VK-1:NS (*green*), the clones aggregate in distinct clusters. **c** *Entamoeba invadens* IP-1 labeled with both *green* and *red* dyes; trophozoites mix equally and look *yellow* under the microscope. **d** *Entamoeba invadens* VK-1:NS labeled with both *green* and *red* dyes. In all trials, cells were labeled with CellTracker *Green* and *Red* CMFD (Invitrogen, Carlsbad, CA). All images taken at 36 h, scale bar = 100 µm, ×10 magnification. (Color figure online)

clone-, and kin-discrimination ability of protists in the wild, or the origin and evolutionary significance of these traits, remain unknown. In such a large, polyphyletic group of unicellular eukaryotes, convergence in trait acquisition for discrimination ability, combined with ubiquitous horizontal gene transfer (HGT), must be common (Paz-y-Miño-C and Espinosa 2010; Bruto et al. 2013).

Spatio-temporal co-occurrence of genetic closely related individuals might be a byproduct of opportunistic colonization of patchy/ephemeral resources, where first clones arrive and proliferate while outcompeting others, or in asexual propagation of cells during vegetative cycles, such as those described in *Dictyostelium* and *Tetrahymena* (Gilbert et al. 2012), or driven by immune responses by a host, like in *Plasmodium* (Nkhoma et al. 2012). When being sampled, such clones could give us the impression of intrinsic kin-biased

aggregation, kin-discrimination, or even kin-recognition, when in fact their close genetic proximity results from epigenetic phenomena, i.e. environmental events inducing the sequence and development of habitat colonization, opportunities for asexual or sexual propagation in the free-living milieu or inside hosts (Kaushik et al. 2006). At the same time, it is important to acknowledge that genes involved directly in aggregation behavior (e.g. *csA*, *FLO1*; Queller et al. 2003; Smukalla et al. 2008), some as function of genetic distance (e.g. *tgr/lagB1* and *tgr/lagC1*; Benabentos et al. 2009; Hirose et al. 2011), have been documented and provide evidence that genetic mechanisms do exist to modulate behavior when cells require to discriminate between taxa, clones, close- and distant-genetic relatives. In this respect, the *FLO1* gene is illustrative of a "recognition allele" role (Hamilton 1964); it facilitates aggregation of cells that carry the gene although the rest of the genome differs (Smukalla et al. 2008). The adaptive value of such type of gene (which appears to behave "selfishly"; Dawkins 1976) is intriguing, and the phenotype it encodes for in the transgenic *FLO1+* yeast offers interesting possibilities for laboratory experimentation.

From an epidemiological and health-care application perspectives, the ecological/evolutionary implications of the interaction between pathogens and their kin-biased behaviors inside hosts require systematic investigation, including: evolution of pathogen virulence as function of the ability to discriminate/recognize kin; sexual and nonsexual reproduction of pathogens as function of vector and host immune responses; effects and costs of inbreeding/outbreeding during pathogen propagation; modeling of the interaction between pathogen relatedness structure and host resistance evolution; and cooperation and intra-host dynamics and population genetics of co-infecting pathogens (Foster 2005; Pollitt et al. 2011; Mideo and Reece 2012; Nkhoma et al. 2012).

Conclusions

Protists open significant opportunities to develop comprehensive research programs to study the origin and evolution, at the organismic unicellular level, of complex forms of cell–cell communication, including social behavior and the scientific paradigms in kin-selection theory (i.e. cooperation to maximize food intake, colonize environments, form reproductive assemblages, or infest hosts and manipulate vectors for infection; altruism/cheating in programmed cell death during cysts-/fruiting-body formation, or during infestation of hosts; Penn and Frommen 2010; Strassmann and Queller 2011; Gardner and West 2010; Ghoul et al. 2014). Because unicellular eukaryotes are precursors of multicellular life, we can infer that the mechanisms of species and kin discrimination/recognition documented in today's Bikonta (including plants) and Unikonta (including fungi and animals; Lecointre and Le Guyader 2006) coalesce evolutionarily to the ability of ancient cells to distinguish between close and distant genetic relatives. The sophisticated anatomical, physiological, behavioral and cognitive traits associated with species and kin discrimination/recognition in plants, fungi, invertebrates and vertebrates have evolved and diverged gradually from ancestral unicellular features.

Population structure as function of kinship, in both parasitic and free-living taxa, is virtually unknown in protists and deserves detailed analyses (Kamel and Grosberg 2013). Laboratory and field studies need to carefully examine the ecology (i.e. of the free-living, commensal, or parasitic lineages) and the degree of genetic distance, between and within cell-lines used in experiments, prior to inferring, conclusively, that unicellular eukaryotes can discriminate taxa, clones or kin. The epidemiological implications and health-care

applications of taxa-, clone-, and possibly kin-discrimination/recognition ability in protists also need further investigation.

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