Journal of Eukaryotic Microbiology ISSN 1066-5234

SYMPOSIUM ARTICLE

Kin Discrimination in Protists: From Many Cells to Single Cells and Backwards[1]

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Keywords
Antirecognition strategies; cheaters and resisters; communication problem; green-beard effect; honest signals; inclusive fitness; kin selection; phenotype matching; recognition alleles.

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[1]Presentation delivered at the symposium Evidence of Taxa-, Clone- and Kin-Discrimination in Protists: Ecological and Evolutionary Implications, VII European Congress of Protistology – University of Seville, 5–10 September 2015, Seville, Spain.

Received: 20 December 2015; revised 3 February 2016; accepted February 7, 2016.

doi:10.1111/jeu.12306

ABSTRACT

During four decades (1960–1990s), the conceptualization and experimental design of studies in kin recognition relied on work with multicellular eukaryotes, particularly Unikonta (including invertebrates and vertebrates) and some Bikonta (including plants). This pioneering research had an animal behavior approach. During the 2000s, work on taxa-, clone- and kin-discrimination and recognition in protists produced genetic and molecular evidence that unicellular organisms (e.g. *Saccharomyces*, *Dictyostelium*, *Polysphondylium*, *Tetrahymena*, *Entamoeba* and *Plasmodium*) could distinguish between same (self or clone) and different (diverse clones), as well as among conspecifics of close or distant genetic relatedness. Here, we discuss some of the research on the genetics of kin discrimination/recognition and highlight the scientific progress made by switching emphasis from investigating multicellular to unicellular systems (and backwards). We document how studies with protists are helping us to understand the microscopic, cellular origins and evolution of the mechanisms of kin discrimination/recognition and their significance for the advent of multicellularity. We emphasize that because protists are among the most ancient organisms on Earth, belong to multiple taxonomic groups and occupy all environments, they can be central to reexamining traditional hypotheses in the field of kin recognition, reformulating concepts, and generating new knowledge.

MOST of the theoretical framework and experimental settings to elucidate the mechanisms of kin discrimination come from studies with multicellular eukaryotes (i.e. insects, amphibians, fish, reptiles, birds, mammals, and, to a much lesser extent, plants; Biedrzycki and Bais 2010; Dudley et al. 2013; Fletcher and Michener 1987; Hepper 1991; Holmes and Sherman 1983; Penn and Frommen 2010; Tang-Martínez 2001). Theory and laboratory work have relied on the assumption that altruistic, nepotistic behavior—adaptive cooperation within family units—has evolved in parallel with an organism's ability to tell apart close- from distant- or nonrelated conspecifics. Darwin (1859) speculated about the "puzzle of the sterile social insects," in which female workers at a nest dedicate their lives to the persistence of the colony (structured around a large progeny), via assisting a fertile queen to reproduce with the available males. Darwin suggested that, in such cases of apparent sacrifice—by the workers—for the good of all, "selection may be applied to the family." But, in the late 1800s, he could not offer a detailed mechanistic explanation for the latter.

Fisher (1930) and Haldane (1932, 1955) wrestled with the genetics and mathematics of altruism and the anecdotic expression "I would lay down my life for two brothers or eight cousins" became legacy of their work. Hamilton (1964) and Maynard-Smith (1964) further reasoned that the ability to discriminate between close and distant genetic relatives could be directly linked to survival and reproductive success, and, ultimately, to kin selection (Maynard-Smith 1964, 1977). Close relatives would engage in altruistic cooperation to pass on the shared genes and minimize competition with kin. According to Hamilton (1964), the fitness benefits (*b*) of helping another individual correlate with the coefficient of genetic relatedness (*r*) and are closely dependent on the costs (*c*) of

helping. If the benefits ($_rb$) surpass the costs (c), then altruistic cooperation could evolve ($_rb - c > 0$).

The "field of kin recognition," which conceptual foundations can be traced back to the 1960s, has no consensus on definitions or proposed mechanisms, possibly due to the vast diversity of life histories across organisms and their phylogenetic complexities (reviews in Penn and Frommen 2010; Tang-Martínez 2001). Here we refer to "recognition" as an organism's ability to identify kin versus non-kin; in addition, we use the term "discrimination" as the capacity to distinguish one clone from another. Because we discuss instances of taxa-, clone-, and kin-discrimination/recognition in single-celled organisms capable of both discriminating between same and different, and discriminating/recognizing among clones of distinctive value of r, we use these terms together (but see Penn and Frommen 2010; Tang-Martínez 2001).

Recent studies with unicellular eukaryotes (protists) have uniquely enriched the field of kin recognition, particularly after characterizing the genes involved in discrimination-mediated aggregation (mostly for flocculation or biofilm-like formation, starvation-triggered dormancy, or reproduction; below) or in clone-versus-clone competition to colonize hosts in parasitic taxa (Espinosa and Paz-y-Miño-C 2014a). The last decade of kin recognition studies with protists has focused on the molecular biology and genetics of cell–cell discrimination and kin recognition abilities in *Saccharomyces*, *Dictyostelium*, *Polysphondylium*, *Tetrahymena*, *Entamoeba* and *Plasmodium* (Table 1; Espinosa and Paz-y-Miño-C 2014a). Here we review these studies succinctly and remark on the type of scientific progress (i.e. basic and applied science) that can be achieved by using protists as model organisms in investigations of taxa-, clone- and kin-discrimination. We highlight that

Table 1. Evidence of taxa-, clone-, and kin-discrimination/recognition in protists

Organisms	Behavioral trait reported	Experimental observation	Proposed mechanism
Dikarya			
Saccharomyces cerevisiae	Flocculation biofilm-like clusters	*FLO1*+ cells cluster with carries of gene	*FLO1* gene[a]
Mycetozoa			
Dictyostelium discoideum	Fruiting-body formation	*csA*+ cells form fruiting bodies with same	*csA* gene[b]
D. discoideum	Fruiting-body formation	Highly related (r) fruiting-body formation	Unknown[c]
D. discoideum	Mound formation, slug migration	Clonal aggregation/migration in cultures	*lagB1 lagC1* genes[d]
D. discoideum	Fruiting-body formation	Clonal fruiting bodies form in mixed cultures	*tgrB1 tgrC1* genes[e]
Dictyostelium purpureum	Fruiting-body formation	Highly related (r) fruiting-body formation	Unknown[f]
Dictyostelium giganteum	Fruiting-body formation	Clonal/non-clonal fruiting-body formation	Unknown[g]
Polysphondylium violaceum	Fruiting-body formation	Clonal fruiting-bodies form in mixed cultures	Unknown[h]
Ciliophora			
Tetrahymena thermophila	Aggregation in clusters	Motility toward and aggregation with clones	TPAF molecules[i]
Archamoebae			
Entamoeba invadens	Aggregation in clusters	Clonal aggregation in mixed cultures	Unknown[j]
Entamoeba moshkovskii	Aggregation in clusters	Clonal aggregation in mixed cultures	EPAF molecules[k]
Apicomplexa			
Plasmodium chabaudi	Among-clone competition	Selfing to outcompete unrelated	Unknown[l]
Plasmodium falciparum	Within-clone competition	Kinship patterns of infection in host	Unknown[m]

[a]Smukalla et al. (2008).
[b]Queller et al. (2003).
[c]Ostrowski et al. (2008).
[d]*lag* and *tgr* are synonymous for the genes *lagB1*/*lagC1* and *trgB1*/*tgrC1*; Benabentos et al. (2009). There are other genes involved in social cooperation in *D. discoideum*, however, indirectly linked to cell–cell discrimination/recognition, including: *fbxA* (targets proteins for ubiquination), *dimA bZIP*/*bRLZ* (transcription factor); *chtC* (putative transmembrane protein, null mutants are facultative cheaters), *rccA* (resists exploitation by *chtC* and does not cheat on wild type; Li and Purugganan 2011).
[e]Hirose et al. (2011).
[f]Mehdiabadi et al. (2006).
[g]Kaushik et al. (2006).
[h]Kalla et al. (2011).
[i]*Tetrahymena* Proliferation Activating Factors TPAFs; Chaine et al. (2010).
[j]Espinosa and Paz-y-Miño-C (2012).
[k]*Entamoeba* Proliferation Activating Factors EPAFs; Espinosa and Paz-y-Miño-C (2014a,b). Our lab has identified six putative cell-signals excreted 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; these putative EPAFs are likely linked to clone–clone discrimination/recognition (Espinosa et al. in press).
[l]Reece et al. (2008).
[m]Nkhoma et al. (2012).

protists are robust systems to test multiple hypotheses in overlapping research programs in kin recognition, which can also be extended to exploring the origins of multicellularity. Some of these hypotheses include: recognition alleles and "green-beard" genes (Dawkins 1976; Hamilton 1964); the communication problem in honest signaling for kin recognition (Johnstone 1997); cheating and concealed identity by signal senders during kin recognition (antirecognition strategies; Beecher 1991); origin of multicellularity under the inclusive fitness principle (Hamilton 1964), or as a byproduct of the interactions between cheaters and resisters in situations of low-genetic relatedness (Buss 1987; Levin et al. 2015). We discuss these topics from the perspective that an integrated, multidisciplinary approach (i.e. molecular, genetic, physiological, behavioral, ecological, and evolutionary) is needed to make significant progress in the field.

Note that important scientific literature exists on kin discrimination/recognition in prokaryotes and its implications for the origin and evolution of multicellular-prokaryotic assemblages. Although we do not discuss prokaryotes in this article, we advise the reader to search for information elsewhere (e.g. Celiker and Gore 2013; Kraemer and Velicer 2011; Lyons and Kolter 2015; Mitri and Foster 2013; Pathak et al. 2013; Rumbaugh et al. 2012; West et al. 2006).

THE GENETICS OF KIN RECOGNITION: FROM MANY CELLS TO SINGLE CELLS

Hamilton (1964) suggested that recognition of conspecifics—prior to engaging in adaptive cooperation or altruism—would ultimately depend on genes (i.e. recognition alleles). In retrospect, his "super gene" model referred to a pleiotropic gene capable of: (i) influencing the expression of a trait for recognition, which would allow (ii) carriers of the trait to recognize similar traits in others, and (iii) induce the carriers to behave altruistically only toward other carriers of the gene. Dawkins (1976) called this phenomenon the "green-beard effect" (i.e. green-beard genes) in which any carrier of the green-beard-cue for recognition would spot the cue in conspecifics (regardless of their genetic proximity in the rest of the genome; Gardner and West 2010). The major problem with this model was that it would be evolutionarily unstable and prone to erode cue diversity (but see Ho and Shaulsky 2015), since the popular cue (i.e. the green beard) would quickly outnumber other cues in the population and become ineffective for the high advantage it initially had: selective cooperation and altruism toward the carriers of the super gene(s). Alternatively, green-beard genes could be polymorphic, or multiple genes could potentially adopt comparable functions (for recognition), and the system become evolutionarily stable under the relative adaptive value of such genes in their genomes and populations (Gardner and West 2010).

The recognition alleles model remained a theoretical supposition for thirty years, until Keller and Ross (1998) reported—via indirect evidence—its possible existence in the red fire ant *Solenopsis invicta*. These authors described that in ant colonies Bb workers were able to distinguish BB from Bb queens via odor cues (pheromones), and with the peculiarity that Bb workers killed BB queens; a behavior induced by the allele $Gp\text{-}9^b$, which was hypothesized to be linked to a green-beard allele. Because the bb genotype had low viability and/or fertility (i.e. zero representation in the population), the authors were able to quantitate with confidence the proportion of queens of each of the viable $Gp\text{-}9^b$ genotypes that were attacked by the workers. They found that, at age 1 (early in life) and age 2 (later in life), the workers consistently killed the BB queens (61% of queens were killed by workers of age 1, and 91% of queens were killed by workers of age 2) but never killed the Bb genotype. In addition, the proportion of workers of each $Gp\text{-}9^b$ genotype (either BB or Bb workers) that recruited around attacked or nonattacked queens (also of each genotype, BB or Bb queens) allowed the authors to infer the possible existence of recognition alleles that functioned via a green-beard effect: BB workers had low recruitment around attacked BB queens (21% of BB workers) or nonattacked Bb queens (34% of BB workers). However, 78% of Bb workers recruited around and killed BB queens (i.e. suggesting high drive to recruit around BB queens to kill them). In contrast, 62% of Bb workers recruited around and never killed the Bb queens (i.e. suggesting usual drive to recruit around Bb queens and coexist with them). Ever since the Keller and Ross (1998) study came out, additional inference-based research on recognition alleles in multicellular eukaryotes has emerged, but skepticism about its actual empirical demonstration in invertebrates and vertebrates continues (Gardner and West 2010; Penn and Frommen 2010).

Direct substantiation of specific genes involved in cell–cell discrimination or kin recognition came during the 2000s, from experiments with protists, particularly the common yeast *Saccharomyces cerevisiae* (Dikarya) and the social ameba *Dictyostelium discoideum* (Mycetozoa; Table 1; Espinosa and Paz-y-Miño-C 2014a). As follows:

Free-living yeast, *S. cerevisiae*, depends 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 (above) in which a single gene promotes cooperation toward other carriers of the gene even if they are non-kin (Espinosa and Paz-y-Miño-C 2014a).

The social ameba *D. discoideum* has also become exemplar of green-beard genes. 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 globular structure (inside of

which, cells differentiate into spores); once released, under favorable conditions, the spores mature into free-living, propagating ameba (Espinosa and Paz-y-Miño-C 2014a; Romeralo et al. 2012). To remain in intimate proximity, amebas 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 amebas cluster preferentially with those equipped with fully functional adhesion polypeptides (Espinosa and Paz-y-Miño-C 2014a; Queller et al. 2003). Analogous experiments (wild-type versus 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; Espinosa and Paz-y-Miño-C 2014a). However, distinctive from *csA+,* which function is primarily adhesive (although clone specific), the *tgr* genes work in complementary pairs directly involved in cell–cell discrimination and possible recognition (Benabentos et al. 2009; Espinosa and Paz-y-Miño-C 2014a; 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 (Espinosa and Paz-y-Miño-C 2014a; 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*, *Dictyostelium purpureum*, *Polysphondylium violaceum*; Table 1) collected in diverse localities (Espinosa and Paz-y-Miño-C 2014a; Kalla et al. 2011; Mehdiabadi et al. 2006; Ostrowski et al. 2008).

Despite this evidence, many questions still remain about the actual need of social ameba to discriminate kin from non-kin in the wild, or the adaptive value of such ability for the population structure of *Dictyostelium* or *Polysphondylium* (Espinosa and Paz-y-Miño-C 2014a). These concerns are justifiable considering that fruiting bodies in the wild are often composed of clonal clusters (below; Gilbert et al. 2012; Espinosa and Paz-y-Miño-C 2014a). Moreover, because studies with another social ameba, *Dictyostelium giganteum* (Table 1), have generated intriguing 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 (Espinosa and Paz-y-Miño-C 2014a; Kaushik et al. 2006). Thus, the optimal balance between the benefits and costs of kin discrimination/recognition in social amebas remains unclear. Although clonal reproduction secures high relatedness ($r = 1.0$), chimerism allows cooperation among unrelated clones (a benefit), but it can also promote conflict, competition and cheating (below).

CAN PROTISTS LEARN PHENOTYPIC CUES TO DISCRIMINATE KIN?

In 1899, H.S. Jennings wrote: "*Paramecium*... an animal that learns nothing, that exercises no choice in any respect, that is attracted by nothing and repelled by nothing, that reacts entirely without reference to the position of external objects, that has but one reaction [movement] for the most varied stimuli... can hardly be said to have made the first step in the evolution of mind, and we are not compelled to assume consciousness or intelligence in any form to explain its activities." Except for mind, consciousness, and intelligence, which are not prerequisites for kin discrimination or recognition (both can also operate in a reflex manner: stimulus-response), Jennings was mistaken about his entire characterization of *Paramecium*. Since the early 1900s, sensitization, trial-and-error learning, and classical or operant conditioning (relevant attributes among some of the multicellular eukaryotes that learn to recognize kin) have been documented in *Paramecium*; for example, micro-tube-escape swimming behavior via discrimination learning (1910s), habituation to approach baited and un-baited targets using bacteria as food-reinforcer (1950s), and swim-approach behavior toward mild-electrically charged fields in learning discrimination tasks using positive and punishment reinforcements (2000s; Armus et al. 2006; French 1940; Gelber 1958; Hennessey et al. 1979; Jensen 1957; Mingee 2013; Mirsky and Katz 1958). Moreover, associative learning, via classical conditioning, has been suggested to take place in human macrophages in vitro (i.e. macrophage avoidance/approach to bacterial-food stimuli with or without streptomycin negative reinforcement; Nilsonne et al. 2011). But, to our knowledge, there is no direct, experimental evidence that protists can rely specifically on sensitization (i.e. the enhancement of a response to an incremental exposure to a stimulus, e.g. the differential frequency exposure to kin versus non-kin during a life cycle), trial-and-error learning (i.e. repeated attempts to solve a task until success, e.g. attempts to behave altruistically toward kin, and the benefits it entails, versus the costs of maladaptive altruism toward non-kin), or classical or operant conditioning to discriminate between kin and non-kin (i.e. learning to associate a behavioral or chemical cue with the advantages/disadvantages of aggregating, cooperating or reproducing with conspecifics of distinctive value of *r*). All these topics, remain open areas of investigation and experimentation with protists since, like *Paramecium*, they possess basic sensory perception capabilities, which could have been co-opted during evolution to function in kin discrimination/recognition.

Because unicellular eukaryotes are diverse, occupy all environments and belong to old phylogenetic lineages, it is conceivable that other mechanisms of kin recognition (besides recognition alleles) might operate in protists. Holmes and Sherman (1983) suggested that kin recognition could be mediated by learning cues correlated with genetic relatedness. These authors described two mechanisms: association and phenotype matching. Both relied

on learning cues of an organism's extended phenotype (as per Dawkins 1982; the effects that genes have on the immediate environment of an organism via its behavior; in this case, chemo-signals for recognition that persist, or change predictably, outside the soma and to which kin and non-kin react). In the case of recognition by association, organisms learn unique, individually distinctive phenotypes of kin members with which they associate, particularly during early life (e.g. at a place of common hatching or nest). In future encounters, subjects recognize as kin only those specific individuals (this implies chemical or, when the organism is capable of, memory for recognition). Recognition by association is common in mammals, which natural histories determine predictable association with kin during early life. In contrast, in recognition by phenotype matching an organism learns (or imprints on) the *generic* traits of its own phenotype, or of the phenotypes of known kin, and builds a generic template for future comparisons (= matching). During later encounters with conspecifics, it recognizes as kin only those who match the kin-template, regardless of having or not interacted with them in the past. In this respect, phenotype matching resembles recognition alleles, but it differs from them in that the former relies on learning generic kin cues and building a template for future contrast (note that recognition alleles trigger an immediate, innate response, with no learning involved; but this also has problems since it is almost impossible to prevent an organism from learning kin cues from itself). Phenotype matching has been documented experimentally in fish, amphibians, reptiles, birds, and some mammals (reviews in Penn and Frommen 2010; Tang-Martínez 2001).

There is no indication that protists would not be able to rely on kin recognition via associative, learning-like mechanisms (association with chemical cues or phenotype matching based on the formation of chemical templates) since their immediate environments are saturated with chemo-signals, with which subjects interact in space time; thus, having the opportunity to form chemical-memory-like experiences (comparable to *Paramecium*'s sensitization, trial-and-error learning, retention, or conditioning; Armus et al. 2006; Hennessey et al. 1979; Mingee 2013). But even if protist–protist interactions operate just as simple chemical reflexes (limited to stimulus-response, above), or sensitizations, characterizing such mechanisms would help us understand the molecular physiology of cell–cell discrimination/recognition. For example, *Tetrahymena thermophila* is a ciliate which genetic polymorphisms encode for differential levels of cell clustering and dispersal (Table 1). Aggregation can be costly in these organisms 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; Espinosa and Paz-y-Miño-C 2014a). Cells exude *Tetrahymena* Proliferation Activating Factors (TPAFs) that are used by con- or heterospecifics to detect each other in the environment (i.e. extended phenotype). 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 ($r = 0$) 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; Espinosa and Paz-y-Miño-C 2014a). Therefore, *T. thermophila* seems capable of discriminating between self and different (based on sensitivity to detecting chemosignals in the environment), and also of modulating dispersal behavior as function of aggregative genotype (Espinosa and Paz-y-Miño-C 2014a).

Thus, kin discrimination or recognition take place in a multifaceted context of communication, in which signals are exchanged between senders and receivers, and proper responses and actions are subject to selection. Because signals operate within the sensory perception capabilities of organisms, receivers can make false positive (type I error) or false negative (type II error) identifications (Penn and Frommen 2010). Alongside, signal senders can benefit from concealing their identity (cheat) and profit from altruistic cooperation directed at them by honest signalers (i.e. the antirecognition hypothesis; Beecher 1991). This imposes a challenge on signal senders, as the broadcast of genuine identity would be evolutionarily constrained by the adaptability of cheating (i.e. the communication problem hypothesis; Johnstone 1997; but see Ghoul et al. 2014), which persistence in the population is often frequency-dependent. Only during the past decade, studies with the facultative multicellular social ameba, *D. discoideum* (which exists in both free-living cells and large aggregations that resemble multicellular bodies), have allowed investigators to experiment with scenarios in which the dynamics in the interaction between cheaters and noncheaters take place (below). And such interactions are relevant to our understanding of how kin discrimination and kin recognition occur at the cellular level, and how they relate to the origin and evolution of multicellularity (Espinosa and Paz-y-Miño-C 2014a; Ghoul et al. 2014).

CELL–CELL RECOGNITION AND THE ORIGIN OF MULTICELLULARITY

Multicellularity is a major evolutionary transition in which single-celled organisms switched from living individually to permanent assemblages (Herron et al. 2013; West et al. 2015). It is possible that multicellularity originated—more than once—in clonality, via a gradual aggregation of closely related cells, capable of recognizing one another by means of chemical cues, and which lived consistently in intimate proximity and benefited from specialized division of labor (i.e. distinctive tissues and organs with given functions; reviews in Ruiz-Trillo and Nedelcu 2015). Such specialization included the full allocation of soma-reproduction to a small population of cells within the soma, the gametes. Because sexually reproducing multicellular organisms experience a generational single-celled bottleneck (the zygote), which destines all its descendants to be clonal ($r = 1.0$), the evolution of multicellularity has

been traditionally explained under inclusive fitness theory (Hamilton 1964). However, research with protists is helping us to reexamine this classical view, as well as propose alternative scenarios under which multicellularity could have evolved (Herron et al. 2013; Queller and Strassmann 2012; Ratcliff et al. 2012; Wegener-Parfrey and Lahr 2013). The natural history of *D. discoideum* is illustrative in this respect. Depending on environmental conditions, this organism can experience three codependent cycles: vegetative, sexual, and social (Romeralo et al. 2012). During the vegetative phase, free-living haploid cells (*n*) proliferate in nutrient-rich environments via mitotic divisions (Li and Purugganan 2011). During the sexual phase (when resources are limited), these haploid cells merge in zygotes (2*n*), which attract, chemically, nearby solitary vegetative cells and feed on them; the zygotes grow and mature into macrocysts, inside of which meiosis occurs and multiple recombinant haploid cells are produced; the latter are then released into the environment as new vegetative cells (Li and Purugganan 2011). The social cycle (also triggered by food scarcity) is quite complex and particularly relevant to clone–clone- or kin-recognition and, as described above, it involves vegetative cells, which aggregate in mounds, form motile slugs, stalks that support spore-producing globular structures, and spores that develop inside of them (Espinosa and Paz-y-Miño-C 2014a; Li and Purugganan 2011; Romeralo et al. 2012). When the constituents of a social cycle are clones ($r = 1.0$), their slugs travel longer distances than chimeric slugs ($r < 1.0$) of comparable sizes; the clones invest more cells into longer stalks (where active cell death is inevitable); and have no gain in cheating their own kin either in stalk or spore formation. The opposite is characteristic of chimeric aggregations: short-distance traveling of slugs, short stalks; competition for allocating cells to spore formation, and cheating in the allocation of cells to the stalks (Strassmann and Queller 2011; but see Nedelcu et al. 2010 for a contrasting view on the paradigm of altruistic suicide in the unicellular world). However, there are some advantages to chimerism: chimeric slugs and fruiting bodies can grow quite large in contrast to clones; large slugs move farther, thus overcoming the tendency of chimeras to move short distances; and large fruiting bodies have higher allocation of cells to them than small fruiting bodies (Strassmann and Queller 2011). These observations suggest that, although there are advantages to associate with clone members and produce spores with them (inclusive fitness perspective, above), *D. discoideum* seems to also benefit from chimeric aggregations, possibly induced by environmental conditions, in which the benefits of chimerism surpass—or are equal to—the costs (Celiker and Gore 2013; Wyatt et al. 2013).

This plasticity in *D. discoideum* has allowed investigators to experimentally manipulate cell lines in the laboratory and test an old proposal (Buss 1987), that multicellularity can potentially evolve under low relatedness ($r < 1.0$) as a byproduct of the dynamics in the interaction between cheater cells and resistant-to-cheater cells (Levin et al. 2015). Note that in *D. discoideum* cheaters monopolize their representation in the spore-production part of the fruiting body and minimize their presence in the stalk (where cells die). In principle, if obligate cheaters are experimentally selected and freed in cell cultures of highly altruistic cells (noncheaters in which r is close or equal to 1.0), cooperation to form multicellular aggregations (i.e. slugs, stalks, and fruiting bodies) would eventually drive the evolution of resistance to cheaters, and cheaters would be outcompeted by altruists. However, Levin et al. (2015) have demonstrated that if relatedness is kept low, and several generations of cheaters and noncheaters grow together, the noncheaters can evolve resistance to cheating before cheating sweeps through the population and the overall ability to aggregate in multicellular structures is lost. Thus, offering a mechanism—other than inclusive fitness—by which simple forms of cell aggregation could become stable as a byproduct of the evolution of resistance to cheating. And testing such scenario has become possible only due to the versatility of experimenting with protists, like *D. discoideum*, in the laboratory (Queller and Strassmann 2012).

FROM UNICELLULAR BACK TO MULTICELLULAR EUKARYOTES

The field of kin discrimination and recognition needs a readjustment in which protists—and possibly all unicellular organisms—become central to the revision of concepts (e.g. discrimination versus recognition, which historically have included multicellular eukaryotes but disregarded protists), reassessment of mechanisms (e.g. genetic and/or developmentally acquired mechanisms of kin discrimination/recognition under predictable environmental influence), and hypotheses (e.g. reflex-response-like chemical recognition versus associative-learning-like recognition; origin of multicellularity; honest signaling versus cheaters; above). The "problem of cooperation," with kin or non-kin (i.e. the high cost of investing on others), requires careful reexamination using social, gregarious and nonsocial unicellular organisms of diverse phylogeny and life histories (Ghoul et al. 2014; West et al. 2006). Because unicellular eukaryotes are the precursors of multicellularity, we infer that the mechanisms of taxa, clone and kin discrimination/recognition in today's Unikonta (including fungi and animals) and Bikonta (including plants; Lecointre and Le Guyader 2006; Paps et al. 2013) coalesce evolutionarily to the ability of ancient cells to distinguish between same (self or clone) and different (diverse clones), as well as between close versus distant genetic relatives (as function of r; Espinosa and Paz-y-Miño-C 2014a). The sophisticated anatomical, physiological, behavioral, and cognitive traits associated with taxa, clone, and kin discrimination/recognition in multicellular organisms have evolved and diverged gradually—after extinctions and exaptations—from ancestral unicellular features (Espinosa and Paz-y-Miño-C 2014a). And the academic journey to reexamine these processes needs to bear in mind that the conceptual framework of kin recognition has relied (1960–1990s) on exploring a minute fraction of organisms on Earth: animals

and some plants. Of course, studying taxa, clone, and kin discrimination in protists has its own challenges (Herron et al. 2013; Montagnes et al. 2012); some are intrinsic to working with microscopic organisms in the field, others to culturing cell lines in the laboratory, which can lead to confounding interpretations of results in discrimination or recognition tests (Espinosa and Paz-y-Miño-C 2012, 2014a, b). For example, in our research on taxa and clone discrimination in *Entamoeba* (Table 1), we have alerted about some procedural difficulties that could be generalized to other protists (Espinosa and Paz-y-Miño-C 2012, 2014a,b): Laboratory strains customarily classified within single taxonomic lineages might actually belong to distinctive taxa (Espinosa and Paz-y-Miño-C 2012, 2014a,b). The strains of *Entamoeba 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 and Diamond 1997; Clark et al. 2006; Stensvold et al. 2010), although 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 ameba; moreover, each strain can be characterized by its distinctive morphology and pattern of aggregation (i.e. cell size within cluster, number of amebas per cluster, size of and distance between clusters; Espinosa and Paz-y-Miño-C 2012, 2014a,b). 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 versus distant genetic relatedness among clones). *Entamoeba* spp. probably exude *Entamoeba* Proliferation Activating Factors (EPAFs, analogous to the TPAFs, above) that are used to detect each other in the environment (Espinosa and Paz-y-Miño-C 2012, 2014a,b; Espinosa et al. in press).

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* case, above) between and within the cell lines to be used in laboratory trials (Espinosa and Paz-y-Miño-C 2012, 2014a,b). This is a challenge considering that protists' taxonomy and genomes are still superficially understood (Caron 2013; Heidel et al. 2011; Pawlowski 2013; Stoeck and Stock 2010).

KIN RECOGNITION IN THE CONTEXT OF PARASITISM

A remarkable example of both basic and applied science in which kin discrimination/recognition in protists is directly relevant to understanding the ecology and evolution of parasitism comes from *Plasmodium* spp. (Table 1; Espinosa and Paz-y-Miño-C 2014a). Malaria-causing protists 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 (Espinosa and Paz-y-Miño-C 2014a; Reece et al. 2008). When single *Plasmodium 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 nonadaptive 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 (Espinosa and Paz-y-Miño-C 2014a; 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, that is, more males are needed to probabilistically encounter and fertilize same-clone females in a multiple-clone milieu (Espinosa and Paz-y-Miño-C 2014a; 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 *Saccharomyces* or *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 (Espinosa and Paz-y-Miño-C 2014a).

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 *Plasmodium falciparum*, which infects humans (Espinosa and Paz-y-Miño-C 2014a; 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% versus 3%); thus, suggesting an intrinsic ability of *P. falciparum* to col-

onize 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 (Espinosa and Paz-y-Miño-C 2014a; 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"; different from "kin recognition via phenotype matching," above Espinosa and Paz-y-Miño-C 2014a; Medeiros et al. 2013; Mitta et al. 2012; Théron and Coustau 2005). 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 (Espinosa and Paz-y-Miño-C 2014a; Medeiros et al. 2013; Mitta et al. 2012).

In addition, clone–clone competition within multiple infections are expected to result in increased virulence (i.e. more infectious clones maximize transmission), in contrast to high relatedness among co-infecting clones, which favor female-biased gametocyte sex ratios (above) and reduced virulence (Nkhoma et al. 2012). But field studies in high- and low-malaria transmission areas (Malawi versus Thailand, respectively) have consistently reported high-kinship of multiple sporozoites originated in single mosquito bites, which suggests not only immune suppression of *Plasmodium* subpopulations within infections, or serial transmission of related sporozoites among hosts, but also multiple, unknown environmental factors influencing malaria virulence, host immunity, intrahost dynamics of co-infecting clone genotypes, and *Plasmodium* resistance to drugs (Nkhoma et al. 2012; Schneider et al. 2012).

Recent studies have documented that another insect-transmitted protistan pathogen, *Trypanosoma brucei*, relies on "social motility" to migrate from the vector's midgut to the salivary glands (i.e. to complete development into mammalian-infective trypomastigotes; Imhof and Roditi 2015; Oberholzer et al. 2010). Like *Plasmodium*, single- or multiple-clone *T. brucei* infections face resource allocation trade-offs between maintaining the infection (survival) and investment into transmission (reproduction; Pollitt et al. 2011). This scenario of large cell aggregations competing to navigate through the tsetse-fly tissues (= high mortality) and to arrive at the salivary glands likely requires clone–clone recognition/discrimination ability to warrant adaptive cooperation and minimize cheating, but this is something yet to be characterized in *Trypanosoma*.

THE SPATIOTEMPORAL EFFECT OF KIN RECOGNITION ON FITNESS

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 spatiotemporal effect of kin discrimination/recognition on fitness (i.e. the kin population structure resulting from the mechanisms of discrimination/recognition; Espinosa and Paz-y-Miño-C 2014a; Kamel and Grosberg 2013). The relationship between parasites 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 antimalaria drugs or undergoing an anemic drop in red-blood-cells; Espinosa and Paz-y-Miño-C 2014a; Pollitt et al. 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 antimalaria drugs), it reduces production of gametocytes and relies chiefly on asexual proliferation of sporozoites (Espinosa and Paz-y-Miño-C 2014a; 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 (Espinosa and Paz-y-Miño-C 2014a; Pollitt et al. 2011).

CONCLUSIONS

Unicellular eukaryotes, protists, the ancestors of all multicellular life, offer us opportunities to study taxa-, clone-, and kin-discrimination/recognition at the genetic and molecular levels; with the potential to expand this research to the spatiotemporal effect of cell–cell kin discrimination/recognition on fitness. Protists are central to the reevaluation of the theoretical framework and concepts in the field of kin recognition, and to research about the origins and evolution of multicellularity. Because unicellular eukaryotes belong to ancient and highly diverse phylogenetic lineages, occupy all environments on Earth, and participate in complex interactions with other organisms (as hosts, symbionts, or parasites), they can be robust model systems to study the implications of taxa, clone, and kin discrimination/recognition in ecological and evolutionary contexts, and with emphasis on basic or applied sciences (Espinosa and Paz-y-Miño-C 2014a). An integrated, multidisciplinary approach (i.e. molecular, genetic, physiological, behavioral, ecological, and evolutionary) is needed to make impactful contributions to the field of kin recognition. But there are some challenges to working with microscopic, unicellular eukaryotes in the wild or the laboratory. The natural histories of most taxa are still unknown (in numerous cases only fragmentary, although distinctive genetic sequences have been cataloged as likely belonging to new species),

and researchers worldwide struggle to culture or keep them alive in the lab, particularly marine species, the most diverse.

ACKNOWLEDGMENTS

We thank the International Society of Protistologists and the organizers of the VII European Congress of Protistology for sponsoring the symposium "Evidence of Taxa-, Clone-, and Kin-Discrimination in Protists: Ecological and Evolutionary Implications," and the University of Seville for hosting the Congress. G. Paz-y-Miño-C is sponsored by New England Science Public and the New England Center for the Public Understanding of Science and A. Espinosa by NIH-NIGMS grant # 2P20GM103430. An associate editor and two anonymous reviewers provided valuable comments to improve the manuscript.

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