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SYMPOSIUM ARTICLE

Entamoeba Clone-Recognition Experiments: Morphometrics, Aggregative Behavior, and Cell-Signaling Characterization

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ABSTRACT

Studies on clone- and kin-discrimination in protists have proliferated during the past decade. We report clone-recognition experiments in seven *Entamoeba* lineages (*E. invadens* IP-1, *E. invadens* VK-1:NS, *E. terrapinae*, *E. moshkovskii* Laredo, *E. moshkovskii* Snake, *E. histolytica* HM-1:IMSS and *E. dispar*). First, we characterized morphometrically each clone (length, width, and cell-surface area) and documented how they differed statistically from one another (as per single-variable or canonical-discriminant analyses). Second, we demonstrated that amebas themselves could discriminate self (clone) from different (themselves vs. other clones). In mix-cell-line cultures between closely-related (*E. invadens* IP-1 vs. *E. invadens* VK-1:NS) or distant-phylogenetic clones (*E. terrapinae* vs. *E. moshkovskii* Laredo), amebas consistently aggregated with same-clone members. Third, we identified six 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. To our knowledge, this is the first multi-clone characterization of *Entamoeba* spp. morphometrics, aggregative behavior, and cell-signaling secretion in the context of clone-recognition. Protists allow us to study cell–cell recognition from ecological and evolutionary perspectives. Modern protistan lineages can be central to studies about the origins and evolution of multicellularity.

THE ability to discriminate/recognize self (clone) from different (distinctive clones), or kin from non-kin (as function of genetic relatedness *r*), can be advantageous from an individual's inclusive fitness perspective (Hamilton 1964). Organisms that preferentially cooperate with close, rather than with distant genetic relatives, are more likely to pass on the shared genes (Hamilton 1964; Maynard-Smith 1964). These predictions, which are central to the field of "kin-recognition," have been historically examined in multicellular eukaryotes (animals and plants; Biedrzycki and Bais 2010; Penn and Frommen 2010; Tang-Martínez 2001). During the past decade, studies on taxa-, clone-, and kin-discrimination/recognition in unicellular eukaryotes (protists) have proliferated, particularly in laboratory work with five major lineages: Mycetozoa (*Dictyostelium*, *Polysphondylium*), Dikarya (*Saccharomyces*), Ciliophora (*Tetrahymena*), Apicomplexa (*Plasmodium*) and Archamoebae (*Entamoeba*; Espinosa and Paz-y-Miño-C 2014a; major lineages follow phylogenetic classification by Worden et al. 2015). Protists have given us the opportunity to identify specific genes involved in cell–cell discrimination/recognition (e.g. *FLO*, *csA*, *tgrB1*, *tgrC1*) that encode for plasma membrane proteins for cell–cell adhesion, which allow organisms to aggregate, propagate and disperse together, or cope with starvation via encystation (dormancy) or formation of social multicellular assemblages for spore production (Benabentos et al. 2009; Espinosa and Paz-y-Miño-C 2014a; Hirose et al. 2011; Queller et al. 2003; Smukalla et al. 2008; Strassmann and Queller 2011). Ultimately, studies on taxa-, clone-, and kin-discrimination/recognition in protists are giving us hints about how individual cells cooperate altruistically and form large assemblages that resemble those of multicellular organisms (Espinosa and Paz-y-Miño-C 2014a; Herron et al. 2013; West et al. 2015).

Here we report clone-recognition experiments with seven *Entamoeba* lineages (Fig. 1). First, we characterize morphometrically each clone and document how these microscopic organisms are distinctive from one another. Second, we demonstrate how amebas themselves discriminate/recognize clone-members from non-clone members in behavioral trials. And third, we identify putative cell-signals secreted by the amebas, which seem to induce preferential cell aggregation and proliferation with clone members. We discuss these results in the context of clone- and kin-discrimination/recognition in protists, and highlight the relevance of this research for our understanding about the origins and evolution of multicellularity.

Note that in this article we use the terms clone-recognition and clone-discrimination together (i.e. recognition/discrimination), as the ability of protists to distinguish one clone from another and/or, in some instances, the capacity to distinguish among clones of different degree of genetic relatedness. However, there is much discrepancy among authors about the proper use of these terms (see Penn and Frommen 2010; Tang-Martínez 2001).

MATERIALS AND METHODS

Cell cultures, growth conditions and reagents

Entamoeba strains were grown at 23 °C (*E. invadens* IP-1, *E. invadens* VK-1:NS, *E. terrapinae*, *E. moshkovskii* Laredo and *E. moshkovskii* Snake) or 37 °C (*E. histolytica* HM-1:IMSS and *E. dispar*) under axenic conditions in flat-bottomed 48-well plates or 16-ml borosilicate glass tubes containing: media TYI-S-33 (Diamond et al. 1978), NYU (same as TYI-S33 but tryptone replaces casein digest peptone) or LYI-S-2 (Clark and Diamond 2002); all media were enriched with 10% ABS (Sigma-Aldrich, St. Louis, MO; Espinosa et al. 2001, 2009, 2012; Espinosa and Paz-y-Miño-C 2012). In each clone culture, the 48-well plates were inoculated with 900 trophozoites per well and the 16-ml tubes with 5×10^3 trophozoites. Aliquots of cells were harvested when reaching confluency by chilling (following Espinosa et al. 2012; Espinosa and Paz-y-Miño-C 2012); the cell density was determined using a Cellometer Vision HS RF-150 (Nexcelom BioScience LLC, Lawrence, MA). Additional media components were purchased from Fisher Scientific (Agawam, MA), Sigma-Aldrich, and Atlanta Biologicals (Atlanta, GA).

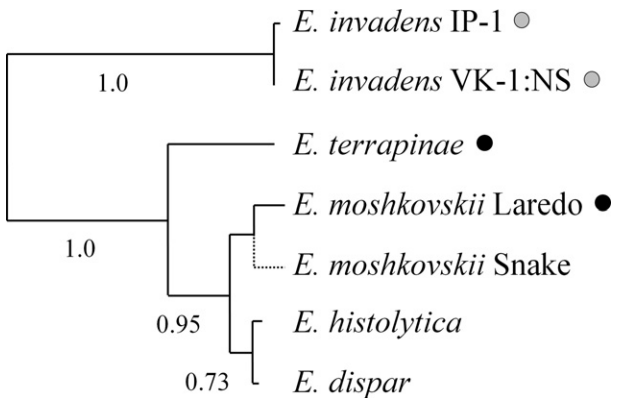

Figure 1 Schematic phylogeny based on ssrRNA sequences of the *Entamoeba* clones discussed in this article. The gray circles indicate two relatively-close related clones (*Entamoeba invadens* IP-1 and *E. invadens* VK-1:NS) used in the aggregation experiments described in Fig. 4. The black circles indicate two relatively-distant related clones (*Entamoeba terrapinae* and *Entamoeba moshkovskii* Laredo) used in the aggregation experiments described in Fig. 5. Accession numbers NCBI: *E. invadens* IP-1 AF149905.1; *E. terrapinae* AF149910.1; *E. moshkovskii* Laredo AF149906.1; *Entamoeba. histolytica* X56991; and *Entamoeba dispar* Z49256.1. The ssrRNA sequence of *E. invadens* VK-1:NS was provided by Graham C. Clark. The placement of *E. moshkovskii* 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.

Morphometrics

To characterize morphometrically each of the *Entamoeba* clones (Fig. 1), we estimated cell length, width and surface area by using a Zeiss Axiovert 40 CFL fluorescent microscope (X-Cite Series-120 Q; Microtech Optical, New England Inc., Bloomfield, CT). We photographed amebas at 12, 24, 36, and 48-h of growth (10X and 32X) and analyzed the photos using Image Pro Software (Micro-Tech Optical; New England Inc., Bloomfield, CT), which allows to click-mark points on an image, delineate contours of individual cells, and estimate measurements. The number of cells measured per clone were: *E. invadens* IP-1 = 399, *E. invadens* VK-1:NS = 399, *E. terrapinae* = 400, *E. moshkovskii* Laredo = 400, *E. moshkovskii* Snake = 400, *E. histolytica* HM-1:IMSS = 396, and *E. dispar* = 383.

Aggregative behavior

To assess aggregative behavior (as indicator of clone-discrimination ability) in mixed-cell-line vs. single-cell-line cultures, we labeled amebas fluorescently with CellTracker Green-CMFD and Red (Invitrogen, Carlsbad, CA; Espinosa and Paz-y-Miño-C 2012, 2014a,b). As follows: 1×10^5 trophozoites per strain were harvested by chilling and centrifuged at 4,000 *g* for 20-min. Trophozoite pellets were resuspended in CellTracker Red (1:3 dilution in DMSO) and CellTracker Green CMFD (1:100 dilution in DMSO). Two incubation periods (30-min each) followed by a 5-min PBS + formaldehyde fixing period, and a final resuspension in 1.4-ml media were performed according to the manufacturer's protocol. All cells were grown at 23 °C for 12, 18, and 36-h. We expected that in mixed-cell-lined cultures, cells would aggregate in distinct color clusters (indication of discrimination ability), and that in single-cell-line cultures, trophozoites would mix and look yellow under the microscope (indication of full mixing among same-clone amebas, with no side-effects of coloration on behavior; Espinosa and Paz-y-Miño-C 2012, 2014a,b). We followed these procedures in two separate pairings: *E. invadens* IP-1 vs. *E. invadens* VK-1:NS (the closest phylogenetic clones we have in our laboratory; Fig. 1), and *E. terrapinae* vs.

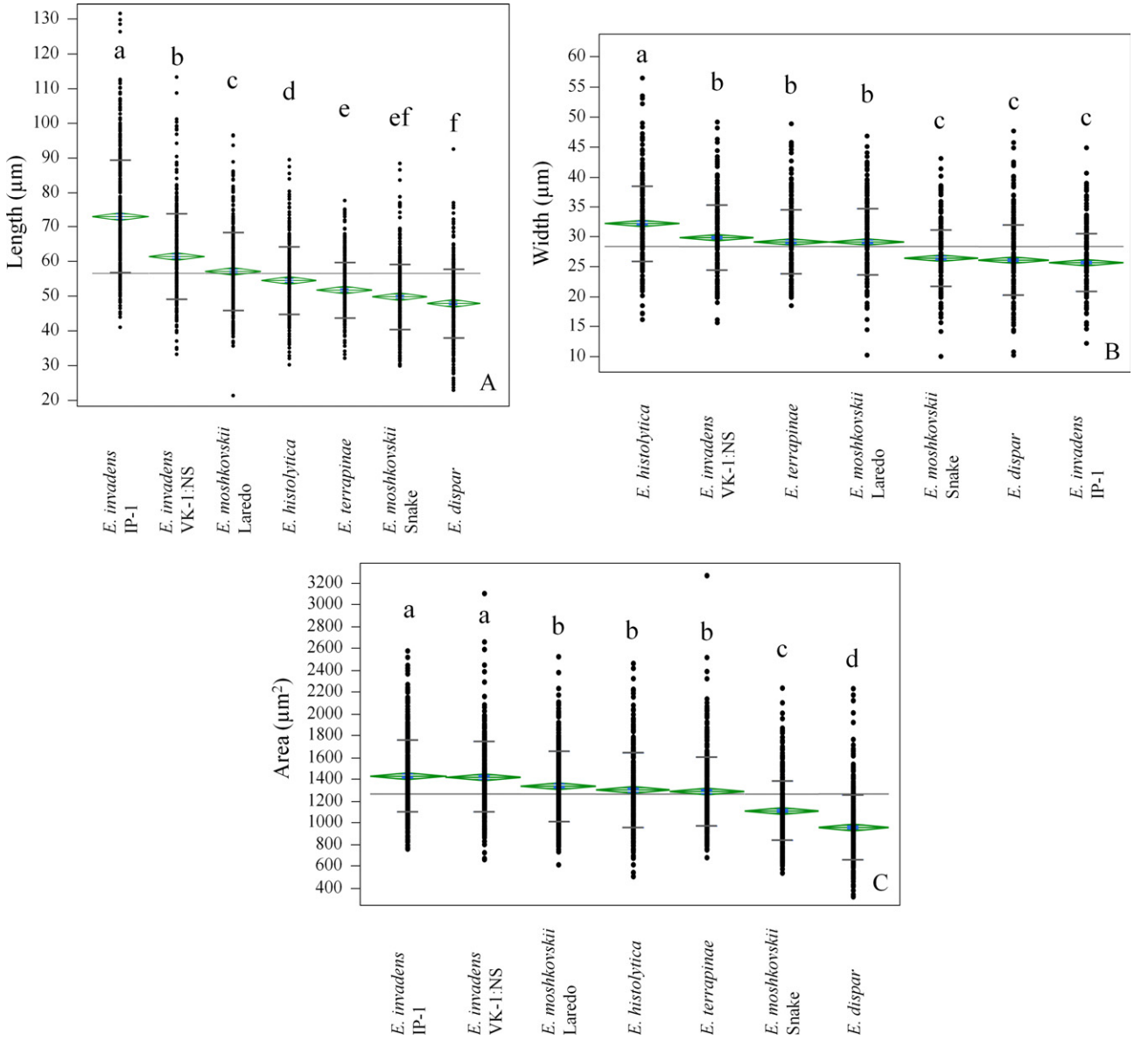

Figure 2 Morphometric analyses of *Entamoeba* clones. **A.** Length: data-point distributions per clone (each point corresponds to an individual ameba) with flat diamonds representing confidence intervals; the line in the center of a diamond depicts the group's mean (the mean of all groups is marked by the horizontal line across groups); the vertical span of the diamond represents the 95% confidence interval for the mean in each group (inside are the standard errors, barely visible); the standard deviations are marked by two small lines above and below each diamond; the extent of the diamond in the *x* axis is proportional to the number of amebas per clone (*Entamoeba invadens* IP-1, $N = 399$; *E. invadens* VK-1:NS, $N = 399$; *Entamoeba moshkovskii* Laredo, $N = 400$; *Entamoeba histolytica*, $N = 396$; *Entamoeba terrapinae*, $N = 400$; *E. moshkovskii* Snake, $N = 400$, and *Entamoeba dispar*, $N = 383$); one-way ANOVA, $F_{6,\ 2,776} = 229.51$, $p < 0.0001$; lowercase letters indicate Tukey–Kramer HSD test pair-wise comparisons, $p < 0.05$. **B.** Width: caption analogous to (A); one-way ANOVA, $F_{6,\ 2,776} = 76.91$, $p < 0.0001$. **C.** Area: caption analogous to (A); one-way ANOVA, $F_{6,\ 2,776} = 114.50$, $p < 0.0001$.

E. moshkovskii Laredo (two more distantly related amebas; Fig. 1; for extensive phylogenetic analyses of *Entamoeba* lineages, see Stensvold et al. 2010, 2011).

Cell-signaling characterization

To identify cell-signaling secretion for aggregation and in the context of clone-recognition, we followed two strategies. First, we grew *Entamoeba* clones in fresh (Fr) or sterile but aged culture-media (age 1 = 12; 2 = 24; and 3 = 48-h) in which same-clone trophozoites had grown and released their chemosignals (i.e. *Entamoeba* Proliferation Activating Factors, EPAFs; Espinosa and Paz-y-Miño-C 2014a). We knew that single-cell lines labeled with green and red dyes would aggregate, mix fully and look yellow under the microscope (above), a feature we used

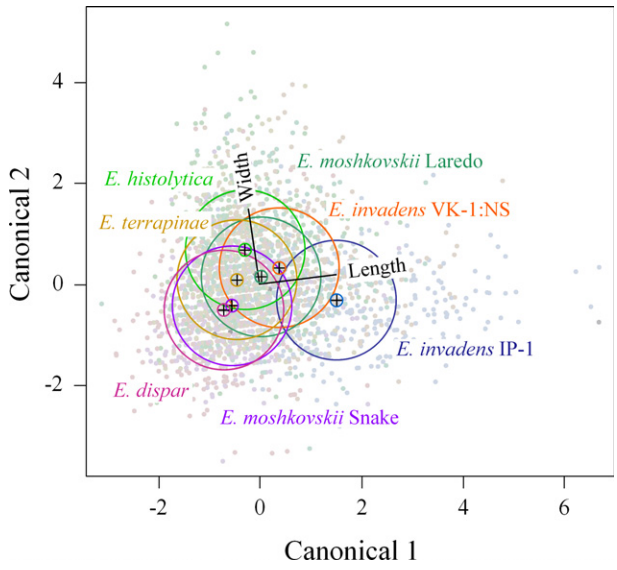

Figure 3 Canonical plot depicting the spatial dimensions for maximum morphometric separation among seven *Entamoeba* clones. The labeled rays (length and width) show the directions of the covariates in the canonical space; the rays branch out from the point 0,0, which corresponds to the grand mean (N = 2,777); 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 *Entamoeba invadens* IP-1 (right-bottom circle) is the most elongated ameba in respect to the other taxa; *Entamoeba dispar* is the smallest (short and thin; left-bottom circle), and *Entamoeba 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. Number of amebas per clone is provided in caption of Fig. 2.

to quantitate surface area of aggregation. We expected that inoculates of equal green-/red-dye mixes would increase growth and aggregation as function of media age, because older-age media would have accumulated more EPAFs than younger media. We conducted these experiments separately with 3-d old *E. invadens* IP-1 and *E. invadens* VK-1:NS, which were processed and colored (prior to re-growth in aged media) as in above. The 1, 2 and 3 aged culture-media were collected via aspiration, placed in separate, sterile 15-ml tubes, filtered through 0.45-μm pore Nalgene filters (to exclude all amebas), and then mixed with fresh media in a 1:1 ratio for incubation. Measurements of cell aggregates were taken from photos (same as in above) at 12, 18, and 36-h of growth. Experiments were conducted in triplicate (total nine repetitions).

Second, we identified the putative cell-signals secreted by the amebas from 1-ml filtered samples of aged culture-media (at +36-h). Aliquots of these samples were run in 10% PAGE gels and stained with Coomassie brilliant blue R-250. Distinctive gel bands were cut into 1-mm cubes, washed with 50% (v/v) acetonitrile in 50-mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. These dehydrated gel slices were rehydrated with 10-μl of 50-mM ammonium bicarbonate containing 50-ng trypsin (Pierce Trypsin Protease MS Grade; Pierce Biotechnology, Rockford, IL) and incubated overnight at 37 °C. Peptides resulting from the trypsin digestion were recovered by serial extractions with 5% (v/v) formic acid / 50% (v/v) acetonitrile, pooled in a vacuum centrifuge, and resuspended in 6-μl of 0.1% (v/v) trifluoroacetic acid plus 5% (v/v) acetonitrile in water. The resuspended samples were analyzed using microcapillary LC/MS/MS Orbitrap LC-MS reverse-phase chromatography, which generated raw-peptide sequences (proteomics conducted at Taplin Biological Mass Spectrometry Facility, Harvard University Medical School). The raw sequences were compared to the *E. invadens* genome/proteome database (Ehrenkaufer et al. 2013; http://www.ncbi.nlm.nih.gov/nuccore/AANW00000000) and aligned with homologs (e.g. *Dictyostelium*, *Plasmodium*) to determine sequence similarities. For the latter we also searched NCBI (http://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/) databases. To infer protein function in *Entamoeba* spp., in reference to protistan homologs, we combined information from the NCBI and UniProt databases with the literature.

Statistical analyses

Measurements of individual amebas' length, width and surface area were plotted as data-point distributions per clone and analyzed with one-way ANOVA (null hypotheses rejected at $p < 0.001$). To characterize the morphometric identity of each clone, we determined the 95% confidence interval for the mean values of each clone in each of the three, independent measurements. *Post-hoc* pairwise comparisons for each ANOVA were done by applying two-tailed Tukey–Kramer HSD test (null hypotheses rejected at $p < 0.05$).

To add confidence to our morphometric characterization of the clones, we combined the variables length and width (the most informative of the three measurements) in a canonical analysis to establish the degree of association between these variables and clone identity. We used the Wilks' Lambda (λ) method for conversion of the canonical model into F statistics (null hypothesis rejected at $p < 0.001$; Sherry and Henson 2005). We relied on the Wilks' value of lambda ($1-\lambda = R^2_c$) to estimate the magnitude (R^2_c converted to percentage) of the relationship between both variables and their combined effects of variance on clone identity. To further establish the magnitude of the relationship between each separate variable (length or width) in respect to clone identity, we obtained the square value of each canonical correlation and converted it to percentage (Sherry and Henson 2005).

In the case of the aggregation experiments of distinctive *Entamoeba* clones in fresh or aged culture-media at 12, 18 and 36-h of growth (above), we used mean values of

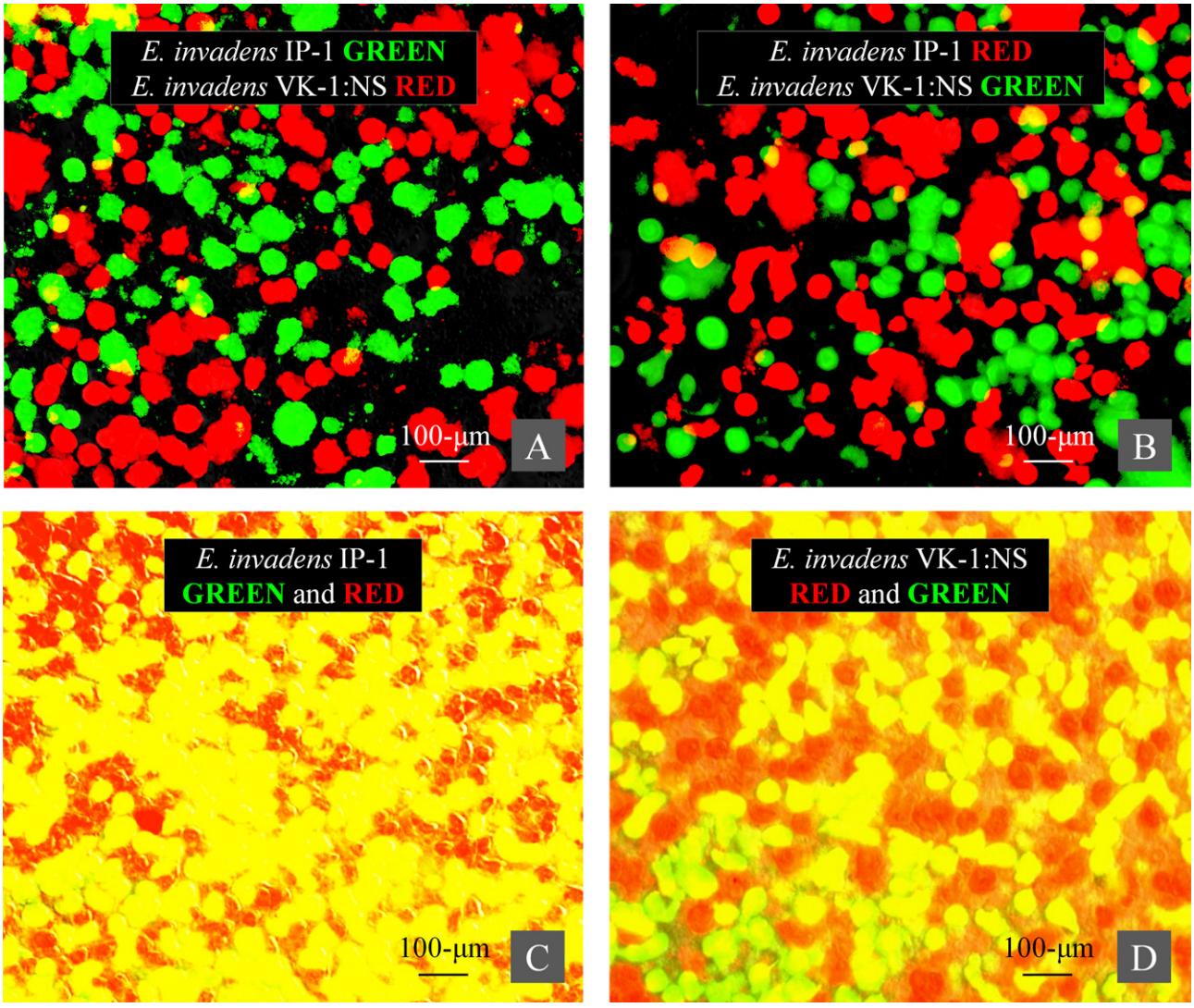

Figure 4 Clone-aggregation preference shown by *Entamoeba invadens* IP-1 and *E. invadens* VK-1:NS in mixed- (top) or single-cell-line (bottom) cultures. **A.** Fluorescent micrograph of *E. invadens* IP-1 labeled green and *E. invadens* VK-1:NS labeled red, each clone aggregates in distinct clusters. **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.** *E. invadens* IP-1 labeled with both green and red dyes; trophozoites mix equally and look yellow under the microscope. **D.** *E. invadens* VK-1:NS labeled with both green and red dyes. In all trials, cells were labeled with CellTracker Green CMFD and Red (Invitrogen). All images taken at 36-h, scale bar = 100-µm, X10 magnification.

surface area of aggregation per condition (Fr, 1, 2 or 3) in chi-squared analysis (3 × 4 contingency table). Null hypotheses were rejected at $p < 0.05$.

RESULTS

Morphometrics

Entamoeba clones were morphometrically different from one another as per cell length, width and soma surface area (Fig. 2). Of these three measurements, cell length was the most informative for clone-morphometric discrimination purposes (Fig. 2A). Length allowed us to distinguish statistically among most clones, except for *E. terrapinae* that was statistically indistinguishable from *E. moshkovskii* Snake, which, in turn, was statistically indistinguishable from *E. dispar* (Fig. 2A). Cell width (Fig. 2B) and soma surface area (Fig. 2C) 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* Snake, *E. dispar* and *E. invadens* IP-1 were thin-bodied amebas (Fig. 2B). According to soma

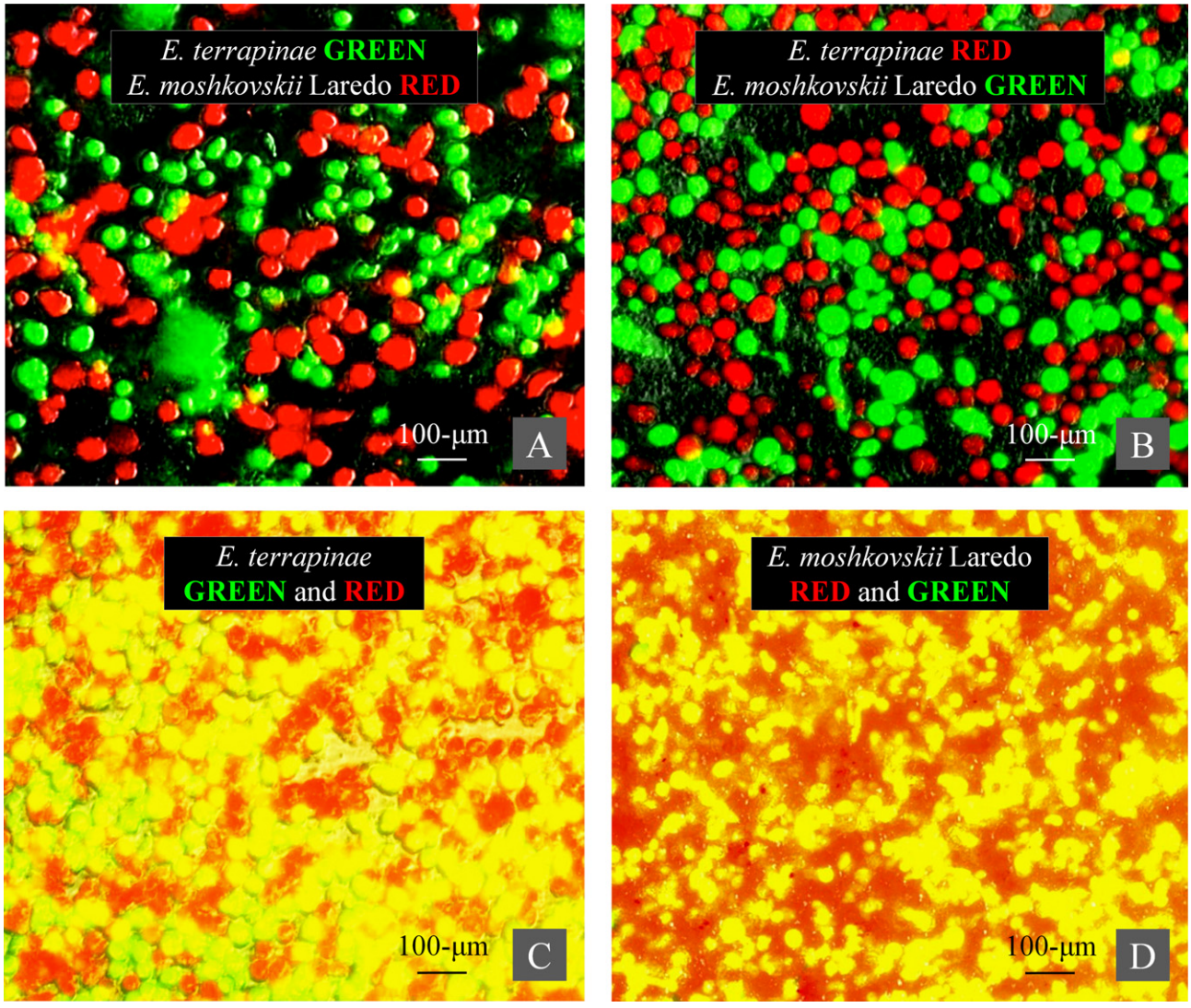

Figure 5 Clone-aggregation preference shown by *Entamoeba terrapinae* and *Entamoeba moshkovskii* Laredo in mixed- (top) or single-cell-line (bottom) cultures. **A.** Fluorescent micrograph of *E. terrapinae* labeled green and *E. moshkovskii* Laredo labeled red, each clone aggregates in distinct clusters. **B.** Reverse-color labeling of trophozoites of *E. terrapinae* (red) and *E. moshkovskii* Laredo (green), the clones aggregate in distinct clusters. **C.** *Entamoeba terrapinae* labeled with both green and red dyes; trophozoites mix equally and look yellow under the microscope. **D.** *Entamoeba moshkovskii* Laredo labeled with both green and red dyes. In all trials, cells were labeled with CellTracker Green CMFD and Red (Invitrogen). All images taken at 36-h, scale bar = 100-µm, X10 magnification.

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* Snake was a small-area-bodied ameba; and *E. dispar* was the smallest-area-bodied ameba (Fig. 2C). When we combined length and width in a canonical discriminant analysis, we could tell each ameba clone apart with 95% confidence (Fig. 3), except for *E. moshkovskii* 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.

Aggregative behavior

Amebas themselves were able to discriminate between self (same clone) and different (themselves vs. another clone). And this was the case for both the closest phylogenetic clones in our pair-testing (i.e. *E. invadens* IP-1 vs. *E. invadens* VK-1:NS; Fig. 4) and the more distant phylogenetic clones (i.e. *E. terrapinae* vs. *E. moshkovskii* Laredo; Fig. 5). When *E. invadens* IP-1 was labeled green and *E. invadens* VK-1:NS was labeled red, and both clones were grown together (i.e. mixed cell-line cultures), each ameba aggregated in distinct clusters of a single color (i.e. green or red; Fig. 4A,B). However, when each ameba

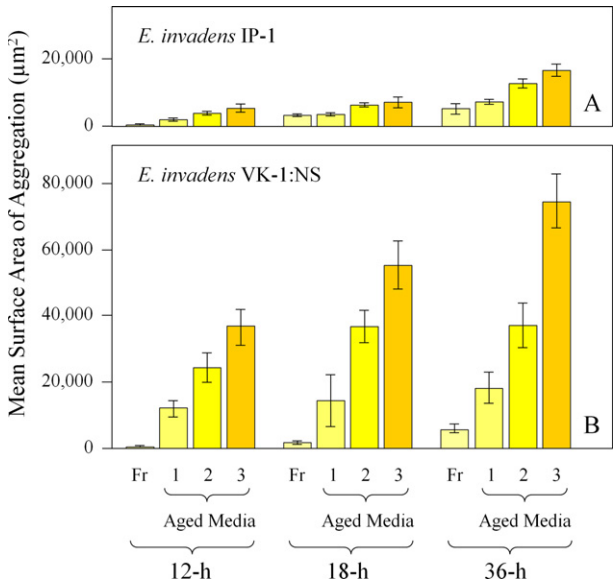

Figure 6 Quantification of aggregative behavior of distinctive *Entamoeba* clones in fresh or aged culture-media at 12, 18 and 36-h of growth. As shown in Fig. 4C–D, trophozoites of single-cell lines, labeled with both green and red dyes, aggregated and looked yellow under the microscope (an indication that cells had fully mixed). When equal green-/red-dyed mixes of 3-d-old amebas were inoculated in fresh (Fr) or sterile but aged media (1, 2 or 3), in which same-clone trophozoites had previously grown and released their chemosignals (*Entamoeba* Proliferation Activating Factors, EPAFs), the amebas grew and aggregated faster as function of media age (1 = 12-h; 2 = 24-h, and 3 = 48-h). **A.** *Entamoeba invadens* IP-1, χ^2 = 1,692.50, *df* = 6, $p < 0.0001$. **B.** *E. invadens* VK-1:NS, χ^2 = 5,653.42, df = 6, $p < 0.0001$. Surface area of ameba aggregates was determined by Image-Pro software (Micro-Tech Optical; New England Inc.). Measurements were averaged from three replicate wells and three separate experiments (methods followed Espinosa and Paz-y-Miño-C 2012). Standard errors are shown.

(*E. invadens* IP-1 or *E. invadens* VK-1:NS) was labeled with both dyes (green and red) and grown alone (i.e. single-cell-line cultures) they aggregated and fully mixed with themselves (i.e. they looked yellow under the microscope; Fig. 4C,D). We obtained identical results with the pairing *E. terrapinae* vs. *E. moshkovskii* Laredo (Fig. 5A–D).

Cell-signaling characterization

As we expected, equal green-/red-dyed mixes of amebas grew and aggregated faster when inoculated in sterile but aged media (1, 2 or 3), in which same-clone trophozoites had previously grown and released their EPAFs (Fig. 6). Both *E. invadens* IP-1 (Fig. 6A) and *E. invadens* VK-1:NS (Fig. 6B) increasingly grew and aggregated as function of incubation time (12, 18 or 36-h) in fresh (control) or aged media; however, growth and aggregation in aged media consistently surpassed growth and aggregation in the control condition (Fig. 6A,B). Although we did acknowledge that the aged media contained a variety of enzymes secreted by the amebas (e.g. metabolic proteins mixed with EPAFs), we focused our attention on the likely candidates involved in proliferation, aggregation and movement.

In Table 1, we report the proteomics characterization of putative cell-signals (EPAFs) identified in liquid and gel samples of aged media 3 from cultures of both *E. invadens* IP-1 and *E. invadens* VK-1:NS. We found six proteins common to both clones: 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). Comparable protein-functions have been identified in protistan homologs, particularly in *Dictyostelium* and *Plasmodium* (Table 1; references therein). Note that these extra- and intracellular signals likely interact in the overall physiology of *Entamoeba*, 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 *Dictyostelium discoideum*, are directly involved in cell–cell adhesion, regulation of proliferation, or cell aggregation (proteomic analyses; Bakthavatsalam and Gomer 2010; Biller et al. 2014). This suggests that the proteins we identified in our cell-signal characterizations possess similar functions in our *Entamoeba* clones. We acknowledge that this area of our work needs further identification of the genes involved in these cell–cell interactions, but it is evident that both *E. invadens* IP-1 and *E. invadens* VK-1:NS excrete to their surroundings (and secrete intracellularly) chemical cues for recognition and aggregation.

DISCUSSION

Entamoeba clones of diverse taxa can be characterized morphometrically (length, width and cell-surface area) and with high statistical confidence (Fig. 2, 3). Most significantly, ameba clones themselves can discriminate between self (same clone) and different (themselves vs. another clone) and they do it by excreting (and secreting intracellularly) chemical cues (EPAFs; Table 1) that induce amebas to aggregate preferentially with clone members rather than with members of a different clone (Fig. 4, 5). The differential behavioral treatment of clone- vs. non-clone members, and between phylogenetically close (*E. invadens* IP-1 and *E. invadens* VK-1:NS) or distant taxa (*E. terrapinae* vs. *E. moshkovskii* Laredo), demonstrates that the capacity to discriminate between same and different in these unicellular eukaryotes is an ancient trait (amebas, themselves, as protists, are ancient). But we still need to determine the specific gene(s) involved in this ability, and if amebas are capable of discriminating among conspecifics of gradually-decreasing genetic relatedness (*r*), which would be an indication of possible kin-discrimination/recognition (Espinosa and Paz-y-Miño-C 2014a,b). Keep in

Table 1. Proteomics characterization of putative cell-signals identified in *Entamoeba invadens* IP-1 and *E. invadens* VK-1:NS aged media 3 (as described in caption of Fig. 6)

Proteins identified in *Entamoeba*[a]	Function in *Entamoeba* spp.[b]	Homolog carrier	Function/mutation in homolog of protein
RasGap/Ankyrin A0A0A1TXR8_ENTIV	Cell proliferation, actin polymerization, migration[c]	*Dictyostelium*	Directional migration/deficient cell polarity, aberrant sorting stalk formation[d]
Coronin-WD40 A0A0A1UAI2_ENTIV	Actin binding protein Cytokinesis, cell motility[f]	*Dictyostelium* *Plasmodium*	Chemotaxis/forms spores not stalks[d,e] Directional motility during invasion[g]
Actin L7FMW8_ENTIV	Adhesion, locomotion[c], encystation[g]	*Dictyostelium*	Mound formation[d]/reduced cell motility[d]
Protein kinases A0A0A1UES6_ENTIV	Cytokinesis, G-protein modulation[c], encystation[h]	*Dictyostelium*	Controls aggregation[e]/deficient aggregation, decreased chemotaxis[d]
Heat shock 70 A0A0A1U467_ENTIV	Protein folding, stress response, epigenetic regulation[i]	*Dictyostelium*	F-actin stabilization[e]
Ubiquitin O96339_ENTIV	Cell proliferation, development protein turnover[j]	*Dictyostelium*	Facilitates aggregative stage[d, e]/defective slugs[d]

[a]Proteins' identification numbers (IDs) come from *E. invadens* (source UniProtKB http://www.uniprot.org/uniprot).
[b]*Entamoeba* spp. corresponds to characterizations of function in *E. histolytica* and *E. invadens* as reported in the literature citations in this table.
[c]Bosch and Siderovski (2013a).
[d]Dictybase; http://dictybase.org/Downloads/multiple-mutants.html.
[e]Loomis (2015).
[f]Eckert et al. (2011).
[g]Olshina et al. (2015).
[h]Herrera-Martínez et al. (2013).
[i]Weber et al. (2006).
[j]Bosch and Siderovski (2013b).

mind that, in nature, aggregation behavior in our seven *Entamoeba* clones is directly linked to survival (i.e. group-access to resources or cyst-formation under starvation) and reproduction (i.e. quorum sensing) of the free-living (*E. moshkovskii* Laredo), commensal (*E. terrapinae* and *E. dispar*) and parasitic taxa (*E. invadens* IP-1, *E. invadens* VK-1:NS, *E. moshkovskii* Snake and *E. histolytica* HM-1: IMSS; Eichinger 2001; Espinosa and Paz-y-Miño-C 2012, 2014a,b; Faust and Guillen 2012; Weedall and Hall 2015).

Taxa-, clone-, and kin-discrimination have been reported in other protists. For example, the ciliate *Tetrahymena thermophila* can discriminate between self and different based on its sensitivity to detecting *Tetrahymena* proliferation activating factors in the environment (TPAFs, analogous to EPAFs). *Tetrahymena thermophila* modulates dispersal behavior as function of aggregative genotype (Chaine et al. 2010; Espinosa and Paz-y-Miño-C 2014a). 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). Similar to *Entamoeba* spp., *T. thermophila* aggregation with self can increase survival by gaining access to patchy, ephemeral resources and/or by inducing cell encystation, with the right partners, during food scarcity (Chaine et al. 2010; Espinosa and Paz-y-Miño-C 2014a); isolated cells do not survive.

Genetic characterization of kin-discrimination/recognition in protists comes from studies with the common yeast (*Saccharomyces cerevisiae*) and social amebas (*Dictyostelium spp. and Polysphondylium sp.*). In *S. cerevisiae*, *FLO* genes encode for plasma membrane proteins that allow cell adhesion during aggregation (yeast cells form biofilms during starvation; Smukalla et al. 2008). Interestingly, cell carriers of this gene aggregate exclusively with other cells that also carry it (Espinosa and Paz-y-Miño-C 2014a; Smukalla et al. 2008). In *D. discoideum*, multiple genes, also expressed in the plasma membrane, encode for cell–cell adhesion proteins, which can result in kin-discrimination/recognition for aggregation; these genes include *csA* (which works alone and analogously to *FLO* in *S. cerevisiae*, encoding for cell-adhesive polypeptides; Espinosa and Paz-y-Miño-C 2014a; Queller et al. 2003) and *tgrB1* and *tgrC1* (which work in complementary pairs specifically involved in cell–cell discrimination; Benabentos et al. 2009; Espinosa and Paz-y-Miño-C 2014a; Hirose et al. 2011; Strassmann and Queller 2011). Studies with phylogenetically distant social amebas (i.e. *D. discoideum*, *D. purpureum*, *Polysphondylium violaceum*) suggest that recruitment behavior for mound, slug and fruiting-body formation correlates with the genetic distance among clones (i.e. higher aggregation among clones sharing higher values of *r* within the same species), which indicates that the cellular ability to discriminate/recognize kin from non-kin might be common in other gregarious protists (Espinosa and Paz-y-Miño-C 2014a; Kalla et al. 2011; Mehdiabadi et al. 2006; Ostrowski et al. 2008).

All these studies suggest that taxa-, clone-, and kin-discrimination/recognition ability benefits more

close-genetic relatives than distantly related individuals (i.e. inclusive fitness; Hamilton 1964). Altruistic cooperation for biofilm formation (*Saccharomyces*), aggregation for encystation (*Entamoeba*, *Tetrahymena*), or mound-, slug- and fruiting-body formation (*Dictyostelium*, *Polysphondylium*) involve complex cell–cell communication mechanisms, gene regulation, physiology and behavior. All these cellular interactions during complex life cycles entail both selective cell survival and active cell death (Espinosa and Paz-y-Miño-C 2014a; Li and Purugganan 2011; Romeralo et al. 2012); scenarios in which cheating at the cellular level can take place, as it has been documented experimentally (e.g. *Dictyostelium*; Levin et al. 2015; Queller and Strassmann 2012).

Protists might offer us exciting opportunities to study taxa-, clone-, and kin-discrimination from ecological and evolutionary perspectives (Espinosa and Paz-y-Miño-C 2014a). Unicellular eukaryotes exist in all environments, occupy all possible ecological niches (from free-living to symbiotic and pathogenic), and belong to ancient lineages. Some of the ancestors of these lineages were, themselves, the common ancestors of all multicellular organisms (Herron et al. 2013; West et al. 2015). In this respect, protists should be central to current and future studies about the origins and evolution of multicellularity.

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