CHAPTER TWELVE

EXAMINING CRYPTICITY IN *ENTAMŒBA*: A BEHAVIOURAL AND BIOCHEMICAL TALE

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Biologists often face difficulty in establishing whether the organisms they study belong to single or multiple lineages. The challenge derives from conceptual (Type I) and methodological (Type II) errors when attempting to resolve cryptic diversity: either organisms belong to as many—or as few—lineages as researchers infer, or the diversity that investigators think to exist is illusory. Insufficient ecological data and natural history information, or still limited technology, can lead scientists to over- or underestimate lineage richness (Caron 2013, Finlay 2004, Pawlowski and Burki 2009, Pawloski et al. 2012). How can this problem be resolved? Integrated investigative approaches can help us answer this question.

By combining behavioural analysis, colour tagging of individual cells, and pair-mix-culturing of some *Entamœba* varieties, we have previously resolved apparent crypticity in lineages from diverse natural histories, i.e. free-living/opportunistic (*E. moshkovski* Laredo), commensal (*E. moshkovski* snake) and parasitic (*E. invadens* IP-1, *E. invadens* VK-1:NS, *E. terrapinae*, *E. histolytica*) (Espinosa and Paz-y-Miño-C 2012). In this chapter, we expand on these studies with *Entamœba* and discuss how simple behavioural- and growth-mixed-culture trials can assist any researcher in identifying *Entamœba* taxa—and potentially other protists—customarily thought to be "cryptic." For this, we rely on *Entamœba*s' own ability to discriminate one another. We also discuss how unravelling phylogenetic relations among unicellular eukaryotes, usually confounded by Horizontal Gene Transfer (HGT), extinctions or highly variable genetic distances, can help us understand the environmental complexity in which vast unicellular diversity evolved.

Surveying Protistan Diversity

Protists comprise the most abundant and diverse eukaryotes in the planet. Researchers rely on a variety of molecular tools to assemble these organisms into "operational taxonomic units" (OTUs, clusters based on DNA sequence similarity and, therefore, informative of possible relatedness). High-throughput sequencing techniques (Next Generation Sequencing - NGS: Single-molecule real-time sequencing, Ion semiconductor, 454 Pyrosequencing, Sequencing by synthesis, Sequencing by ligation, Chain termination; Barcoding, DNA-based FISH -fluorescent in situ hybridization) have accelerated the magnitude of discovery and characterization of protists (Caron 2013, Pawlowski et al. 2012). Small subunit ribosomal RNA genes (18S) are also frequently used when reconstructing protistan phylogenies. Taxonomic studies in many protists (e.g. diatoms, amœba and heterotrophic flagellates) rely as well on mitochondrial gene sequences or expanded genomic regions analysis (Caron 2013).

Most studies with high-throughput sequencing identify gene fragments that underestimate or overestimate the richness of protistan communities. The former derives from placing ecologically and/or behaviourally distinct lineages in a single OTU due to limited community sampling (Caron 2013). The latter results from placing, in two or more OTUs, populations with highly variable DNA sequences (e.g. highly variable individuals) that belong to the same OTU (Caron 2013, Pawloski et al. 2012). Few studies, however, have linked protistan physiology/morphology directly with OTUs (Caron 2013, Medinger et al. 2010). Extensive genome/proteome laboratory trials are, therefore, needed to expand the genetic analysis of gene fragments into whole genes and multigenes, i.e. numerous OTUs that are representative of protistan lineages (Dawson and Hagen 2009). Ensuring that OTUs have ecological meaning requires the matching of molecular sequences with physiological, behavioural, and biochemical data (this chapter). Only then will such "informational unification" help us establish natural protistan assemblages with confidence.

Holobiont Communities and Networks of Gene Exchange

Tree-like representations of life's diversification are didactically useful, but they ignore the significance of HGT in evolutionary history (Paz-y-Miño-C and Espinosa 2010). Genes can be transferred from/to and/or exchanged among all organisms, with higher frequency and magnitude among prokaryotes and single-cell eukaryotes than among multicellular

taxa (Paz-y-Miño-C and Espinosa 2010). In bacteria, closely related lineages show high levels of gene exchange, comparable to a *highway of gene sharing* (Bansal et al. 2013). Expanding these studies of gene highways to protistan communities could, in consequence, reveal past and present ecological associations between closely and distantly related phylogenetic groups.

High frequency and magnitude of inter- and intra- domain HGT in protistan genomes have been confirmed through phylogenetic studies (Bruto et al. 2013). For example, genomes from extracellular mucosal parasites (*E. histolytica, G. lamblia, T. vaginalis*) have a noticeable number of horizontally acquired metabolic genes from resident microbionts in vertebrates (Alsmarck et al. 2013), thus suggesting that HGT has contributed significantly to protistan evolution (i.e. genetic adaptation to anoxic conditions in the vertebrate/invertebrate gut).

Holobionts, as ecological units composed of macro-organisms and the viruses, microbes and protists living in them (Margulis 1991, Paz-y-Miño-C and Espinosa 2013), constitute model ecosystems to explore the genetic interconnectedness between/among their resident bionts. The *network exchange* hypothesis (Bapteste et al. 2012) posits that higher-order entities (interconnected "genome units" or collective reproducers) profit from the combined products encoded by the genes of the partners (Bapteste et al. 2012). Protists can be excellent models of higher order entities to detect the interconnectedness of genes from/to their own resident bionts, and to the host holobiont. Therefore, these expanded network analyses can provide the knowledge for a comprehensive assembly of protistan lineages.

Discrimination in Protists

Behavioural cues displayed among closely related single or multicellular organisms can differ from those directed at unrelated individuals (Hamilton 1964, Kalla et al. 2011, Rumbaugh et al. 2012). Aggregation and/or discrimination have been reported among close relatives in bacteria (Kraemer and Velicer 2011, Rumbaugh et al. 2012) and protists (*Dictyostelium*, *Entamœba*, *Polysphondylium violaceum*; dictyostelids; *Plasmodium, Trypanosomas*; Espinosa and Paz-y-Miño-C 2012, Kalla et al. 2011, Li and Purugganan 2011, Reece et al. 2008, 2011, Romeralo et al. 2012). Phylogenetic and mating analyses in a dictyostelid suggest higher cooperation among organisms from the same lineage than between two or more varieties mixed together, which has helped biologists to

characterize taxonomically *Polysphondylium* natural groups (Kalla et al. 2011). The genes and G-coupled receptor signal transduction proteins involved in *Dictyostelium* aggregation have broadened our understanding of cell-to-cell communication among genetically related dictyostelids (Heidel et al. 2011). Aggregative migration in other protists has been examined in the context of feeding, defense, invasiveness, reproduction or programme cell death (PCD)(e. g. marine eukaryotes; dinoflagellates; algae, parasitic amœba ; Brodsky 2009; Paul et al. 2007; Strom et al. 2007; Zaki et al. 2006).

Natural selection could favour clonal lineages of unicellular parasites in which some clones undergo PCD (an active and genetically regulated type of cell death) if it increases the later transmission of their clone-mates (Kaczanowski et al. 2011). Here, the speculation has been that parasites engage in PCD based on both population density and relatedness. If *Trypanosoma brucei* clone-mates infect a host, the parasites that die apparently facilitate the transmission of their genotype (Kaczanowski et al. 2011, Pollitt et al. 2011), but this could simply be a byproduct of aggregation rather than an "altruistic" trait. *Plasmodium falciparum* haplotypes recovered from patients that suffered multiple infections shared higher genetic relatedness within than between infections (Nkhoma et al. 2012). This genotypic relatedness of malaria haplotypes suggests that they were inoculated through single mosquito bites and spread by serial transmission between people (Nkhoma et al. 2012). *Plasmodium chaubaudi* parasites facultatively alter their sex ratio in response to changes in host anæmia. Infective processes tested in genetically related *P. chaubaudi* revealed an adjustment of parasite sex allocation in response to the presence of unrelated genotypes (Pollitt et al. 2011, Reece et al. 2008, 2011). Although these studies suggest a potential capacity of several single-cell eukaryotes to discriminate conspecifics that belong to "similar" versus "different" genotypes in respect to "self," further studies are needed to determine the mechanisms and discrimination levels upon controlled experimentation of genetic relatedness (r).

Entamœba: a behavioural and biochemical tale

Entamœba is an ideal model to examine discrimination. By using aggregative, biochemical and morphological cues, we have demonstrated that *E. invadens* IP-1 and VK-1:NS trophozoites aggregate with members of their own variety and maintain separation from clusters of non-alike amœba (Espinosa and Paz-y-Miño-C 2012). Measurements of individual

cells (average length, width, and surface area) showed that the strains were morphologically distinguishable when combined traits were examined (Espinosa and Paz-y-Miño-C 2012).

We broadened this laboratory strategy to study the free-living/opportunistic *E. moshkovski* Laredo (*E. m*L) and the commensal *E. moshkovski* Snake (*E. m*S). Pair combinations of *E. m*L / *E. m*S labeled with green/red fluorescent dyes, or in the reciprocal red/green fluorescent dyes (Table 1), were grown together.

Table 1. Experimental combinations of *E. moshkovskii* Laredo (*E. m*L) and *E. moshkovskii* Snake (*E. m*S) labeled with CellTracker Red and / or Green CMFD fluorescent tags (Invitrogen)

Unlabelled	Labelled (Green or Red)
*E. m*L / *E. m*S	*E. m*L (Green) / *E. m*S (Red) *E. m*L (Red) / *E. m*S (Green)
*E. m*L alone	*E. m*L (Green) / *E. m*L (Red)
*E. m*S alone	*E. m*S (Green) / *E. m*S (Red)

*Entamœba m*L trophozoites formed distinct separate colour clusters, which increased after 12, 18, and 36 h without mixing with members of the other variety; a similar pattern of fluorescent single colour clusters was observed for *E. m*S trophozoites. As shown in Figure 1 (a-c), *E. m*L aggregated in green clusters, *E. m*S in red clusters, or vice versa (Figure 1 d-f).

In contrast, when *E. m*L trophozoites were labelled with green and red dye and grown together, yellow clusters were observed between all trophozoites, indicating strong variety associative behaviour. Large fluorescent yellow clusters (green + red) increased gradually at 12, 18, and 36 h (Figure 2 a-c). Pair combinations of *E. m*S trophozoites that were labelled with green and red dyes showed similar behaviour (Figure 2 d-f). There was no detectable toxicity in the trophozoites with either dye for the length of the experiments (36 h, control data not shown). All *Entamœba* varieties were morphologically distinguishable when combined traits were examined (length, width and surface area of cells; Table 2).

Thus, combining biochemical and behavioural cues with morphological traits allowed us to resolve apparent crypticity in *Entamœba* (as in, for example, Stensvold et al. 2011). Comparable studies have also demonstrated behavioural clumping as function of genetic relatedness in dyctiostelids (Kalla et al. 2011, Romeralo et al. 2012). It is, therefore, possible to unravel illusory crypticity and, by doing it, contribute to a more detailed understanding of the evolutionary histories of other protists.

Table 2. Phenotypic characterization of *Entamœba* lineages

	E. dispar	*E. invadens* IP-1	*E. invadens* VK-1:NS	*E. histolytica* HM1:IMSS	*E. moshkovskii* Laredo	*E. moshkovskii* Snake	*E. terrapinae*
Avg cluster surface area (µm)	976 ± 256	2986 ± 584	1767 ± 477	4078 ± 863	588 ± 122	1659 ± 738	2976 ± 459
Avg cell length (µm)	45 ± 9	78 ± 13	59 ± 11	103 ± 17	33 ± 6	55 ± 19	78 ± 9
Avg cell width (µm)	24 ± 3	56 ± 10	45 ± 9	54 ± 9	22 ± 4	38 ± 5	47 ± 8
No. amoeba per cluster	8	30	22	13	21	16	13
Distance between clusters (µm)	110	20	50	70	40	60	25
In vitro culture (°C)	37	23	23	37	23	23	23
Host	human	snake	Komodo dragon/ salamander	human	ponds, lakes	snake	turtle

Conclusion

Aggregative behaviours attributed to genetic relatedness have been documented in prokaryotes and single cell eukaryotes (Kraemer and Velicer 2011, Rumbaugh et al. 2012, Espinosa and Paz-y-Miño-C 2012, Kalla et al. 2011, Li and Purugganan 2011, Reece et al. 2008, 2011, Romeralo et al. 2012), yet the levels of discrimination and mechanisms involved still need to be elucidated. Our studies linking behaviour with lineage ancestry in protists are particularly informative at times when

prevalent large-scale molecular sampling of Earth's life continues to unmask new organisms, in which behavioural diversity—hidden in apparent "crypticity"—continues to be undervalued. Expanded network analyses that include holobionts with their microbionts, in an ecologically relevant "informational unification", will help us discover natural and vast protistan assemblages.

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