Genetic diversity and gene family expansions in members of the genus *Entamoeba*

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Abstract (<250)

Amoebiasis is the third-most common cause of mortality worldwide from a parasitic disease. Whilst the primary aetiological agent of amoebiasis is the obligate human parasite *Entamoeba histolytica*, other members of the genus *Entamoeba* can infect humans and may be pathogenic. Here, we present the first annotated reference genome for *Entamoeba moshkovskii*, a species that has been associated with human infections, and compare the genomes of *E. moshkovskii*, *E. histolytica*, the human commensal *Entamoeba dispar* and the non-human pathogen *Entamoeba invadens*. Gene clustering and phylogenetic analyses show differences in expansion and contraction of families of proteins associated with host or bacterial interactions. They intimate the importance to parasitic *Entamoeba* species of surface-bound proteins involved in adhesion to extracellular membranes, such as the Gal/GalNAc lectin and members of the BspA and Ariel1 families. Furthermore, *E. dispar* is the only one of the four species to lack a functional copy of the key virulence factor cysteine protease CP-A5, whilst the gene's presence in *E. moshkovskii* is consistent with the species' potentially pathogenic nature. *Entamoeba moshkovskii* was found to be more diverse than *E. histolytica* across all sequence classes. The former is approximately 200 times more diverse than latter, with the 4 *E. moshkovskii* strains tested having a most recent common ancestor nearly 500 times more ancient than the tested *E. histolytica* strains. A four-haplotype test indicates that these *E. moshkovskii* strains are not the same species and should be regarded as a species complex.

Key Words

Entamoeba, gene family, genome diversity, species complex

Introduction

Amoebiasis affects up to 50 million people annually, resulting in up to 100,000 deaths (Walsh 1986). The aetiological agent of amoebiasis in humans is the obligate human parasite *Entamoeba histolytica*, which is transmitted between hosts by a faecal-oral route. The outcome of infection ranges from asymptomatic carriage (in the majority of cases) to dysentery, characterised by bloody stools and, in some cases where parasites escape the gut, abscesses in the liver and other organs that are fatal if untreated. Amoebiasis is particularly prevalent in areas of poor sanitation and people living in these conditions are the most commonly affected. Outside of these settings, risk groups are travellers returning from endemic regions, people who engage in risky sexual practices (Stark *et al.* 2007; 2008) and institutionalised populations (Rivera *et al.* 2006; Nishise *et al.* 2010).

The low proportion of infections that result in invasive amoebiasis remains unexplained. Our understanding of the epidemiology of the disease was complicated, in part, by the existence of a second, non-invasive, member of the genus *Entamoeba* - *Entamoeba dispar* (Diamond & Clark 1993). Morphologically identical to *E. histolytica* and closely related, *E. dispar* is infective to humans but is thought to be avirulent (Diamond & Clark 1993; Bansal *et al.* 2009) despite liver-derived clinical isolates of *E. dispar* bringing its avirulence into question (Ximénez *et al.* 2010). Invasive disease is deleterious to the parasite as trophozoites passing into the blood or tissues will not go on to form cysts and infect new hosts. Therefore, 'virulence' should not be selected for and may be considered as a negative interaction for the host and parasite.

The differences in virulence capabilities seen between *E. dispar* and *E. histolytica* have been exploited by various groups attempting to determine which proteins may enable virulence

capabilities in *E. histolytica* but not in *E. dispar* (Davis *et al.* 2009; Leitsch *et al.* 2006). Two key families, which we investigate here in relation to host-parasite interactions in a greater number of *Entamoeba* species, are the cysteine proteases and the Gal/GalNAc lectin proteins.

To invade the intestinal epithelium, trophozoites must first degrade and cross the mucosal layer that covers and protects it. The cysteine proteases are a group of at least 50 endopeptidases, 36 of which form 3 major clades - 'A', 'B' and 'C' (Clark *et al.* 2007; Casados-Vázquez *et al.* 2011). Whilst, collectively, the cysteine proteases are regarded as virulence factors, evidence suggests that approximately 90% of *E. histolytica*'s cysteine protease-derived proteolytic activity is provided by just three proteins – EhCP-A1, EhCP-A2 and EhCP-A5 (Bruchhaus *et al.* 1996; Ankri *et al.* 1999; Stanley *et al.* 1995; Meléndez-López *et al.* 2007). EhCP-A5 is of particular interest as no functional orthologue exists in the non-pathogenic *E. dispar* (Jacobs *et al.* 1998) and expression of the protein is thought to be necessary for *E. histolytica* to invade the human intestinal mucosa (Thibeaux *et al.* 2014). In concert with amoebic glycosidases, an undefined number of cysteine proteases degrade the MUC2 polymers that constitute much of the mucosal layer (Moncada *et al.* 2003; 2005). Trophozoites employ surface-bound proteins to bind to host mucins as a natural part of a commensal lifecycle and, once they have degraded the mucosal layer, epithelial cells. One such protein is the Gal/GalNAc lectin, a heterodimer comprising a 170 kDa heavy subunit and a 35 kDa light subunit, associated with a 150 kDa intermediate subunit (Petri *et al.* 2002). The lectin binds to galactose and N-acetyl-D-galactosamine on host cell membranes. Without it, *E. histolytica*'s ability to adhere to host cells is significantly diminished, as is its cytotoxic impact upon the host cells, leading to the understanding that the cytokine cascade induced by *E. histolytica* that ultimately leads to the degradation of host cells is contact-dependent (Stanley 2003; Li *et al.* 1988; 1989; Ravdin *et al.* 1980; 1989). However, despite the wealth of knowledge that exists regarding gene families potentially responsible for causing invasive amoebiasis such as the cysteine proteases and Gal/GalNAc lectins, much uncertainty remains regarding which of these families play essential

roles and what key differences exist between those species and strains capable of causing pathology and those that cannot.

A more distantly related species, *Entamoeba moshkovskii*, was originally thought to be free-living and therefore non-pathogenic (Tshalaia 1941; Neal 1953; Clark & Diamond 1997). However, as with *E. dispar*, human-derived clinical isolates (Clark & Diamond 1991) and cases of diarrhoea directly associated with *E. moshkovskii* infection (Fotedar *et al.* 2008; Shimokawa *et al.* 2012) have challenged this assumption. As such, the ability of *E. moshkovskii* to cause invasive amoebiasis is of increasing interest, with multiple studies presenting further evidence that *E. moshkovskii* is human-infective and potentially pathogenic (Hamzah *et al.* 2006; ElBakri *et al.* 2013; Khairnar & Parija 2007; Ayed *et al.* 2008; Lau *et al.* 2013).

Despite its evolutionary distance from *E. histolytica*, *E. dispar* and *E. moshkovskii* (Stensvold *et al.* 2011), the reptile-infective *Entamoeba invadens* is also known to be pathogenic and can cause fatal disease in a wide range of reptiles (Meerovitch 1958; Kojimoto *et al.* 2001; Chia *et al.* 2009). This species is also of interest for research into lifecycle development because it is the only member of the genus for which encystation can be successfully induced *in vitro* in axenic culture, using various methods (Avron *et al.* 1986; Vázquezdelara-Cisneros & Arroyo-Begovich 1984; García-Zapién *et al.* 1995). Through genome sequencing, it was found that *E. invadens* has an average sequence identity with *E. histolytica* of 60% (Wang *et al.* 2003; Ehrenkaufer *et al.* 2013).

Several reports, focusing on single nucleotide polymorphisms (SNPs), have found evidence to support the theory of limited genetic diversity amongst *E. histolytica* strains (Beck *et al.* 2002; Weedall *et al.* 2012; Bhattacharya *et al.* 2005). Initially, this was thought to indicate a clonal species, however evidence of meiotic recombination has been discovered, suggesting that *E. histolytica* actually reproduces sexually (Weedall *et al.* 2012). There is a relative paucity of

studies into diversity in other members of the genus *Entamoeba*. In the case of *E. moshkovskii*, this is because, until now, there was no reference genome with which to compare different strains. In spite of this, there is support for the theory that *E. moshkovskii* is, in fact, highly variable and may be a species complex, rather than an individual species (Clark & Diamond 1997; Jacob *et al.* 2016). If we are able to more accurately identify which isolates are capable of infecting humans or causing disease, it may afford us a greater understanding of the genetic and molecular mechanisms behind these traits.

Here we present the first annotated genome for *E. moshkovskii*. We have compared this to the sequenced genomes of other members of the genus *Entamoeba*, offering greater insight into the evolution of gene families involved in host-parasite interactions. We focus particularly on the evolution of the cysteine proteases and Gal/GalNAc lectins. We observe that the expansion and contraction of these gene families appears to reflect their rapid evolution and the different host ranges of the various species. We also analyse divergence between the genomes in order to gain evidence of selective pressures acting upon genes within them. We have identified genes under diversifying selective pressures within each species, indicating sequences that are important for survival in the host. Finally, we compare genome-wide diversity levels between and within *E. histolytica* and *E. moshkovskii*. Comparisons of variability between the species and different sequence classes are also made, particularly with a view to establishing the variability of the *E. moshkovskii* genome and whether or not it exists as a species complex (Clark & Diamond 1997; 1991; Heredia *et al.* 2012).

Methods

Whole genome sequencing of *E. moshkovskii* strains

Previously described *E. moshkovskii* strains Laredo (ATCC 30042) and FIC (ATCC 30041) are compared alongside two other strains described here for the first time – '15114' and 'Snake'. Strain 15114 was received in London from Dr Rashidul Haque (ICDDR,B, Bangladesh), via Dr Bill Petri (UVA), in October 1999 as *E. histolytica*, but was identified as *E. moshkovskii* in August 2000. Strain Snake was received in London from Prof Jaroslav Kulda (Charles University, Prague, where it had been kept for over 50 years and was thought to be *E. invadens*) in April 2008. Both were adapted to grow axenically by standard methods.

Axenic cultures of *E. moshkovskii* strains Laredo, FIC, 15114 and Snake were grown and maintained in LYI-S-2 media (liver extract, yeast extract, iron and serum) with 15% adult bovine serum (Clark & Diamond 2002). To culture high cell counts, strains were incubated at room temperature, in darkness, for 7 days. Once at a high density, the cells were centrifuged, washed twice in phosphate-buffered saline (PBS) solution and lysed with QIAGEN cell lysis buffer, before an adapted version of the previously described CTAB method (Clark & Diamond 1991), as employed by Weedall *et al.* (2012), with two rounds of the phenol:chloroform:isoamyl alcohol (25:24:1) extraction, was used. The extracted and purified DNA was suspended in nuclease-free water. For strains FIC, 15114 and Snake, 100 bp libraries were pooled and sequenced using the 'TruSeq DNA sample prep low throughput protocol' (Illumina), using the in-line control reagent and gel-free method. Libraries were size selected for total fragment lengths between 400 and 600 bp using a Pippin Prep machine (Sage Science) with a 1.5% agarose gel cassette. A 150 bp Paired End (PE) library was similarly generated for the Laredo strain. Alignment of the resulting reads for each strain is described below in the section entitled 'Variant calling and analysis in *E. histolytica* and *E. moshkovskii*'.

E. moshkovskii Laredo was also sequenced using the 454 method in order to generate a *de novo* assembly. Two single-end fragment libraries, a 3 kb insert PE library and an 8 kb insert PE library were constructed using the manufacturer's protocols and sequenced using the 454 GS

FLX Titanium system (Roche). The Newbler Assembler v2.3 (Margulies *et al.* 2005) was used to carry out a *de novo* assembly of the total 3,812,076 generated reads > 150 bp using default parameters. The resulting scaffolds, and contigs no smaller than 500 bp, were concatenated to produce an un-ordered draft assembly.

Annotation of the *E. moshkovskii* Laredo genome

A training set of 197 models, including 57 multi-exon models, was manually curated for annotation software AUGUSTUS v2.5.5's training script autoAug (Stanke & Waack 2003). The set was informed using three datasets. Open reading frames 150 amino acids or greater in length were cross-referenced with 'hits' generated by entering a 3.5 Mb section of the assembly into a BLASTX search (Altschul *et al.* 1990) against the *E. histolytica* HM-1:IMSS protein set with an Exponent Value (E-value) threshold of 1e-10. Finally, transcriptomic data generated using a previously published protocol (Hon *et al.* 2013) were used, although default cutoff scores were used with HMMSplicer v0.9.5 (Dimon *et al.* 2010). AUGUSTUS was then run using default parameters and a set of 'hints', consisting of weighted intron positions inferred from the splice junction data (Bonus = 10; Penalty = 0.7; un-weighted values = 1).

Proteins encoded by putative coding sequences (CDSs) in the AUGUSTUS output were entered into a reciprocal BLASTP search against the protein set of *E. histolytica* HM-1:IMSS, using default parameters. Predicted sequences with a reciprocal best hit (RBH) were included in the final annotation set. Those without a definite orthologue were included if their total exon length exceeded 350 bp and if they were attributed an AUGUSTUS confidence score of at least 0.75 or they 'hit' an *E. histolytica* HM-1:IMSS gene in a one-way BLASTP search using an E-value threshold of 1e-5.

To add functional annotations to gene models, the *E. moshkovskii* Laredo protein set was entered into reciprocal BLASTP searches against the protein sets of *E. histolytica* HM-1:IMSS and

E. dispar SAW760, using default parameters. Where an *E. moshkovskii* Laredo protein had an RBH against a protein from either of the other species' sets with a minimum bit-score of 10, the gene by which it was encoded was annotated with the same function as its orthologue. CDSs with RBHs in both *E. histolytica* HM-1:IMSS and *E. dispar* SAW760 were thus functionally annotated twice.

As a measure of completeness, the annotated protein set, along with the protein sets of *E. histolytica* HM-1:IMSS, *E. dispar* SAW760 and *E. invadens* IP-1, was compared with the Benchmarking Universal Single-Copy Orthologs (BUSCO) v3 Eukaryota *obd9* sequence set (Simão *et al.* 2015) using the BUSCO v3 virtual machine with default settings.

Reference strain data in other species

Genomic, CDS and protein sequences, as well as genomic feature files, for *E. histolytica* HM-1:IMSS, *E. dispar* SAW760 and *E. invadens* IP-1 were downloaded from AmoebaDB v2.0 (Aurrecoechea *et al.* 2010; 2011). Average fold coverage values were acquired from the NCBI Whole Genome Sequence Project pages. The accession numbers for the versions of the three projects used are as follows (with original project accession numbers in parentheses): *E. histolytica* HM-1:IMSS: AAFB02000000 (AAFB00000000); *E. dispar* SAW760: AANV02000000 (AANV00000000); and *E. invadens* IP-1: AANW03000000 (AANW00000000).

Non-reference read data in *Entamoeba histolytica*

Existing sequence data for *E. histolytica* strains were used (Weedall *et al.* 2012; Gilchrist *et al.* 2012). Strains MS96-3382 and DS4-868, sequenced using Illumina technology, were downloaded from the European Nucleotide Archive (http://www.ebi.ac.uk/ena). Their Run accession numbers are SRR368631 and SRR369427, respectively. We used our existing SOLiD-derived read data for *E. histolytica* strains Rahman, 2592100, PVB-M08B, PVB-M08F, HK-9, MS27-5030, MS84-1373 and a cell line derived from the reference strain, HM-1:IMSS-A.

Defining orthologues and gene families

OrthoMCL v2.0.3 (Chen *et al.* 2006) was used to identify gene families with orthologues in *E. histolytica* HM-1:IMSS, *E. dispar* SAW760, *E. invadens* IP-1 and *E. moshkovskii* Laredo. Default parameters were used, though an E-value threshold of 1e-5 was applied to the All-vs-All BLASTP search stage. MySQL served as the relational database. A 50% cutoff value was applied. All proteins from all four species were included in the comparison. MCL was run using a clustering granularity value of 3.0.

Identification of orthologues within virulence factor gene families

E. histolytica HM-1:IMSS genes encoding cysteine proteases and Gal/GalNAc lectin subunits were identified using AmoebaDB and NCBI's Gene Database. Corresponding protein sequences were entered into a TBLASTN search against the complete gene sets of *E. histolytica* HM-1:IMSS, *E. dispar* SAW760, *E. invadens* IP-1 and *E. moshkovskii* Laredo to identify orthologues. An E-value threshold of 1e-5 and a limit of 50 hits per search were applied to limit the number of poor quality hits and computational expense incurred in analysing them.

Where 50% or more of a query sequence's length was cumulatively matched across all hits to a particular reference sequence, that reference sequence and all genes with which OrthoMCL clustered it were added to its respective virulence factor family. Clusters or individual genes present in 2 families were manually investigated to determine to which family the gene and their cluster should be added. Any identified orthologues lacking functional annotations on AmoebaDB were entered into a BLASTP search against the NCBI's nr database, using default parameters, to subjectively identify any high-quality hits against a member of the virulence factor family to confirm their annotation. In addition to this, any informative or requisite domains or functions were identified using the InterPro and ProtoNet subsections of UniProt. In groups containing noticeably fewer genes in one species, an *E. histolytica* HM-1:IMSS gene within the clade, or an *E. dispar* SAW760 gene in the absence of an *E. histolytica* gene, was entered into a

TBLASTX search against the genome of the 'missing' species, using default parameters. High-quality hits were determined subjectively, using the E-values of known family members. Non-pseudogenous hits were added to their respective virulence factor family.

Phylogenetic analyses of virulence factor families

MUSCLE v3.8.31 (Edgar 2004) was used, with default parameters, to align sequences within each family. Bootstrapped Maximum Likelihood phylograms, were generated for each virulence factor family using PHYLIP v3.69 (Felsenstein 1989). Default parameters were used unless otherwise stated. Seqboot was run to generate 1,000 bootstrap pseudo-replicate alignments. Protdist was then run to generate distance matrices for each bootstrap replicate alignment, using the Jones-Taylor-Thornton matrix as well as the gamma distribution of evolution rates among amino acid positions, and proportion of invariant sites if greater than 0, as determined using values calculated by MEGA v5.2.1 using default parameters (Jones *et al.* 1992; Tamura *et al.* 2011). Fitch estimated phylogenies with the Fitch-Margoliash criterion for the 1,000 randomised data sets before Consense output bootstrapped trees. To apply branch lengths that represent evolutionary distances to the trees, the first two PHYLIP programs described above were run again, using the same parameters, but for 1 dataset rather than 1,000. Bootstrapped trees were input to Fitch with their respective single data set trees, applying branch lengths to the relationships. Statistical comparisons of branch lengths, representative of evolutionary distances between genes, were manually calculated. Mann-Whitney-Wilcoxon tests (with continuity correction) were performed for each data set using alpha values of 0.05.

In the cysteine protease A subfamily, all incomplete CDSs were entered into a BLASTN search against their species' complete gene set, with an E-value threshold of 1e-4. Query sequences and sequences hit by them were accepted as members of the family. Phylogenetic trees for such nucleotide sequence sets were generated using a method similar to the one above but implementing PHYLIP's DNAdist as opposed to Protdist and using the F84 distance matrix.

Variant calling and analysis in *E. histolytica* and *E. moshkovskii*

Reads from the reference strains (*E. moshkovskii* Laredo reads sequenced for this project; *E. histolytica* HM-1:IMSS-A reads downloaded, as described above) were aligned to the existing assembled reference sequences, downloaded from AmoebaDB v2.0 (Aurrecoechea *et al.* 2010; 2011), using the Burrows-Wheeler Aligner (BWA) v0.5.9 (Li & Durbin 2009). Default parameters were applied to the 'aln' command except in two cases. Firstly, suboptimal alignments were permitted for reads that could be mapped to multiple sites provided that there were no more than 10 equally best potential sites. Secondly, maximum edit distances of 4 and 12 were applied to the SOLiD reads and longer Illumina reads, respectively. The 'samse' and 'sampe' commands were used to align the SOLiD and Illumina reads, respectively, using default parameters. Unmapped and non-uniquely mapped reads were filtered out.

SNPs in the aligned reference strains' reads were called using the SAMtools v0.1.18 (Li *et al.* 2009) mpileup command (default parameters were used apart from forcing the output of per-sample read depths) and bcftools view command (default parameters were used except for setting it to output both bases and variants). High quality SNPs were defined as those that met the following parameters: Phred quality score >= 20; read depth >=5 and <= 95th percentile of all depths seen in assembly; and farther than 5 bp from a gap, using a window of 30 bp. High quality homozygous SNPs were inserted in place of their respective original bases within the original reference sequences. The updated reference sequences were then used in place of the original genomes when reads from non-reference strains were mapped, and SNPs called, using the method outlined above.

Total counts of SNPs per gene, excluding pseudogenes and sequences with an incomplete triplet codon, were calculated per strain, distinguishing between synonymous and non-synonymous SNPs in coding regions and SNPs in non-coding regions. Programs from the

Phylogenetic Analysis Using Maximum Likelihood (PAML) package v4.5 (Yang 1997; 2007) were used to calculate pN and pS values for each gene relative to each strain's respective reference strain. A pairwise calculation amongst all strains within a species would have made the unlikely assumptions that all SNPs were called in each strain and that any base not called as a SNP was definitely the same as in the reference strain. The Probabilistic Alignment Kit (PRANK) v.111130 was run using an empirical codon model with other parameters set to default values, followed by codeml, run using default parameters.

TMRCA analysis

To generate Time to Most Recent Common Ancestor (TMRCA) values for *E. histolytica* and *E. moshkovskii*, all 4-fold degenerate (4D) sites at which only homozygous SNPs were located, and to which reads were mapped at a depth of 35x or greater in all strains of each species, were identified and concatenated. This amounted to 339,091 bases in *E. histolytica* and 641,223 bases in *E. moshkovskii.* The pairwise SNP rates, calculated as fractions of the total number of concatenated 4D sites in *E. histolytica* and *E. moshkovskii*, were used to calculate final 'distances', as well as to visualise, for the first time, the phylogenetic relationships between the strains of *E. moshkovskii*. The generic eukaryotic rate of 2.2 e-9 substitutions per base per annum was considered an acceptable approximation given its use in a similar previous study (Kumar & Subramanian 2002; Neafsey *et al.* 2012).

PHYLIP v3.69 (Felsenstein 1989) was used to generate neighbour-joining phylograms for nucleotide positions of common 4D sites in *E. moshkovskii* strains and *E. histolytica* strains, using the additive tree model. Default parameters were used unless otherwise stated. Seqboot was run with 1,000 bootstrap replicates. DNAdist was then run using the Jukes-Cantor model, which does not take codon position into account (Jukes & Cantor 1969). Neighbor was subsequently run for the 1,000 data sets, the output of which was processed by Consense. To apply branch lengths that represented evolutionary distances to trees, a distance matrix, consisting of differences between

pairs of strains per 4D site, was submitted to Neighbor for a single data set. Branch lengths were manually added to Consense output files.

Four-haplotype test in *E. moshkovskii*

This test was employed to detect meiotic recombination signals between the 4 tested strains of *E. moshkovskii* in order to determine whether or not they belong to one species or a species complex. One million pairs of high quality SNPs, defined as nucleotide positions called in every strain and existing as homozygotes in every strain, but varying between them, were randomly sampled. Within groups of 10,000 pairs, proportions of SNP pairs existing as four haplotypes were calculated and the group's average distance between pairs of sites was calculated. This test was carried out with a previously used Perl script (Weedall *et al.* 2012).

Results and Discussion

Assembly and annotation of the *E. moshkovskii* Laredo genome

We sequenced the genome of *E. moshkovskii* Laredo. Of the four DNA libraries sequenced on the 454 GS FLX Titanium system (Roche), the two single-end fragment libraries together yielded 2,211,151 reads (86% > 150 bp). The 3 kb and 8 kb insert PE libraries generated 743,770 (86% > 150 bp) and 857,155 (90% > 150 bp) reads, respectively. Assembly of the combined total of 3,812,076 reads generated 12,880 contigs. When assembled into scaffolds, 3,352 contigs were included in 1,147 scaffolds. The scaffolds were concatenated, along with 3,460 contigs of at least 500 bp in length, to give a total assembly length of 25,247,493 bp. This is slightly larger than the genomes of the closely related *E. histolytica* and *E. dispar*, but far shorter than that of the more distant *E. invadens* (Table 1). However, *E. moshkovskii* is the only *Entamoeba* reference genome that includes contigs not mapped to scaffolds and each of the other genome projects have used

different size filtering strategies. The total length of the *E. moshkovskii* genome represented by scaffolds alone is similar to those of the *E. histolytica* and *E. dispar* genomes.

The average sequence depth for the *E. moshkovskii* assembly is inflated by a relatively small number of contigs and scaffolds with uncommonly high coverage depths (Figure S1). The modal depth of the assembly was 27x with a mean depth of 82.65x. Exclusion of contigs with coverage depths greater than 2 standard deviations from the mean lowered the average depth to 54.41x. It is likely that such inflated coverage depths are the result of repeat regions in the genome (Sipos *et al.* 2012; Treangen & Salzberg 2011). The GC content of *E. moshkovskii* is similar to those of the other three species, and the narrow range of GC contents seen across the genome is normally distributed (Table 1; Figure 1). Notably, the *E. invadens* genome has an unusual GC distribution compared to the other species, suggesting different regions of the genome may have different nucleotide biases.

E. moshkovskii is predicted to possess 12,449 genes. A total of 216 Eukaryota-lineage BUSCO sequences, including fragmented and duplicated sequences, are represented by this gene content, out of a 303-strong set (Table 2). This includes only 2 fewer complete single-copy BUSCO sequences than in *E. histolytica* and suggests a slightly more complete assembly than is seen for *E. dispar* and *E. invadens*, for which there are a greater number of missing BUSCO sequences. Therefore, we assume that the lack of a complete BUSCO Eukaryota gene set is due to the large evolutionary distance between these protists and the species used to construct the BUSCO gene set. A total of 9,495 genes in the *E. moshkovskii* gene set are predicted to be complete gene models, with the genome containing 2,765 partial genes and 189 pseudogenes. In total, 50.2% of predicted genes are functionally annotated. The final set of gene models, and the concatenated assembly upon which they were based, have been made publicly available as part of AmoebaDB v2.0, released on 11th March 2013. Functional and structural annotations were included in AmoebaDB v4.0.

As the manually curated gene set used to train AUGUSTUS was based upon gene models in *E. histolytica*, it is unsurprising that the statistics relating to the *E. moshkovskii* gene set are similar to those seen in *E. histolytica*. *Entamoeba histolytica* possesses the best studied of the genomes here and is the only one to have a manually curated assembly and gene set. This encourages confidence in the gene set predicted for *E. moshkovskii*. However, it does also come with the caveat that mistakes in the *E. histolytica* gene set could be carried into the *E. moshkovskii* set.

Entamoeba species show extensive expansion and contraction of gene families

In order to identify gene families unique to each species OrthoMCL v2.0.3 was used to cluster sequences in the reference genomes of *E. histolytica*, *E. dispar*, *E. invadens* and *E. moshkovskii*. A total of 4,704 gene families comprising 21,741 genes were shared by all four species (Figure 2). The number of genes unique to each species positively correlates with the total number of genes in their genomes, as do the number of gene families to which those unique genes belong (Table 3).

In the gene set unique to *E. histolytica*, three of the four most prevalent families encode surface proteins. The largest group of genes encodes a 22-gene subset of the BspA family (however, other members of the BspA family are orthologous to sequences in the other species studied here, demonstrating a role to play in all 4 species). Totalling 115 sequences in *E. histolytica* alone, the large family lies within one of seven subfamilies containing leucine-rich repeat regions. Multiple BspA-like proteins in *E. histolytica* are located on the plasma membrane of trophozoites (Davis, Zhi Zhang, *et al.* 2006; Silvestre *et al.* 2015) and BspA proteins are known to play roles in adhesion to extracellular membranes in both *Bacteroides forsythus* and *Trichomonas vaginalis* (Sharma *et al.* 1998; Hirt *et al.* 2002; Noël *et al.* 2010). It is likely that

members of the BspA family are similarly involved in adherence to host cells in *Entamoeba* species. However, the reason for the expanded set of unique BspA genes in *E. histolytica* is unclear.

Eighteen Ariel1 surface antigen family proteins are found in the *E. histolytica*-exclusive gene set, as well as 2 orthologous serine-rich antigen proteins, whilst there are no Ariel1 genes unique to *E. dispar* and *E. invadens*. The only gene in *E. moshkovskii* annotated as encoding an Ariel1 surface protein (EMO_091800) is, according to our analysis, unique to *E. moshkovskii*, and forms a cluster with 5 other unannotated genes. The annotated gene was found, during genome annotation, to potentially possess an incomplete coding sequence and, as such, its ability to be expressed and the overall function of this gene cluster remains unclear without expression analysis. To a degree, this confirms past research, which noted that the Ariel1 family was present in *E. histolytica* but not in *E. dispar* (Willhoeft *et al.* 1999). The family belongs to the same larger family as the SREHP protein (Mai & Samuelson 1998), which has been shown to be antigenic (T Zhang *et al.* 1994), however the reason for its absence in *E. invadens* and potential lack of functionality in *E. moshkovskii* cannot be determined without further investigation.

In a further comparison of *E. histolytica* and *E. dispar*, 12 members of the AIG1 family are present only in *E. histolytica*, whilst 13 are found only in *E. dispar*. These GTPases, originally isolated in *Arabidopsis thaliana*, are thought to confer resistance to bacterial infections (Reuber & Ausubel 1996; Gilchrist *et al.* 2006), and have been shown to be more highly expressed in virulent *E. histolytica* cell lines (Biller *et al.* 2010). The presence of commensal gut microbiota in the species' trophozoites' environment makes it logical for them to have a large number of genes encoding AIG1 proteins (49 in total in *E. histolytica*). The different numbers of these genes and the fact that they are undergoing lineage specific expansions suggest that they are evolving rapidly which is consistent with coevolution with microbial species in the gut.

E. histolytica possesses 7 species-specific cysteine proteases and 3 species-specific peroxiredoxins. These genes have roles in invasion and protection from reactive oxygen species (ROS), respectively, abilities that are known to be key parts of *E. histolytica*'s pathogenic repertoire (Davis *et al.* 2006; Lidell *et al.* 2006; Poole *et al.* 1997). However, all of these peroxiredoxin sequences unique to *E. histolytica* are pseudogenes, as are 5 of the 7 cysteine proteases. It is possible that these expansive families are not as significant as once thought, though we consider the cysteine protease families in greater detail below.

There are many more unique genes and families in *E. invadens* than in *E. histolytica* and *E. dispar*. *Entamoeba invadens* possesses genes that encode a number of unique cysteine proteases, thioredoxin proteins, heat shock proteins and lysozymes. Much of this is likely a direct result of *E. invadens*' larger gene complement. However, unique expansions of gene families in *E. invadens* may be indicative of a broader host range, an argument strengthened by *E. dispar* – capable of colonising a range of wild primates (Rivera & Kanbara 1999; Tachibana *et al.* 2000) – possessing almost twice as many unique genes and families as *E. histolytica*.

Whilst genes unique to *E. moshkovskii* remain unannotated, given their inherent lack of orthologues, a BLAST search against the NCBI database revealed putative functions for many of them (Table 3). As in *E. histolytica*, the most prevalent family (in terms of gene numbers) in these species-specific genes is the BspA family (or genes including a leucine-rich repeat region). Kinases also form a large proportion of the gene families unique to *E. moshkovskii*. It is interesting to note that this species possesses larger unique gene clusters than the other species despite it being more closely related to the human-infective species than *E. invadens*. Notably, its 3 largest unique gene clusters contain 261, 121 and 110 genes, whilst *E. histolytica*'s largest unique gene cluster contains 13 genes, and *E. dispar*'s and *E. invadens*' contain 19 and 34 genes, respectively. This large number of hypothetical gene sequences is further evidence of large species-specific gene expansions.

Comparison of key families involved in host-parasite interactions suggest gene loss in *E. dispar* and increased diversity in *E. invadens*

In the course of trying to understand how amoebic lifecycles progress, and occasionally develop into symptomatic disease states, many genes have been identified, including numerous putative virulence factors (Lejeune *et al.* 2009; Mortimer & Chadee 2010; Wilson *et al.* 2012). Two major families described above are of particular interest due to their interactions with host cells – the cysteine proteases and Gal/GalNAc lectins. Both families are comprised of 3 sub-families and are heavily implicated in the development of infections, making them exciting targets in the search for potential treatments of amoebiasis. As such, we carried out phylogenetic analyses of these two major gene families and discuss here expansions and reductions within these families in each species in order to assess the their importance in the lifecycles of the four *Entamoeba* species.

Gal/GalNAc lectins

The Gal/GalNAc lectin heavy subunit allows *Entamoeba* species to adhere to cells by binding to Galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) on their membranes (Ravdin & Guerrant 1981). Whilst there are only two *E. dispar* genes in this family, expansions exist in both *E. histolytica* and *E. moshkovskii*, as well as an expansion in *E. invadens* containing approximately twice as many sequences (Figure S2a). The genes in the expanded *E. invadens* clade are significantly more diverse than the genes in the other two expanded species (based upon branch lengths compared with *E. histolytica*, p-value < 0.001; compared with *E. moshkovskii*, p-value = 0.045).

The intermediate and light subunits of the Gal/GalNAc lectin offer considerably fewer differences than the heavy subunit (Figure S2b and S2c). The intermediate subunit group

contains an *E. invadens* expansion only, raising the number of *E. invadens* genes above the number of genes seen in the other species (mean branch length: 2.962247; s = 1.610896). The light subunit family, meanwhile, contains two *E. invadens* expansions and a smaller expansion in both *E. dispar* and *E. histolytica*, but no expansion in *E. moshkovskii.* Again, the *E. invadens* expansions are more variable than those of *E. histolytica* (p-value = 0.002) and *E. dispar* (p-value = 0.002).

Interestingly, all of the *E. invadens* genes encoding Gal/GalNAc lectin heavy subunits have orthologues in the other 3 species. Given that *E. invadens* is capable of causing amoebic infections in a variety of reptilian hosts, one can theorise that the variable Gal/GalNAc lectin heavy subunits are a key family in allowing *E. invadens* to do so. However, regardless of target host, Gal/GalNAc lectin heavy subunit proteins share enough similarities to be considered orthologous.

Conversely, there is a relative lack of heavy subunit sequences in *E. dispar*. As was suggested in the case of the BspA family, a paucity of proteins required for adherence to host cells may explain why symptomatic disease is seen so infrequently in this species (Diamond & Clark 1993; Ximénez *et al.* 2010). A reduced complement of genes encoding proteins involved in host-parasite adherence suggests a diminished requirement for this type of protein, at least relative to the other species studied here. This could be a crucial characteristic of *E. dispar* that distinguishes it from its relatives. Furthermore, the relative lack of variability in the light and intermediate lectin subunits, when compared with the heavy subunit subfamily, suggests that the smaller subunits are less crucial to the success of amoebic infections than the heavy subunit.

Cysteine proteases

The cysteine proteases can be divided into 3 subfamilies – A, B and C. In subfamily A (Figure S2d), there are more *E. invadens* genes than there are genes from the other species, and a notably lower number of *E. dispar* sequences. The higher number of *E. invadens* genes is due to a lineage-specific expansion (mean branch length: 0.814269; s = 0.266459). A pseudogenous *E.*

dispar sequence, meanwhile, lies in a region syntenic to *E. histolytica*'s CP-A5 (Willhoeft *et al.* 1999). This gene has been shown to be important in the virulence phenotype of *E. histolytica* (Bruchhaus *et al.* 2003). There are no other *E. dispar* pseudogenes, whereas there are nine *E. histolytica* pseudogenes.

In subfamily B (Figure S2e), *E. dispar* possesses considerably fewer genes than the other three species, as it is the only species whose genes have not been subject to expansion. The *E. moshkovskii* gene expansion is significantly more diverse (p-value < 0.001) but relatively closely related to the *E. histolytica* expansion, being part of the same clade. Conversely, the expanded *E. invadens* genes are more varied than both the *E. moshkovskii* sequences (p-value < 0.001) and the *E. histolytica* sequences (p-value < 0.001) and have expanded in an independent event. As was seen in subfamily A, *E. invadens* appears to possess a larger, more variable set of cysteine proteases than the other three species. Comparatively, in subfamily C (Figure S2f), there are fewer *E. invadens* genes than there are of the other three species. This is due to a large clade consisting mostly of very similar sequences across those three species (mean branch length: 0.266321; s = 0.351707).

The relative paucity of *E. dispar* cysteine protease sequences (33 CDSs, compared with 42 in *E. histolytica*, 51 in *E. invadens*, and 46 in *E. moshkovskii*) suggests a diminished requirement for these proteins, as was the case with the Gal/GalNAc lectins, above. Taken alongside the fact that *E. dispar* is the only one of the four species to have a pseudogenised orthologue of the important CP-A5 gene (Bruchhaus *et al.* 1996; Ankri *et al.* 1999), it appears that *E. dispar* has experienced a general reduction in a family of genes which are known to be involved in host invasion as well as having generalised proteolytic abilities. This reduction is likely to be at least partly responsible for its apparently reduced impact upon host cells, which has long been recognised in the literature (Diamond & Clark 1993). Conversely, the large number of cysteine proteases in *E. moshkovskii* is consistent with studies that suggest *E. moshkovskii* is capable of

causing symptomatic infection in humans (Shimokawa *et al.* 2012; Fotedar *et al.* 2008). *E. invadens* also appears to require a variety of cysteine proteases further supporting the theory that *E. invadens* requires a greater diversity of virulence factors to allow it to effectively parasitise its wide range of hosts.

Intra-species diversity in *E. moshkovskii* relative to *E. histolytica*

We investigated genomic diversity between strains of *E. moshkovskii* and *E. histolytica*. This required non-reference strains, which were unavailable for *E. dispar* and *E. invadens,* so these species could not be compared here. Reference strains were resequenced using Illumina sequencing and reads were mapped to their respective genomes (Table S1). Existing bases at high quality homozygous positions were replaced with the newly called nucleotides to generate updated and improved reference sequences. Reads from non-reference strains were mapped to these updated reference genomes and SNPs were called within them (Table 4). Every strain except *E. histolytica* strain PVF was sequenced and mapped to a coverage depth higher than 35x, the average necessary to reliably detect 95% of SNPs in a genetic sequence (Ajay *et al.* 2011; Sims *et al.* 2014). The non-reference *E. moshkovskii* strains mapped to coverage depths equivalent to, or higher than, those achieved with the *E. histolytica* strains sequenced on the Illumina platform.

Pairwise SNP rates, including heterozygous and homozygous SNPs, were calculated across all genotype quality scores for each non-reference strain relative to its respective reference genome as a measure of divergence (Figure S3). The average divergence of all *E. moshkovskii* strains from the reference was greater than that demonstrated by *E. histolytica* strains compared with the HM-1:IMSS strain (Wilcoxon signed-rank test: p-value < 0.01), a difference apparently independent of genotype quality. Within *E. moshkovskii*, the three non-reference strains' divergence from Laredo suggested that the only human-infective non-reference strain – 15114 - is the least divergent from the similarly human-infective Laredo (compared with Snake: p-value < 0.01; compared with FIC: p-value < 0.01). The sewage-derived

strain FIC is significantly more divergent than both host-derived 15114 and Snake (compared with Snake: p-value < 0.01). Taken together, these relationships suggest lineages diverging to facilitate, or as a result of, parasitic abilities.

E. moshkovskii strains display greater divergence from their reference strain than *E. histolytica* across all sequence classes

SNP rates in a range of sequence classes were studied in more detail (Figure 3). Both homozygous and heterozygous SNPs were included in this analysis. Mann-Whitney statistical tests were used to compare the average divergence between sequence classes between the species. An alpha level of 0.05 was used for all tests. Statistically significant differences in divergence were found between the *E. histolytica* and *E. moshkovskii* strains in all sequence classes (for 4D sites and intronic regions, p-value = 0.02; for all other classes, p-value < 0.01). This confirms that the greater divergence seen in *E. moshkovskii* is ubiquitous across the genome.

Overall, diversity seen between the four strains of *E. moshkovskii* was 200 times greater than that seen between 10 strains of *E. histolytica* (Figure S4). This higher diversity was seen, to varying degrees, ubiquitously across all sequence classes, including non-coding DNA. Non-coding DNA contains a wealth of regulatory elements involved in the control of such important processes as DNA replication and gene expression (Anbar *et al.* 2005; Bracha *et al.* 2003; 2006; Mar-Aguilar *et al.* 2013; Ludwig 2002; Nelson *et al.* 2004; Wilusz *et al.* 2009), therefore these differences will likely result in important phenotypic differences.

Within *E. moshkovskii* and *E. histolytica*, occurrences of polymorphisms in coding regions were compared with those in a variety of classes of non-coding regions. There were no significant differences in divergence seen in coding regions and those values recorded for the non-coding regions in *E. moshkovskii*. Conversely, coding regions of *E. histolytica* genomes were, overall, significantly more divergent than intronic regions (t = 15.0988, d.f = 7, p-value = 1.34 e-6) and 3'

flanking regions (t = 2.5806, d.f = 7, p-value = 0.036), suggesting that polymorphisms occur at different rates in these regions of *E. histolytica*. This could not be proven convincingly in *E. moshkovskii*, possibly implying a greater importance of some non-coding sequences in *E. moshkovskii*.

As intergenic regions in *Entamoeba* genomes are very short it may be that they are densely packed with regulatory regions. Our findings contradict a previous study that focused on individual genes and associated non-coding regions in *E. histolytica* and which suggested that the latter were more divergent than coding regions due to their being under less selective pressure (Bhattacharya *et al.* 2005). However, it is likely that the difference between the two conclusions is because the analyses featured here were performed across the entire genome, as opposed to selected regions, and so are based upon more data.

The 5'- and 3'-flanking regions of a sequence typically contain promoter and enhancer regions, to which transcription factors sometimes bind (Riethoven 2010). SNPs in 5'-flanking regions are known to affect regulation and expression levels (Hayashi *et al.* 1991; Marcos-Carcavilla *et al.* 2010; Peñaloza *et al.* 2013). The effects of promoter-based SNPs on stress resistance have previously been reported, so it is conceivable that SNPs in 5'- and 3'-flanking regions could facilitate, as an example, survival outside of a human host (Sun *et al.* 2007). However, it is likely that the differences in diversity between *E. histolytica* and *E. moshkovskii* are due to greater divergence within the latter, as opposed to selective pressures acting upon particular regions of the genome such as this argument would require.

TMRCA analysis suggests a recent origin for *E. histolytica*

As stated above, divergence across strains' 4D sites was greater in *E. moshkovskii* than in *E. histolytica* (Figure 3). Such sites have long been thought to be under neutral selective pressure, given that mutations in them do not affect the amino acid that their triplet encodes (Kimura 1968;

King & Jukes 1969). As such, they provide an opportunity to evaluate the overall differences in diversity between species without the added complication of selective pressures influencing results. With this in mind, the 4D sites present in *E. histolytica* and *E. moshkovskii* that were sequenced to depths of 35x or greater in all strains of a species (339,091 and 641,223 bases, respectively) were employed to approximate, for each species, the age of the most recent ancestor shared by the tested strains to further evaluate relatedness between strains of the species. The TMRCA for *E. histolytica* is estimated to be 165,000 years, whilst the TMRCA for the *E. moshkovskii* strains is approximately 81,590,000 years. This suggests an origin of *E. histolytica* that is concurrent with the emergence of modern humans, while *E. moshkovskii* is much more ancient. Indeed, it is likely that this ancestral species, pre-dating as it does mammals, has diverged many times, with descendants co-evolving with mammalian hosts through a myriad of lineages to parasitise the wide range of hosts we see *Entamoeba* species infecting today. This theory is not without precedent, having been suggested previously concerning the infection by basal coccidians of ancestral vertebrates such as elasmobranchs (Xavier *et al*. 2018). The subsequent co-evolution and divergence of these parasites with the dawn of their higher vertebrate hosts is thought to have produced the genus *Toxoplasma* amongst others (Rosenthal *et al*., 2016). Phylogenetic analyses of both *E. histolytica* and *E. moshkovskii* demonstrated that observed variation between strains was not a result of significantly more distant reference strains (Figure 4). It should, however, be acknowledged that the assumed mutation rate is not specific to the *Entamoeba* species so the accuracy of these TMRCA values cannot be validated.

Identification of genes under diversifying selection in *E. moshkovskii* and *E. histolytica*

In order to identify genes under diversifying selective pressures within each species and thereby identify genes that are under positive selection from the host, ratios of pN to pS values (pN/pS) were calculated for each coding sequence in each strain of *E. histolytica* and *E. moshkovskii* relative to their respective reference genomes. Numerous variations on such

comparisons of synonymous and non-synonymous substitution rates have led to the identification of many coding sequences under positive selection in a wide range of species, as summarised by Yang & Bielawski (2000). The concept's history of power and reliability in such cases, and the relatively simplicity of its calculation, made it an ideal choice for detection of selection in *Entamoeba* species. Heterozygous SNPs were omitted as calculation of the impact they have upon a sequence's pN/pS ratio would have been impractical. Annotations were taken from orthologous sequences where none were available for sequences themselves.

In *E. moshkovskii*, the majority of genes identified as being under diversifying selection lacked annotations or known domains (Table S2). A relatively large number of BspA family members were found to be under diversifying selective pressures in all three strains, suggesting a species-wide function. In addition to the BspA family proteins, all three *E. moshkovskii* strains were found to possess genes with similar housekeeping functions in the form of protein kinases, DNA repair proteins and Ras family GTPases. Whilst there are numerous *Entamoeba* proteins involved in cell adherence, including the Ariel1 surface antigen seen to be under diversifying selection in *E. moshkovskii* strain 15114, the BspA family is the only adherence-related family seen to be under such pressures in all three strains. It would be of great interest to study how crucial the BspA family is in enabling adherence to host cells in *E. moshkovskii*.

pN/pS ratios indicating diversifying selective pressures acting upon genes were present in eight of the nine non-reference *E. histolytica* strains, with only HK-9 appearing to lack sequences under such pressures (Table S3). However, the numbers recorded in each strain were, compared with counts in *E. moshkovskii*, very low, with only MS96 featuring more than five such diversified genes. Of those genes identified as being under diversifying selection, one can see that the majority are unannotated, but, as in the *E. moshkovskii* strains, there appear BspA family proteins in strain MS96 as well as AIG1 family members in MS84 and MS96, and serine/threonine protein kinases in the Illumina-sequenced strains.

The comparatively low numbers of genes under diversifying selection in *E. histolytica* are likely the result of a combination of factors. Firstly, every *E. histolytica* strain excluding DS4 and MS96 were sequenced to a relatively low depth, as a result of differing sequence technologies. As such, fewer SNPs were likely to have been detected, thus profoundly affecting the calculation of pN/pS ratios. However, even taking this into account, we do see very few genes in MS96 and DS4 under diversifying selective pressures compared with strains of *E. moshkovskii*. This supports our findings that *E. histolytica* is significantly less functionally diverse than *E. moshkovskii*. Secondly, pN/pS ratios can only be accurately calculated where a sequence contains both synonymous and non-synonymous SNPs. It was likely that many genes containing only non-synonymous SNPs, which would still certainly be classed as being under diversifying selection, would have been omitted. This would, of course, have also affected the pN/pS ratios in *E. moshkovskii*.

Weak signals of meiotic recombination in *E. moshkovskii* suggest it is a species complex

The high level of genetic diversity and ancient estimate of the TMRCA indicates that *E. moshkovskii* may in fact not be a true species but a species complex, a group of genetically isolated lineages brought together under a single species name. This has been previously suggested by Clark and Diamond (1997) using riboprinting. The four-haplotype test was used to check for evidence of meiotic recombination between the four *E. moshkovskii* strains. According to the infinite sites model of evolution, individual nucleotide positions can only mutate once, meaning that the maximum possible number of haplotypes between two physically linked sites is 3, unless recombination between genomes is possible. Furthermore, recombination is more likely to occur between sites the greater the distance between them. As such, the occurrence of 4 haplotypes within a species, combined with a greater prevalence of such haplotypes over greater genomic distances act as reliable indicators of meiotic recombination. Evidence of meiotic recombination

has previously been reported in *E. histolytica*, demonstrating that it can occur in members of the genus *Entamoeba* (Weedall *et al.* 2012). A Spearman's correlation coefficient was applied to test whether, in the *E. moshkovskii* strains tested here, there was any significant correlation between the proportions of physically linked SNP pairs that exist as 4 haplotypes and the distance between members of those pairs (Figure S5). There was no significant correlation, meaning that four-haplotype SNP pairs are not more prevalent over greater distances as would be expected if there was a strong signal of meiotic recombination between these 4 strains. Four distinct haplotypes were observed in *E. moshkovskii*, although at a much lower frequency than in *E. histolytica*, and we assume these are due to ancient recombination or where the infinite sites model does not hold.

Whilst this suggests that the 4 strains of *E. moshkovskii* studied here do not belong to the same species, this result necessitates two important caveats. Firstly, conclusions drawn from these data do not necessarily extend beyond the strains featured and our results do not preclude the probable occurrence of recombination in any of the subspecies that make up the *E. moshkovskii* complex. Secondly, given that the strains compared to identify genes under selective pressures in *E. moshkovskii* have been shown to not all be of the same species, such identified genes may have been selected for in an ancestral population, rather than currently undergoing selection. Finer resolution of these cases will no doubt be provided by future investigations into the species complex.

Conclusions

Through sequencing the genomes of four strains of *E. moshkovskii*, including the generation of an annotated reference genome, we have performed a comparative analysis of *E. moshkovskii* against its relatives *E. histolytica*, *E. invadens* and *E. dispar*. The genome of *E. moshkovskii* reference strain Laredo contains 12,449 predicted coding sequences. Although many

of these are incomplete, the assembly and annotation comprise a good quality first draft of the genome. We have also undertaken a preliminary analysis of genomic diversity in *E. moshkovskii* by sequencing four isolates using short read sequencing technology. This, combined with existing genomic resources for *E. histolytica, E. dispar* and *E. invadens,* has enabled a detailed analysis of genomic diversity and gene family evolution in the different species.

Surface-bound proteins are implicated in playing a major role in the development of amoebiasis. The pathogenic *E. histolytica* possesses a large number of unique surface proteins, which contrasts starkly with the non-pathogenic *E. dispar*. This study also suggests that other surface-bound proteins might play roles similar in importance to the Gal/GalNAc lectins with regards to enabling pathogenic infections in the genus. Furthermore, *E. invadens* was found to possess a greater number of genes in the Gal/GalNAc lectin heavy subunit subfamily and the cysteine protease subfamilies A and B than the other three species studied. The genes comprising the expansions in these families were also often significantly more variable than those genes seen in the other *Entamoeba* species. It is reasonable to conclude that a proportion of the enlarged gene set seen in *E. invadens* (relative to *E. histolytica* and *E. dispar*) consists of genes required to facilitate the amoeba's polyxenous lifestyle. This argument could be extended to *E. dispar* relative to *E. histolytica*, however, the low numbers of surface proteins seen in *E. dispar* are also seen in its cysteine protease virulence factor gene families.

Overall, the genomes of the studied *E. moshkovskii* strains were found to be more diverse than those of the *E. histolytica* strains, with the former species approximately 200 times as diverse as the latter. This greater diversity was found to be the case across multiple sequence classes, demonstrating that it is not restricted to individual regions of the genome. Furthermore, *E. moshkovskii* was found to have diverged from its strains' most recent common ancestor nearly 500 times longer ago than *E. histolytica*'s strains did from theirs. It is likely, therefore, that the reason for the greater diversity within *E. moshkovskii* is that its genome has accrued mutations

over a longer period of time than that of *E. histolytica*, thus suggesting that genetic diversity is very low in *E. histolytica*.

Our data indicate that *E. moshkovskii* strains are probably not the same species. This is important for understanding its relationship to human infection. It may be that only one of these sequence types can be infective and, therefore, to understand the epidemiology of this emerging disease we need to develop better diagnostics that can differentiate between the different sequence types. Also, if there are pathogenic and non-pathogenic types of *E. moshkovskii*, they could act as a useful system for studying the emergence of pathogenicity. Our attempts to identify gene families of importance in survival of the varied lifestyles exhibited by *E. histolytica* and *E. moshkovskii* identified the BspA family as a putatively important family in members of the *E. moshkovskii* complex. Given the family's role in *E. histolytica*, and absence from the genome of *E. dispar*, it is possible that members of the *E. moshkovskii* species complex may be capable of causing disease in human hosts.

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Figure Legends

Fig 1. The range of GC contents in 100 base sections of reference genome assemblies for *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba invadens*. In total, 99.19% of the *E. histolytica* assembly was included, as was 98.49% of the *E. dispar* assembly, 88.75% of the *E. moshkovskii* assembly, and 98.47% of the *E. invadens* assembly.

Fig 2. Venn diagram showing numbers of unique and orthologous genes and families in the genomes of *Entamoeba histolytica, Entamoeba dispar, Entamoeba invadens* and *Entamoeba moshkovskii*. Numbers are based upon OrthoMCL output. Numbers in bold represent gene families; accompanying numbers in regular font represent the number of genes comprising those gene families.

Fig 3 . Divergence of *Entamoeba histolytica* and *Entamoeba moshkovskii* strains, relative to their reference strains (HM-1:IMSS and Laredo, respectively), within different sequence classes. Circles represent *E. moshkovskii* strains, and crosses represent *E. histolytica* strains. SNPs occurring in regions classified as both flanking regions and coding regions were considered to occur in coding regions only. Rates are relative to sites within their respective sequence classes.

Fig 4. Phylogenies of (a) *Entamoeba histolytica* and (b) *Entamoeba moshkovskii* strains based upon diversity in 4D synonymous sites. The trees were generated using a Neighbour-Joining method and are unrooted. Asterisks at all branching points indicate bootstrapping values of 1,000 out of 1,000. Branching points missing values were not supported by bootstrapping.

Fig S1. Frequencies of mean read depths within each scaffold/contig in the *Entamoeba moshkovskii* Laredo genome. The highest fold coverage recorded was 7730.04x.

Fig S2. Phylograms of *Entamoeba* gene families directly involved in virulence that demonstrate differential expansions and reductions across species. Red boxes highlight clades in which pseudogenes were identified. They are linked to red boxes showing the same clades when phylogeny was calculated using nucleotide sequences, including the pseudogenes. Scale bars in red boxes represent nucleotide phylograms. All phylograms are midpoint rooted. Bootstrapping was performed for 1,000 replicates. Bootstrap values of 1,000 are represented by asterisks (*). Bootstrap values below 400 are not shown. a) Heavy Gal/GalNAc lectin subunits; b) Intermediate Gal/GalNAc lectin subunits; c) Light Gal/GalNAc lectin subunits; d) Cysteine protease Family A; e) Cysteine protease Family B; f) Cysteine protease Family C.

Fig S3. Cumulative divergence of *Entamoeba histolytica* and *Entamoeba moshkovskii* strains, relative to their reference strains, as a function of genotype quality up to values of '99'. *E. histolytica* strains are denoted by solid lines and *E. moshkovskii* strains by dotted lines.

Fig S4. Probability-distributed log ratios of diversity in 2,485 *Entamoeba histolytica* and *Entamoeba moshkovskii* orthologue pairs. Ten *E. histolytica* and four *E. moshkovskii* strains were compared. Columns indicate observed counts of pairs at different diversity ratios. Red line represents normal distribution expected in the case of equal diversity of values within 3 standard deviations of the mean.

Fig S5 The proportion of 4-haplotype SNP pairs in *Entamoeba moshkovskii* as a function of the distance between the pairs. SNP pairs were physically linked (i.e. they were on the same scaffold/contig). Each point represents 1,000 randomly selected SNP pairs. The line represents the correlation between distance between pairs and proportions of 4-haplotype SNP pairs. The correlation was statistically insignificant.

Table 1. Statistics relating to the genome assemblies of *Entamoeba histolytica* HM-1:IMSS, *Entamoeba dispar* SAW760, *Entamoeba invadens* IP-1 and *Entamoeba moshkovskii* Laredo.

Statistic	*E. histolytica*	*E. dispar*	*E. invadens*	*E. moshkovskii*
Genome length (bp)	20,799,072	22,955,291	40,888,805	25,247,493
GC content (%)	24.20	23.53	29.91	26.54
Non-ACGT (%)	0.31	0.56	0.93	9.94
Number of scaffolds	1,496	3,312	1,149	1,147
N50 of scaffolds (bp)	49,118	27,840	243,235	40,197
Average scaffold size (bp)	13,903	6,931	35,586	19,190
Number of contigs	-	-	-	3,460
Average contig size (bp)	-	-	-	935
Average coverage depth	12.5x**	4.32x*	4x*	82.65x

Statistics are derived from AmoebaDB v2.0 data, except for asterisked (*) figures, taken from NCBI WGS Projects AANV02 and AANW03; and the double-asterisked (**) figure, taken from [59].

Table 2. Genomic comparison of *Entamoeba histolytica* HM-1:IMSS, *Entamoeba dispar* SAW760, *Entamoeba invadens* IP-1 and *Entamoeba moshkovskii* Laredo.

Statistic	*E. histolytica*	*E. dispar*	*E. invadens*	*E. moshkovskii*
No of CDSs	8,306	8,748	11,549	12,449
Avg Gene size (bp)	1,280	1,259	1,401	1,230
% coding DNA	50.12	46.62	38.01	59.04
Avg protein size (aa)	418	408	449	399
Avg intergenic dist (bp)	1,223	1,365	2,139	798
Proportion of multi-exon genes (%)	24.16	30.73	34.48	26.24
Avg intron size (bp)	74	81	104	89
Avg no of introns per spliced gene	1.27	1.34	1.48	1.31
Number of BUSCO orthologues	220	211	211	216

Annotation files upon which statistics are based were obtained from AmoebaDB v2.0.

Table 3. Functional annotations in genes unique to *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba invadens* or *Entamoeba moshkovskii*

Number of families with function	Number of genes within families	Family function
Entamoeba histolytica		
6	22	BspA family
4	18	Surface antigen ariel1
2	12	AIG1 family
2	12	Mucins
2	7	Cylicin-2
2	7	Cysteine protease (inc 5 pseudogenes)
1	6	Acetyltransferase
Entamoeba dispar		
1	13	AIG1 family
2	5	Heat shock protein
Entamoeba invadens		
46	214	Serine/threonine/tyrosine kinase
9	34	Ras family GTPase
2	32	Ribonuclease
8	27	Heat shock protein
1	21	Cylicin
2	21	Myosin
2	19	Glutamine/asparagine-rich protein pqn-25
5	16	Actin
1	15	Thioredoxin
1	12	Profilin
1	11	Capsular polysaccharide phosphotransferase
2	11	DNA double-strand break repair Rad50 ATPase
1	9	Embryonic protein DC-8
3	8	Serine/threonine protein phosphatase
1	8	Tropomyosin alpha-1 chain
2	7	ADP ribosylation factor
2	7	Cysteine protease
1	7	Elongation factor 1-alpha
1	7	Furin
2	6	Actophorin
1	6	Gal/GalNAc lectin light subunit
1	6	Nitrogen fixation protein nifU
1	5	Calcium-binding protein/Caltractin/Centrin-1
2	5	Chaperone Clpb
1	5	DNA repair and recombination protein rad52
1	5	GRIP domain-containing protein RUD3
2	5	Serpin (serine protease inhibitor)
1	5	Vacuolar protein sorting-associated protein
Entamoeba moshkovskii		
40	753	BspA like family
80	538	Serine/threonine/tyrosine/protein kinase
10	58	Ras family GTPase
5	53	Transposable element / transposase
4	46	Tigger transposable element-derived protein
9	36	Actin
8	26	Heat shock protein
4	17	Leukocyte elastase inhibitor
1	14	Large xylosyl- & glucuronyltransferase 2 isoform X1

2	13	GNAT family N-acetyltransferase
1	12	Enhancer binding protein-2
4	11	DNA double-strand break repair Rad50 ATPase
1	10	TonB-dependent siderophore receptor
1	9	Methionine-tRNA ligase
1	9	Tandem lipoprotein
2	8	DEAD/DEAH box helicase
2	8	Reverse transcriptase
1	8	Chaperone
3	7	Methyltransferase (various)
3	7	Cysteine proteinase
1	7	Putative AC transposase
3	6	DNA mismatch repair protein MsH2
2	6	piggyBac transposable element-derived protein
1	6	Polyphosphate:AMP phosphotransferase
1	6	Primary-amine oxidase
1	6	Surface antigen-like protein
1	6	Translation elongation factor
1	6	Type VI secretion system tip protein VgrG
1	6	Site-specific tyrosine recombinase XerC
2	5	Chaperone protein DNAK
1	5	Diaminobutyrate-2-oxoglutarate transaminase
1	5	Response regulator

Table 4. Mapping and coverage statistics for each strain studied in this project. Grey rows represent reference strains, reads from which were mapped to their existing respective reference genome. Positions at which high quality homozygous SNP calls were made in the reads were replaced in the original reference sequence. All other strains were mapped to the updated versions of their respective reference strains. Underlined sections of strain names represent the shortened versions of the names that will be used henceforth. References: a) Biller *et al*. 2009; b) Biller *et al*. 2010; c) Weedall *et al*. 2012; d) Ungar *et al*. 1985; e) Diamond and Clark 1993; f) Gilchrist *et al*. 2012; g) Ali *et al*. 2007; h) Dreyer 1961; i) Meerovitch, 1958.

Strain	Country of origin	Sequencing platform	Year of isolation	Average coverage depth (x)	No of mapped reads	Coverage of ref (%)
Entamoeba histolytica						
HM-1:IMSS-A [a,b]	Mexico	SOLiD 4	1967	43.53	13,743,197	61.03
2592100 [c]	Bangladesh	SOLiD 4	2005	41.50	13,618,188	68.83
HK-9 [d]	Korea	SOLiD 4	1951	57.41	21,217,510	71.86
PVBM08B [c]	Italy	SOLiD 4	2007	50.02	17,688,152	70.88
PVBM08F [c]	Italy	SOLiD 4	2007	29.61	8,506,016	71.88
Rahman [e]	UK	SOLiD 4	1964	49.43	19,534,522	67.78
MS27-5030 [c]	Bangladesh	SOLiD 4	2006	59.97	20,419,790	63.27
MS84-1373 [c]	Bangladesh	SOLiD 4	2006	63.01	21,499,758	69.57
MS96-3382 [f]	Bangladesh	Illumina GA II	2007	114.03	20,527,917	89.00
DS4-868 [g]	Bangladesh	Illumina GA II	2006	72.15	13,361,613	88.36
Entamoeba moshkovskii						
Laredo [h]	America	Illumina MiSeq	1956	97.61	8,833,683	89.91
FIC[i]	Canada	Illumina MiSeq	1959	162.27	19,750,749	61.58
Snake	France*	Illumina MiSeq	1948*	209.10	25,655,106	76.96
15114	Bangladesh	Illumina MiSeq	1999	265.55	35,292,777	85.24

* Sent from Institut Pasteur, Paris to Charles University, Prague in 1948. Institut Pasteur has no record of origin (personal communication with Dr Graham Clark, London School of Hygiene and Tropical Medicine).

Figure 1

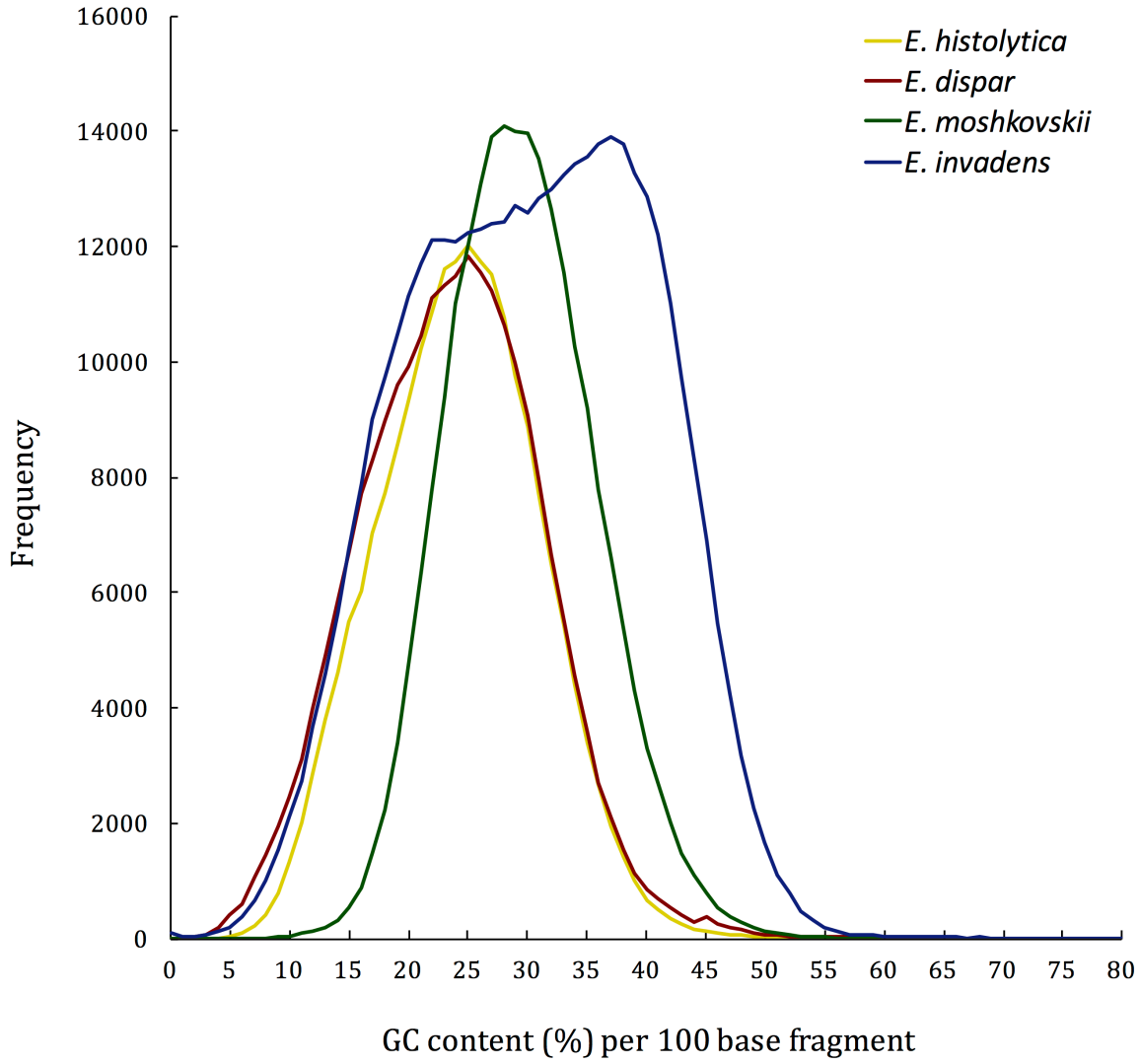


Figure 2

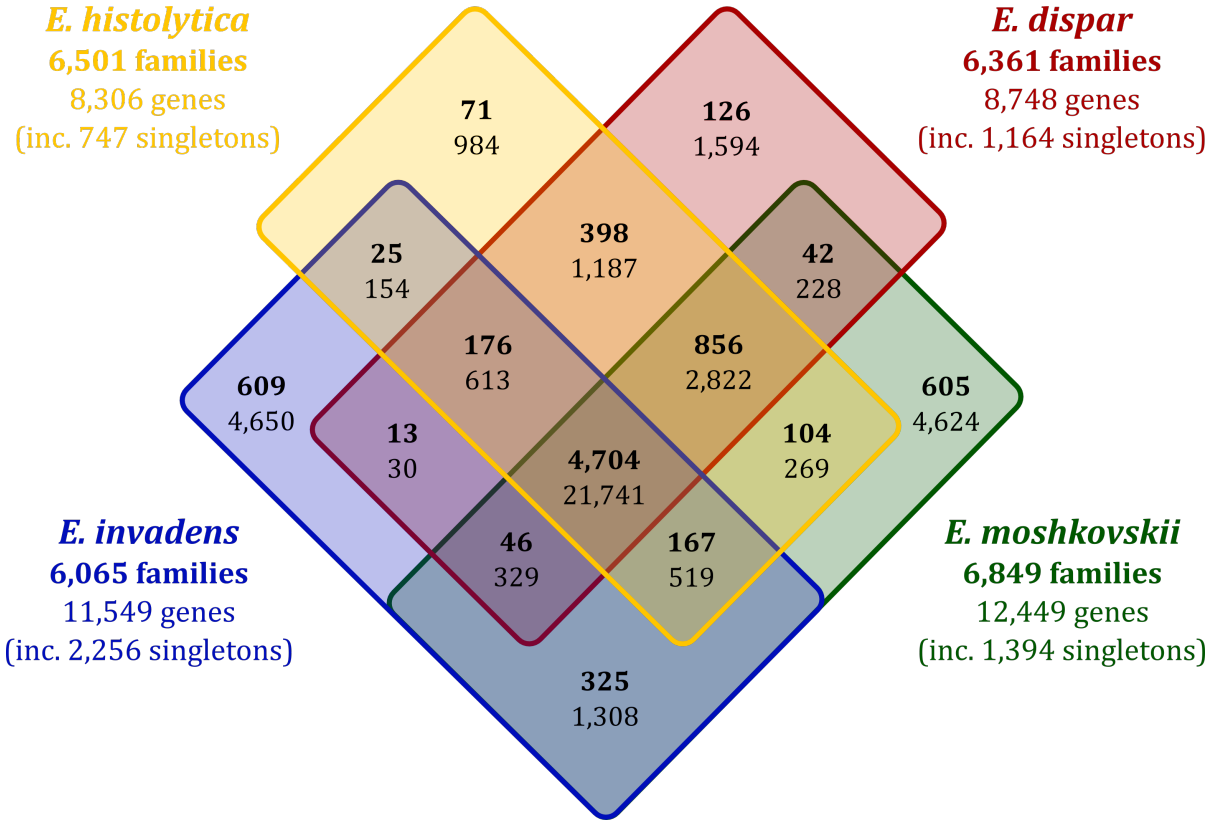


Figure 3

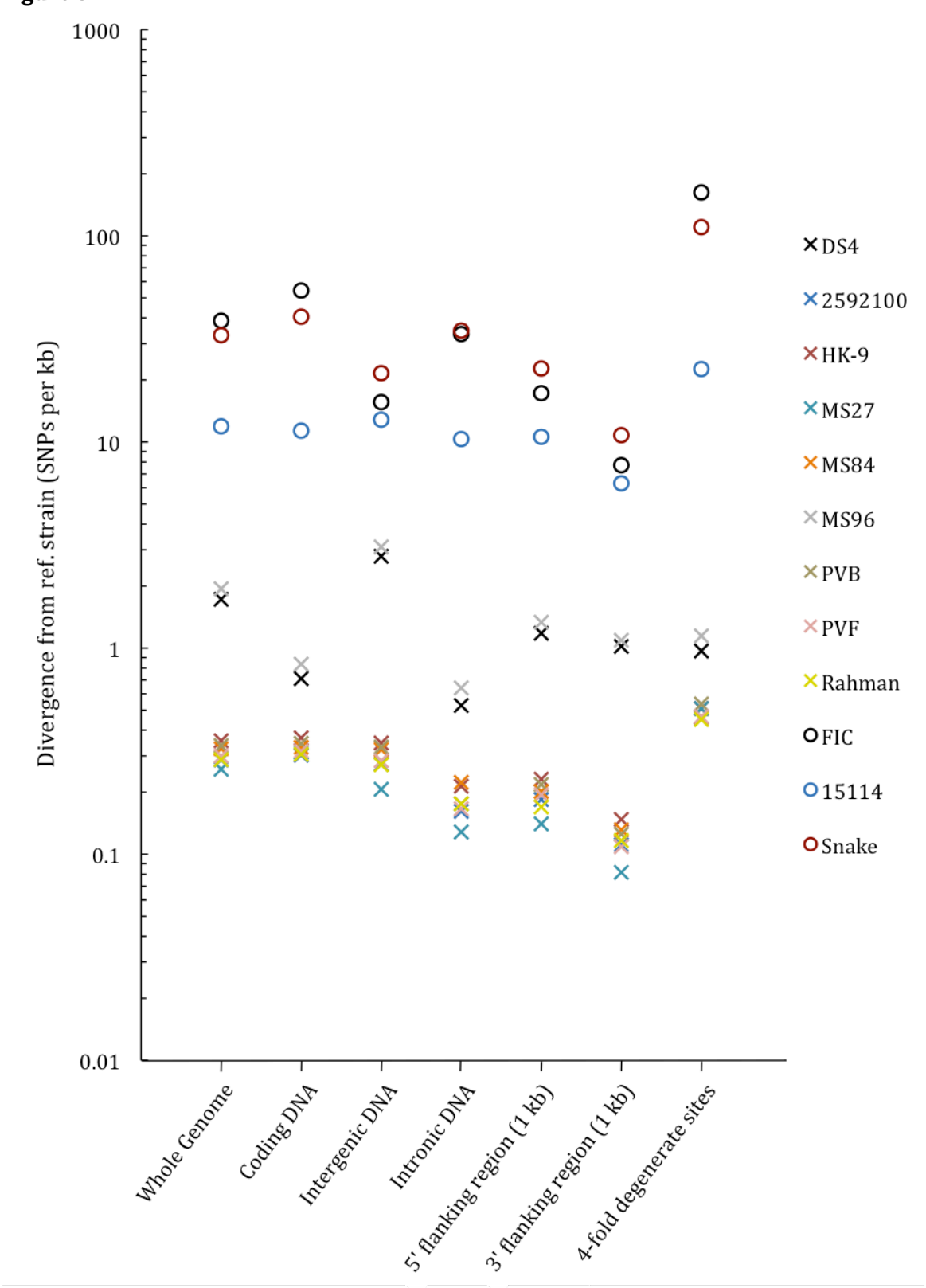
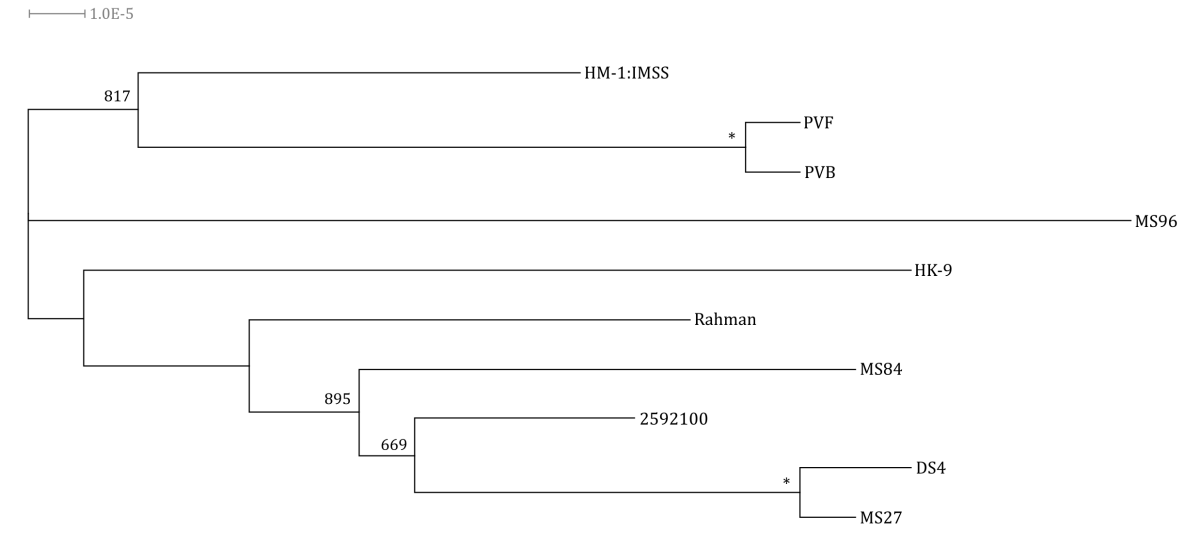


Figure 4

(a)



(b)