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1 **TITLE**

2 Last of the Human Protists: The Phylogeny and Genetic Diversity of *Iodamoeba*

3 **RUNNING HEAD**

4 Diversity and Phylogeny of *Iodamoeba*.

5 **AUTHORS**

6 C. Rune Stensvold^{1*}, Marianne Lebbad², C. Graham Clark³.

7 **CURRENT AFFILIATIONS**

8 ¹Department of Microbiological Diagnostics, Statens Serum Institut, Orestads Boulevard 5,
9 DK-2300 Copenhagen S, Denmark

10 ²Department of Diagnostics and Vaccinology, Swedish Institute for Communicable Infectious
11 Disease Control, SE-171 82 Solna, Sweden

12 ³Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical
13 Medicine, Keppel Street, London WC1E 7HT, United Kingdom

14 ***Corresponding author:** Email: RUN@ssi.dk

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26 ABSTRACT

27 *Iodamoeba* is the last genus of obligately parasitic human protist whose phylogenetic position
28 is unknown. *Iodamoeba* SSU-rDNA sequences were obtained using samples from three host
29 species and phylogenetic analyses convincingly placed *Iodamoeba* as a sister taxon to
30 *Endolimax*. This clade in turn branches among free-living amoeboflagellates of the genus
31 *Mastigamoeba*. Two *Iodamoeba* ribosomal lineages (RL1 and RL2) were detected whose
32 sequences differ by 31%, each of which is found in both human and non-human hosts.

33

34 Keywords:

35 *Iodamoeba*, protist, parasite, genetic diversity, phylogeny, evolution

36 *Iodamoeba* is a genus of intestinal parasitic protist found in humans, non-human primates and
37 other animals. The genus was described by Dobell (1919) who also gave the name
38 *Iodamoeba bütschlii* to the human parasite, and *Iodamoeba* from humans has been assigned
39 to this species ever since. The name *Iodamoeba* derives from the conspicuous iodophilic
40 glycogen mass present in *Iodamoeba* cysts (Supplementary Fig. 1A), often called a vacuole
41 although it is not membrane-bound (Zaman 1972). Cysts are noticeably irregularly shaped,
42 vary in diameter with a mean of ca. 10 μm (Dobell 1919; Taliaferro and Becker 1922), and
43 usually have a single, vesicular nucleus with a large, spherical karyosome. Although
44 mitochondrial structures were reported by Brown (1958) and Dutta (1962), ultrastructural
45 studies did not confirm their presence (Zaman 1972). The life cycle comprises a trophozoite
46 stage, found in the colon where it ingests bacteria and multiplies by binary fission (Dobell
47 1919; Rodenhuis 1919), and a cyst stage responsible for transmission. Although originally
48 placed in the family Entamoebidae together with *Entamoeba*, *Dientamoeba* and *Endolimax*
49 (Chatton 1925), to date DNA sequence data have not been available for *Iodamoeba* and
50 therefore its phylogenetic relationships remain unconfirmed. It is also not known whether
51 humans and non-humans are hosts for the same or different species. In this report we finally
52 answer most of the outstanding questions regarding this, the last genus of human parasitic
53 protist to be investigated.

54 DNA was extracted from purified *Iodamoeba* cysts (Lebbad et al. 2008; Supplementary Fig.
55 1B), directly from faeces, or from primary culture (Table 1). Complete and partial *Iodamoeba*
56 SSU-rDNA sequences were obtained directly from PCR products, or from clones thereof,
57 using a wide range of primers (Supplementary Table 1). Our results indicate a remarkable
58 degree of genetic diversity within *Iodamoeba*. The sequences obtained fall into one of two
59 ribosomal lineages (RLs) (Table 1, Fig. 1) with a genetic divergence of 31%. Even within
60 each RL a substantial degree of diversity exists (Supplementary Fig. 2).

61 No two sequences from *Iodamoeba* DNA samples investigated in the study were identical.
62 Substantial genetic diversity (8%) is seen among six clones from EM080 (Table 1;
63 Supplementary Fig. 3) and the divergence between clones EM081-6 and EM081-3.1 in a
64 1,416 bp overlapping region is 6.7% (not shown). High levels of variation in the SSU-rDNA
65 within strains is uncommon but has been reported previously in, for example, *Dientamoeba*
66 *fragilis* (Silberman et al. 1996) and *Vannella simplex* (Nassonova et al. 2010). However, in
67 this situation we cannot differentiate between two possibilities: each *Iodamoeba* cell may
68 encode several distinct SSU-rDNA variants (intra-genome variation) or most *Iodamoeba*
69 infections are mixtures of multiple strains, each of which has a single SSU-rDNA variant.
70 Whatever the underlying basis of the variation, the remarkable levels of genetic diversity
71 within single *Iodamoeba* infections has implications for the interpretation of boundaries
72 between Operational Taxonomic Units (OTUs). Caron et al. (2009) used a 95% identity level
73 as their boundary between eukaryotic microbial OTUs. Our data indicate that *Iodamoeba*
74 genes can exceed this 5% divergence value even within an individual infection.
75 *Iodamoeba* is well known from pigs and non-human primates, and other examples of natural
76 hosts include rodents, camels and birds (Wenyon 1926; Kessel 1928; Mackinnon and Dibb
77 1938; Levine 1962; Ray and Banik 1964; Sano et al. 1980; Ponce Gordo et al. 2002; Howells
78 et al. 2011). The fact that *Iodamoeba* sequence 215 from *Macaca fascicularis* is closely
79 related to human RL1 sequences (data not shown) and that RL2 is found in both human and
80 pig suggests that existing *Iodamoeba* species names linked to specific hosts may not be valid.
81 More data are needed to clarify the number and host range of RLs in *Iodamoeba*, and until
82 such data are available we suggest that the two lineages identified in the present study be
83 referred to as *Iodamoeba* RL1 and RL2 rather than allocating species names to each, a similar
84 approach to that recently suggested for novel lineages of *Entamoeba* (Stensvold et al. 2011).

85 In our phylogenetic analyses, *Iodamoeba*, *Endolimax* and all mastigamoebids always cluster
86 together to the exclusion of the remaining Amoebozoa with strong support, confirming the
87 placement of *Iodamoeba* within this group (Fig. 1). The respective lengths of the SSU-
88 rDNAs of *Iodamoeba* and *Endolimax* are comparable (2.2—2.4 kbp) and in the range of
89 typical mastigamoebid SSU-rDNAs, giving additional credence to the relationship. However,
90 support for the well established taxon Archamoebae as a whole is only moderate except in
91 Bayesian analysis.

92 The sister taxon relationship of the two genera *Iodamoeba* and *Endolimax* is highly supported
93 but, surprisingly, while monophyly of the two *Iodamoeba* sequences was supported by a high
94 bootstrap value in distance-based analyses, statistical support in Bayesian and maximum
95 likelihood analyses was absent. Manual comparison of the two *Iodamoeba* sequences with
96 the *Endolimax* sequence revealed that shared SNPs were much more frequent between the
97 two *Iodamoeba* RLs than were shared by *Endolimax* and either of the two *Iodamoeba*
98 sequences.

99 In all our analyses *Endolimax* and *Iodamoeba* share a specific common ancestor (Fig. 1) and
100 their branch emerges from within the free-living amoeboflagellate mastigamoebids rather
101 than clustering with the parasitic *Entamoeba* spp. This indicates that adaptation to parasitism
102 occurred independently at least twice in the Archamoebae, in the ancestor of *Entamoeba* and
103 in the *Iodamoeba*+*Endolimax* branch; we cannot be sure whether the common ancestor of
104 *Iodamoeba* and *Endolimax* was a parasite or not.

105 We set out to finally resolve the identity of the last genus of human parasitic protist to be
106 studied at the molecular level – *Iodamoeba*. To fully resolve the phylogenetic position and
107 taxonomic status of *Iodamoeba* and *Endolimax* based on SSU-rDNA, more data on
108 intrageneric diversity for both *Endolimax* and *Iodamoeba*, but also *Mastigamoeba*, are
109 needed. For now, we can conclude: 1) that the genus *Iodamoeba* comprises at least two

110 distinct ribosomal lineages, both of which are found in humans and also occur in non-human
111 hosts, 2) that substantial genetic variation is common in *Iodamoeba* from a single infection,
112 3) that *Iodamoeba* and *Endolimax* share a most recent common ancestor, and 4) that the
113 genera *Iodamoeba* and *Endolimax* have arisen from within the mastigamoebids.

114

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117 positive samples.

118

119 **Supplementary Material**

120 A supplementary table and figures are available at Molecular Biology and Evolution online
121 (<http://www.mbe.oxfordjournals.org/>).

122

123

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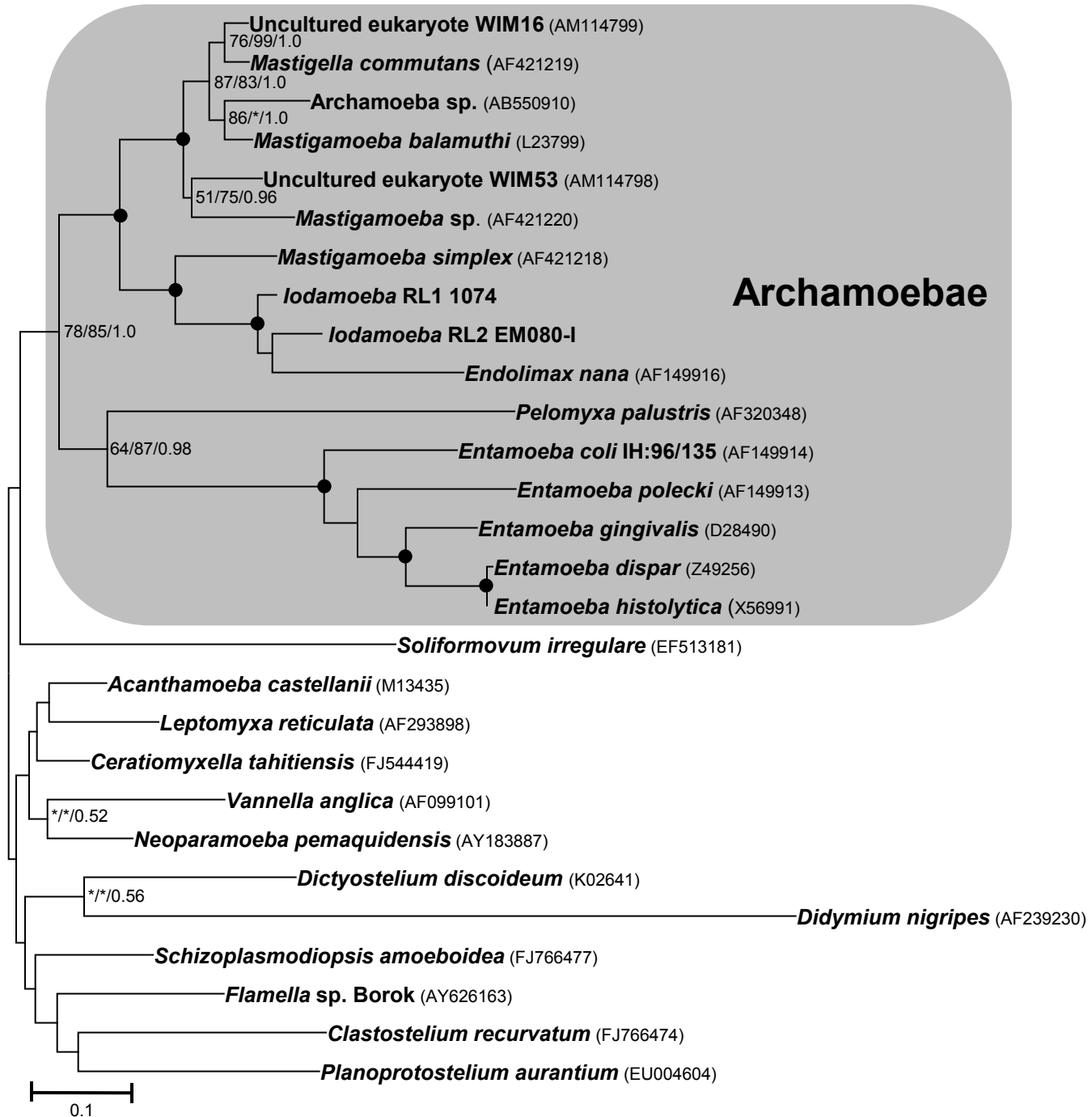
178

179 **FIGURE LEGEND**

180

181 **Fig.1.** Phylogenetic position of *Iodamoeba*. The analysis used 1,430 unambiguously aligned
182 positions from 14 archamoebae, 2 *Iodamoeba* and a broad selection of 12 non-archamoeba
183 amoebozoan sequences. Alignments were generated using MEGA 5 (Tamura et al., 2011)
184 and the inbuilt MUSCLE alignment algorithm then edited. Phylogenetic analyses used three
185 different approaches: distance-based analysis (MEGA 5) used the Neighbor-Joining
186 algorithm and the Maximum Composite Likelihood model, while Bayesian (MrBayes 3.1.2;
187 Huelsenbeck and Roquist 2001) and maximum likelihood (MEGA 5) analyses both used the
188 General Time Reversible (GTR) model of nucleotide substitution with four categories of
189 among-site rate variation and the proportion of invariant sites, selected as best using
190 ModelTest (MEGA5). Statistical support for distance and maximum likelihood trees was
191 evaluated using bootstrapping (1,000 replicates). Bayesian analysis used four Markov chain
192 Monte Carlo (MCMC) strands and 5,000,000 generations, with trees sampled every 100
193 generations. In the Bayesian analysis the final average standard deviation of split frequencies
194 was less than 0.01. A consensus tree was produced after excluding an initial burn-in of 25%
195 of the samples, as recommended. The Maximum Likelihood tree is shown. Bootstrap values
196 and posterior probabilities from the three types of phylogenetic analyses are shown in the
197 following order: Maximum Likelihood/Distance/Bayesian. Nodes where both bootstrap
198 values are >95% and the posterior probability is >0.95 are indicated by black circles.
199 Bootstrap values of <50 or posterior probabilities of < 0.50 are indicated by an asterisk and
200 where all three analyses show these low support values the node is not labelled.

Fig. 1.



201 Table 1. Samples used and sequences produced for phylogenetic analyses.

Sample ID	Source of DNA	Host	Geographical info (Travel/Origin)	Sequence		GenBank Accession No. ^c	Sequence	<i>Iodamoeba</i>
				ID	Length (bp)		Products /Clone	ribosomal lineage (RL)
EM081	Cysts	<i>Homo sapiens</i>	Thailand	EM081-6	1,752	JN635745	Clone	1
				EM081-3.1	1,961	JN635746	Clone	1
1074	Faeces	<i>Homo sapiens</i>	NA ^b	1074	2,376	JN635741	PCR	1
82	Faeces	<i>Homo sapiens</i>	NA	82	2,193	JN635742	PCR	1
28	Faeces	<i>Homo sapiens</i>	NA	28	509	JN635743	PCR	1
215	Faeces	<i>M. fascicularis</i>	NA	215	150	NA	PCR	1
EM080	Cysts	<i>Homo sapiens</i>	Cuba	EM080-I	2,215	JN635740	Clone	2
				EM080-A-H	252-257	JN635747-51	Clone	2
Mabel	Culture ^a	<i>Sus scrofa</i>	UK	Mabel	1,190	JN635744	Clone	2

202 ^aClark et al. 2006.

203 ^bNA: Not available

204 ^cGenBank accession number for sequence 215 was not available since the length of the sequence was < 200 bp.

Supplementary Table 1. Primers used in the study for PCR amplification and sequencing.

Primer name ^a	Primer sequence (5'--3')	Primer specificity ^b	Primer position ^c	DNA sample sequenced
RD5	ATCTGGTTGATCCTGCCAGT	E	1-20	EM080-I, EM081-3.1, 1074, 82
RD3	ATCCTTCCGCAGGTTACCTAC	E	2,194-2,215	EM080-I, EM081-6, 1074
AEMH5.2 ^d	TCTAAGGAAGGCAGCAGGC	E	581-599	EM081-6, EM081-3.1, MABEL
AEMH3.1 ^d	AAGGGCATCACGGACCTGTT	E	1835-1854	EM081-3.1, MABEL
528F	GCGGTAATTCCAGCTC	E	745-760	EM080-I, 1074
528R	GAGCTGGAATTACCGC	E	745-760	EM080-I
1200F	CAGGTCTGTGATGCCC	E	1837-1852	EM080-I
IODAGENUS1580F	ATCGAGTGAGTGTATGGGCTTC	G	1468-1489	1074 EM080-A, EM080-B, EM080-C,
IODAGENUS_F	GGGGTGGTTTATATTTTCATAGCG	G	1199-1222	EM080-E, EM080-H, 215
IODAGENUS_R	TCTCTCTAGGTGCTGGAGGAGTC	G	1443-1465	EM080-I, EM081-6, EM081-3.1
IODAGENUS2300R	CCGAAGCCCATACTCATTC	G	1471-1491	1074, 28
IODAGENUS650F	GTAGTGACGACAAATACCGATG	G	629-650	EM080-I, 82, 28

IODAGENUS780R	CCGCAACAGCTTTAGTATACTC	G	764-787	EM080-I, 82
IODAMOEB100F	AAGGATAACCCTGTTAATTGTAGAG	G	141-165	EM080-I, 82
IODAMOEB2080R	CCCCAGCTTGATGAACATTAC	G	1930-1950	EM080-I EM080-A, EM080-B, EM080-C, EM080-E, EM080-H, EM080-I,
IODAMOEB1610R	CAGCCTTGCGACCATACTC	G	1468-1486	1074
IODAGENUS1230F	AATTGGGGTGGTTTATATTTTCATAGC	G	1172-1197	1074, 82, 215
IODAGENUS2220R	CAAATCCAACATTTTCACCG	G	2074-2093	82
IODAM1200R	ATGCACTACCCACAGCACAC	O	1161-1182	EM081-3.1
IODAM1450R	TACACCCTGTGTTACCAGTGTG	O	911-932	1074
IODAM1400R	GTCTGCAGCGATTGTTTCTATTC	O	1463-1485	1074, 82
IODAM1500R	CAAAACATCACATAAATGTTCTGCC	O	873-897	1074, EM081-3.1
IODAM520R	CACACACAAGTGCGCACTG	O	1149-1166	EM081-6, 1074
IODAM450F	GAAGATATGTCTCGTGGGTGC	O	1066-1086	EM081-6
IODclone3.1_1200F	TGAGCGTCACAACAGTGGC	O	1244-1262	EM081-3.1
IODAM580R	CGTGGTCAATATGCATAGTTTATTATAGAC	O	1208-1237	EM081-6, EM081-3.1, 1074
IODAM600F	CACAACCAGTGCTTAGGAATAGAC	O	1250-1273	EM081-6, 1074

IODAM500F	GTGTCTAGTTGCAGTGCGC	O	1138-1156	EM081-6, 1074
T7	TAATACGACTCACTATAGGG	GSP	NA	EM081-6, EM081-3.1
SP6	ATTAGGTGACACTATAG	GSP	NA	EM081-6, EM081-3.1

^a Primer name does not necessarily reflect position of primer

^b E = Broad-specificity, eukaryotic primer; G = *Iodamoeba* genus-specific primer (with a maximum of 2-3 mismatches in primer and/or conserved 3'-end; O = lineage-/strain-specific (RL1) ; GSP = General sequencing primer (cloning).

^c Primer position relative to EM080-I (non-bold) and 1074 (bold); NA = Not applicable.

^d part of AEMH5/3 pool (Clark et al., 2006).

211 **Supplementary figures**

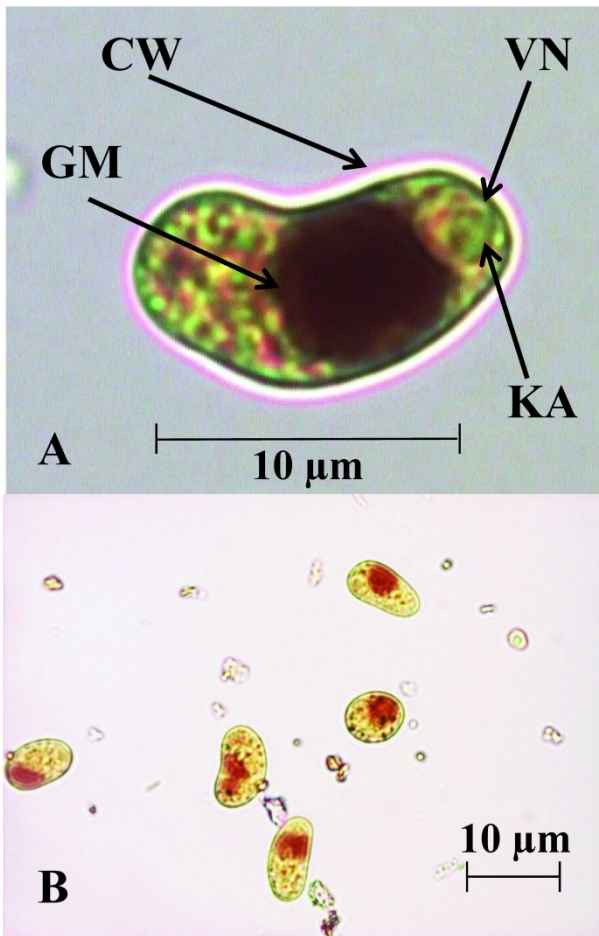
212 **Supp. Fig. 1.** *Iodamoeba* cysts observed by light microscopy of an iodine stained
213 preparation. **A:** Single cyst showing morphological features. Size is indicated. CW = Cyst
214 wall, VN = Vesicular nucleus, KA = Karyosome, GM = Glycogen mass. **B:** Cyst preparation
215 of *Iodamoeba* EM080 showing the absence of other protist cysts.

216 **Supp. Fig 2.** Genetic diversity of *Iodamoeba*. *Iodamoeba* inter-sample phylogeny showing
217 two ribosomal lineages and substantial intra-lineage diversity. A total of 383 unambiguously
218 aligned positions in the region common to all sequences were used in the analysis. Maximum
219 likelihood tree produced as in Fig. 1 is shown.

220 **Supp. Fig. 3.** Alignment of EM080 SSU-rDNA clones showing intra-sample genetic
221 diversity. The sequence shown corresponds to positions 1,192—1,447 in EM081-I. * =
222 identical base in all clones. The diversity detected consisted in two instances of differences in
223 homopolymer length: at position 103 EM080-E has a homopolymer of four Gs, whereas the
224 other clones have five Gs. At position 219, three clones have a homopolymer of three Ts
225 while the others have two Ts. Also, a short region starting at position 182 exhibited single
226 nucleotide polymorphisms (SNPs) and insertions/deletions (indels); in the same region
227 EM080-C is clearly divergent from the other clones although it still belongs to the same
228 ribosomal lineage (RL2).

229

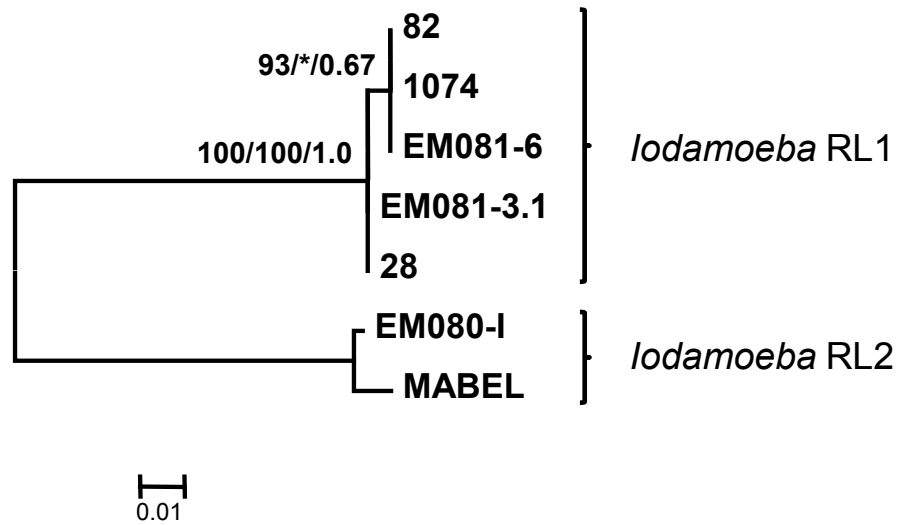
230 Supplementary Fig. 1.



231

232

Supplementary Fig. 2



264 Supplementary Fig. 3.

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1         10        20        30        40        50        60
+-----+-----+-----+-----+-----+-----+
EM080-A  TATTTTCATAGCGAGGGGTAAAATCCTGTGACCTGTGAAAGATAGACAAGAGCGAAAGCATTCCAC
EM080-B  TATTTTCATAGCGAGGGGTAAAATCCTGTGACCTGTGAAAGATAGACAAGAGCGAAAGCATTCCAC
EM080-H  TATTTTCATAGCGAGGGGTAAAATCCTGTGACCTGTGAAAGATAGACAAGAGCGAAAGCATTCCAC
EM080-I  TATTTTCATAGCGAGGGGTAAAATCCTGTGACCTGTGAAAGATAGACAAGAGCGAAAGCATTCCAC
EM080-E  TATTTTCATAGCGAGGGGTAAAATCCTGTGACCTGTGAAAGATAGACAAGAGCGAAAGCATTCCAC
EM080-C  TATTTTCATAGCGAGGGGTAAAATCCTGTGACCTGTGAAAGATAGACAAGAGCGAAAGCATTCCAC
*****

70        80        90        100       110       120       130
-----+-----+-----+-----+-----+-----+
EM080-A  AAAAATGTTTTTCATGTGATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTCGTAG
EM080-B  AAAAATGTTTTTCATGTGATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTCGTAG
EM080-H  AAAAATGTTTTTCATGTGATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTCGTAG
EM080-I  AAAAATGTTTTTCATGTGATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTCGTAG
EM080-E  AAAAATGTTTTTCATGTGATCAAGAACGAAAGTTGGGG-ATCGAAGACGATCAGATACCGTCGTAG
EM080-C  AAAAATGTTTTTCATGTGATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTCGTAG
*****

140       150       160       170       180       190
-----+-----+-----+-----+-----+-----+
EM080-A  TCTCAACTATAAACTATGCCGACCAGGGATTGGAAAAGAAAAGCACGGTTTATCCGTGTTTATT
EM080-B  TCTCAACTATAAACTATGCCGACCAGGGATTGGAAAAGAAAAGCACGGTTTATCCGTGTTTATT
EM080-H  TCTCAACTATAAACTATGCCGACCAGGGATTGGAAAAGAAAAGCACGG-TTAGCCGTGTTTATT
EM080-I  TCTCAACTATAAACTATGCCGACCAGGGATTGGAAAAGAAAAGCACGG-TTATCCGTGTTTATT
EM080-E  TCTCAACTATAAACTATGCCGACCAGGGATTGGAAAAGAAAAGCACGGTTAACCCGTGTTTATT
EM080-C  TCTCAACTATAAACTATGCCGACCAGGGATTGGAAATGAATTCGCAC---TTAT--GTGTGATTA
*****

200       210       220       230       240       250
-----+-----+-----+-----+-----+-----+
EM080-A  TCGAATTATTTAAAACGACAATTTGGCGTTTTAAAATAGACTTCTCCAGCACCTAAGAGAGA
EM080-B  TCGAATTATTTAAAACGACAATT-GGCGTTTTAAAATAGACTTCTCCAGCACCTAAGAGAGA
EM080-H  TCGAATTATTTAAAACGACTATTTGGCGTTTTAAAATAGACTTCTCCAGCACCTAAGAGAGA
EM080-I  TCGAATTATTTAAAACGACAATTTGGCGTTTTAAAATAGACTTCTCCAGCACCTAAGAGAGA
EM080-E  TCGAATTATTTAAAACGACGATT-GGCGTTTTAAAATAGACTACTCCAGCACCTAAGAGAGA
EM080-C  TCGAATTATTTAAAACGACTATTTGGCGTTTTAAAATAGACTTCTCCAGCACCTAAGAGAGA
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