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Authors: Michelle Sait, Ewan M. Clark, Nicholas Wheelhouse, Lucy Spalding, Morag Livingstone, Konrad Sachse, Bryan K. Markey, Simone Magnino, Victoria I. Siarkou, Evangelia Vretou, María R. Caro, Raja Yaga, F. Alex Lainson, David G.E. Smith, Frank Wright, David Longbottom



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1 Genetic variability of *Chlamydophila abortus* strains assessed by PCR-RFLP analysis of
2 polymorphic membrane protein-encoding genes

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4 Michelle Sait^{a,1*}, Ewan M. Clark^{a,1,2}, Nicholas Wheelhouse^a, Lucy Spalding^a, Morag
5 Livingstone^a, Konrad Sachse^b, Bryan K. Markey^c, Simone Magnino^d, Victoria I. Siarkou^e,
6 Evangelia Vretou^f, María R. Caro^g, Raja Yaga^a, F. Alex Lainson^a, David G.E. Smith^{a,h}, Frank
7 Wrightⁱ, David Longbottom^a

8
9 ^a Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh, Midlothian, EH26
10 OPZ, UK

11 ^b Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Naumburger Str.
12 96a, 07743 Jena, Germany

13 ^c School of Agriculture, Food Science and Veterinary Medicine, University College Dublin,
14 Belfield, Dublin 4, Ireland

15 ^d Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno
16 Ubertini", National Reference Laboratory for Animal Chlamydioses, Sezione Diagnostica di
17 Pavia, Strada Campeggi 61, 27100 Pavia, Italy

18 ^e Laboratory of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Aristotle
19 University of Thessaloniki, University Campus, GR-54124, Thessaloniki, Greece

20 ^f Laboratory of Biotechnology, Hellenic Pasteur Institute, Vassilissis Sofias Avenue 127, 11521
21 Athens, Greece

22 ^g Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Murcia, Campus de
23 Espinardo, 30100 Murcia, Spain

24 ^h Institute of Infection, Immunity and Inflammation. College of Medical, Veterinary and Life
25 Sciences, University of Glasgow Garscube Estate, Bearsden Road, Glasgow G61 1QH, UK

26 ⁱ Biomathematics & Statistics Scotland, Invergowrie, Dundee, DD2 5DA, UK

27

28

29 *Corresponding author: Tel.: +44 131 445 5111; fax +44 131 445 6235.

30 *E-mail address:* michelle.sait@moredun.ac.uk

31

32 ¹These authors contributed equally to this work.

33 ² Present address: BigDNA, Wallace Building, Roslin BioCentre, Roslin, Midlothian, EH25 9PP,
34 UK.

35

36 **Keywords:** *Chlamydomonas reinhardtii*, restriction fragment length polymorphism, polymorphic
37 outer membrane protein.

38

39 **Abstract**

40 This study used PCR-RFLP to investigate the genetic variability of *pmp*-encoding genes
41 from fifty-two *Chlamydomphila abortus* (*C. abortus*) strains originating from abortion cases from
42 various geographical regions and host species. Six primer pairs were used to PCR-amplify DNA
43 fragments encoding eighteen *pmps*. PCR products were digested using four restriction
44 endonucleases and Bayesian methodologies were used to compare RFLP profiles and assign
45 strains to a RFLP genotype. Strains could be assigned to 2 genotypes in the region encoding
46 *pmp18D*, 3 genotypes in the regions encoding *pmp1A-pmp2B*, *pmp3E-pmp6H* and *pmp11G-*
47 *pmp15G*, 4 genotypes in the region encoding *pmp7G-pmp10G* and 5 genotypes in the region
48 encoding *pmp16G-pmp17G*. In all regions, the majority of strains (88.4%– 96.1%) had the same
49 genotype as the reference strain S26/3. No correlation could be made between genotype, host
50 species or geographical origin except for the two variant Greek strains, LLG and POS, which
51 formed a discrete genotype in all *pmp*-encoding regions except *pmp18D*. Relative rates of
52 evolution calculated for each *pmp*-encoding gene locus suggest that differing selective pressures
53 and functional constraints may exist on *C. abortus* polymorphic membrane proteins. These
54 findings suggest that although intraspecies heterogeneity of *pmp*-encoding genes in *C. abortus* is
55 low, the sequence heterogeneity should be an important consideration when using *pmps* as the
56 basis for novel diagnostics or vaccine development.

57

58

59 1. Introduction

60 Ovine Enzootic Abortion (OEA) is an economically important disease accounting for
61 approximately 43% of diagnosed abortions in sheep in the UK. The aetiological agent,
62 *Chlamydophila abortus* (formerly *Chlamydia psittaci* serotype 1), can also cause abortion in
63 cattle and pigs and poses a zoonotic risk to pregnant women (Longbottom and Coulter, 2003).

64 The genetic heterogeneity of *C. abortus* has been reported to be low, with methods based
65 on the cross-reactivity of monoclonal antibodies, Restriction Fragment Length Polymorphism
66 (RFLP) and the phylogenetic analysis of 16S rRNA genes, failing to differentiate strains based
67 on host, pathotype or geographical origin (Denamur et al., 1991; Everett et al., 1999; Salinas et
68 al., 1995). More recently, methods such as Multiple Loci VNTR Analysis (MLVA) and Multi
69 Locus Sequence Typing (MLST), have allowed the differentiation of *C. abortus* strains into
70 novel genotypes (Laroucau et al., 2009; Pannekoek et al., 2010) that were partly related to
71 geographical origin (Laroucau et al., 2009).

72 Genes encoding polymorphic membrane proteins (*pmps*) have been identified in all
73 members of the family *Chlamydiaceae*. In *C. abortus* there are 18 *pmp*-encoding genes that are
74 arranged as four loci composed of a single gene, two gene pairs and a large cluster of 13 genes
75 (Thomson et al., 2005). Polymorphic membrane proteins have been implicated in chlamydial
76 adhesion (Wehrl et al., 2004; Crane et al., 2006; Mülleken et al 2010), tissue tropism (Stothard et
77 al., 2003; Gomes et al., 2006) and the induction of immune responses (Mygind et al., 2004;
78 Wehrl et al., 2004; Livingstone et al., 2005) making them potential diagnostic and vaccine
79 targets (Longbottom and Livingstone, 2006; Sachse et al., 2008). The purpose of this study was to
80 analyse the genetic diversity of *pmp*-encoding loci from *C. abortus* isolates using PCR-RFLP
81 and determine whether strains could be distinguished by geographical origin or host species.

82

83 **2. Materials & Methods**84 *2.1 Propagation of C. abortus and gDNA preparation*

85 Fifty-two *C. abortus* strains isolated from various host species and geographical regions
86 were used in this study (Table 1). Strains were propagated as previously described (Graham et
87 al., 1995) and genomic DNA extracted using a Wizard Genomic DNA isolation kit (Promega).

88

89 *2.2 PCR amplification of pmp-encoding loci*

90 Oligonucleotide primers targeting the 18 *pmp*-encoding genes of *C. abortus* S26/3
91 [NC_004552] were designed using Lasergene 8 PrimerSelect software (DNASTAR). Three *pmp*-
92 encoding loci encoding one or two genes in tandem were PCR-amplified as single fragments and
93 are referred to in this study as BA (*pmp1B-pmp2A*), 16-17 (*pmp16G-pmp17G*) and 18D
94 (*pmp18D*). The locus encoding 13 pmps was PCR-amplified as three fragments which are
95 referred to in this study as EH (*pmp3E-pmp6H*), HG (*pmp6H-pmp10G*) and G (*pmp11G-*
96 *pmp15G*).

97 PCR amplification mixes comprised 1 × Expand PCR buffer 3 (Roche Applied Sciences),
98 500µM dNTPs, 300 nm each primer (see Table 2), 0.075 U Expand Long Template Polymerase
99 (Roche Applied Sciences), and 20-100 ng *C. abortus* gDNA made up to a 25 µl final volume
100 using nuclease free water. PCR consisted of 1 cycle at 94°C for 2 min followed by 10 cycles of
101 94°C for 10 s, 51.9 °C for 30 s and 68°C for 15 min. A second amplification stage consisted of
102 20 cycles of 94°C for 10 s, 51.9°C for 30 s and 68°C for 15 min with a 20 s increment per cycle
103 followed by one cycle of 68°C for 7 min. Efficiency of amplification was assessed by agarose
104 gel electrophoresis using 0.8% TAE-agarose gels containing 1 × GelRed (Biotium Inc).

105

106 *2.3 Restriction Fragment Length Polymorphism (RFLP)*

107 Restriction endonuclease (RE) digestions were performed using *Mbo*II (New England
108 Biolabs), *Hae*III (New England Biolabs), *Rsa*I (Roche Applied Sciences) and *Alu*I (Roche
109 Applied Sciences). Restriction endonuclease digestion reactions consisted of 1 × buffer, 5U of
110 the appropriate RE and 5µl of PCR-amplified DNA. Restriction endonuclease digests were
111 incubated at 37°C for 2 h. Five microlitres of each RE digest were electrophoresed on a 1.5%
112 TAE-agarose gel containing 1× GelRed for 180 min or until the dye front had migrated to at least
113 15 cm from the sample wells. Gels were visualised under UV light and imaged using an
114 AlphaImager 2200 (Alpha Innotech).

115

116 *2.4 Data analysis*

117 Restriction fragment length polymorphism gel images were imported into Bionumerics
118 software (version 5.10, Applied Maths) and bands detected using default settings. The migration
119 of each restriction fragment (RF) was expressed as a proportion of the total distance run on the
120 gel, and strains were scored for the presence or absence of each RF generated with each of the
121 four REs. RF positions were normalized to account for inter-gel variation using the RFLP
122 profiles generated by the reference strain S26/3. The variation seen in technical replicates of
123 S26/3 was also used as a general estimate of variation among technical replicates and guided the
124 allocation of fragments to bins. The data were checked for errors and also evidence of horizontal
125 gene transfer (HGT) using the NeighborNet method (Bryant and Moulton, 2004) available in the
126 SplitsTree package (Huson and Bryant, 2006). Seventy of the 542 RFs that could not be
127 unambiguously assigned were removed from the analysis. This resulted in a 0-1 data matrix of

128 52 unique strains by 472 RFs. The number of RFs for each of the six regions was 71 (16-17
129 region), 51 RFs (BA), 54 RFs (18D), 105 RFs (EH), 93 RFs (G), and 98 RFs (HG). As there was
130 no evidence of HGT in the NeighborNet diagram, the application of a phylogenetic tree model
131 was judged to be valid. Bayesian phylogenetic methods have become increasingly important for
132 the analysis of molecular sequence data but can also be applied to RFLP data using the MrBayes
133 package (Huelsenbeck and Ronquist, 2001). The evolutionary relationships among strains at
134 individual *pmp*-encoding loci were therefore analysed in MrBayes using the restriction datatype
135 model. Relative evolutionary rates of the six regions were modelled using a gamma distribution.
136 The Bayesian analysis produced an estimate of an overall tree topology based on all six regions
137 plus a relative evolutionary rate for each region. The settings for the Markov chain Monte Carlo
138 runs were as follows: two simultaneous runs of 625,000 generations with a burn-in period of
139 125,000 generations with trees sampled every 100 generations. The analyses were assessed for
140 convergence of the two runs using the MrBayes potential scale reduction factor (PSRF) values
141 and the standard deviation of split frequencies between simultaneous runs. After an initial run,
142 the value of the rate parameter specified for the exponential prior on branch lengths was altered
143 to be 100 rather than the default value of 10 due to the observation of very high PSRF values for
144 the relative rate parameters for each region. This setting reflects a prior belief that branch lengths
145 are short (the rate parameter is the inverse of its mean). All analyses were then carried out with
146 this altered branch length prior distribution. The PSRF values of 93% (211 out of the 226) of the
147 taxon bipartition parameters were less than 1.20 (the maximum value was 2.04 and 14 other
148 values were between 1.21 and 1.58). In addition, all 25 model parameters (namely, total tree
149 length, the six pairs of parameters for the presence and absence of RFs on each of the six regions,
150 the alpha shape parameter for each of the six regions and the relative rates for each of the six

151 regions) had PSRF values of less than 1.14. These PSRF values suggest good convergence of the
152 two runs (i.e. less than the PSRF threshold of 1.2 as suggested by Gelman et al., 1995). As noted
153 in the MrBayes output, the MrBayes PSRF values should be treated as a rough guide to
154 convergence as not all assumptions are met for a PSRF convergence diagnostic in the
155 phylogenetic context. Another convergence diagnostic, the standard deviation of split
156 frequencies between simultaneous runs, was close to zero (<0.08), confirming convergence. Six
157 additional Bayesian analyses were carried out for each of the six regions individually to produce
158 phylogenetic trees for each region, with similar convergence diagnostics. The statistical support
159 for groups in a Bayesian phylogenetic tree is expressed as a posterior probability ranging from
160 0.0 to 1.0, rather than the zero to 100 scale used in other phylogenetic approaches. RFLP
161 groupings were assigned based on posterior support being present for each grouping.
162 Phylogenetic tree diagrams were produced using the Dendroscope program (Huson et al., 2007).

163

164 **3. Results and discussion**

165 The genetic diversity of *pmp*-encoding genes from fifty two *C. abortus* strains were
166 investigated by PCR-RFLP. Six primer pairs were used to PCR-amplify approximately 55kb
167 spanning the 18 *pmp*-encoding genes and corresponding intergenic regions. PCR products of the
168 expected size were obtained from 48 unique strains using primer set BA and 39, 43, 32, 47 and
169 52 unique strains using primer sets EH, HG, G, 16-17 and 18D respectively. PCR amplification
170 was more variable when primer pairs generating larger PCR fragments (>10 kb) were used,
171 probably due to the shearing of genomic DNA during DNA extraction. Where PCR amplification
172 was poor or absent, the presence of *pmp*-encoding loci was confirmed by PCR amplification of
173 the region as smaller PCR fragments (data not shown).

174 Restriction fragment length polymorphism was performed by digesting PCR amplicons
175 with the four REs *HaeIII*, *AluI*, *RsaI* and *MboII* and resolving RFs within the size range of 50 bp
176 to 10 kb on an agarose gel. RFLP patterns for individual strains were scored based on the
177 presence or absence of each RF and phylogenetic trees were generated to graphically represent
178 strain similarity (Figure 1, Supplementary figures 1-6). Strains were assigned to RFLP groups
179 based on the posterior probability assigned to each clade (Table 3). The number of genotypes
180 assigned at each *pmp*-encoding locus varied from two genotypes observed at the 18D locus to 5
181 genotypes observed at the *pmp16-17* locus. At each of the six loci, the majority of strains
182 belonged to a single genotype, typified by the reference strain S26/3. Based on the assumption
183 that different RFLP patterns indicate that nucleotide changes have occurred in the gene locus,
184 either at the RE recognition site or by nucleotide insertions or deletions in the intervening gene
185 regions, the majority of strains belonging to a single genotype similar to S26/3 suggests a high
186 degree of conservation both of the DNA sequence and the arrangement of the *pmp*-encoding
187 genes.

188 RFLP profiles of the 18D locus were identical in all strains except AB7 and FAG which
189 formed a discrete cluster with a posterior probability of 0.98 based on a single unique restriction
190 fragment which was observed when 18D PCR products were digested with the RE *RsaI* (data not
191 shown). The relative rate of evolution, calculated by modelling RFLP data using a gamma
192 distribution while constraining each region to estimate the same tree topology was 0.135,
193 suggesting that this gene locus was evolving more slowly compared to other *pmp*-encoding gene
194 loci (Table 4). Pmp 18D exhibits high levels of DNA and amino acid sequence conservation
195 across the *Chlamydiaceae*. Putative roles based on the timing of expression *in vitro* and the
196 inhibition of *in vitro* infection using neutralizing antibodies against Pmp18D suggest a role in the

197 adhesion of *C. pneumoniae* and *C. trachomatis* to host cells (Crane et al., 2006; Wehrl et al.,
198 2004; Mölleken et al., 2010). However the expression of the gene early in the replication cycle
199 suggests that this protein may have an additional function during the late replicative/early
200 differentiation phase (Wheelhouse et al., 2009).

201 Other *pmp*-encoding loci showed a greater degree of gene heterogeneity with discrete
202 genotypes observed in each region. Strains S404/5, S507 and S82/3 (posterior probability = 0.70)
203 formed a discrete grouping in region EH (Table 3). Strains Zar-2B and AO-4 (posterior
204 probability = 0.72) and strains AB7 and C94/2 (posterior probability = 0.56) formed a discrete
205 grouping in region HG (Table 3). In *C. trachomatis* PmpH has been implicated in the
206 mechanisms of pathogenesis due to the observation that *pmpH* gene sequences diverged into 3
207 clades that were consistent with the three different disease pathotypes, urogenital, ocular and
208 lymphogranuloma venereum (Stothard et al., 2003; Gomes et al., 2006). It is still unknown if the
209 grouping in the region HG in *C. abortus* has a similar pathophysiological basis.

210 Strains AO-4 and Zar-5 formed a discrete grouping at regions G (posterior probability =
211 0.77) (Table 3) and strains C94/1 and C94/2 (posterior probability = 0.83), strains C1/98, C17/98
212 and C9/98 (posterior probability = 0.55) and strains 15 and 532 (posterior probability = 0.51)
213 formed a discrete grouping in region 16-17 (Figure 1, Table 3). The relative rate of evolution in
214 *pmpG* and *pmp16-17* regions was predicted to be 1.488 and 1.468 respectively. The higher
215 relative rate of evolution of the *pmp* G family of proteins, a family that has a number of
216 paralogues present in *C. abortus*, suggests that the *pmpG* and *pmp16-17* may be under different
217 selective pressures to other *pmp*-encoding genes. It is unclear whether these genes have a high
218 relative rate of evolution because they contribute to antigenic variance or whether they are
219 redundant in the infection cycle.

220 Two strains, LLG and POS, showed a markedly different genotype to other strains with
221 posterior support observed for the discrete grouping of strains LLG and POS at *pmp*-encoding
222 regions BA (posterior probability = 0.59), EH (posterior probability = 0.87), HG (posterior
223 probability = 0.79), G (posterior probability = 0.62) and 16-17 (posterior probability = 0.91).
224 This was the only RFLP group that could be correlated with geographical location, with both
225 strains originating from Greece. However LLG and POS are not typical Greek strains having
226 been identified as variant strains by MLVA (Laroucau et al., 2009) and the DNA sequencing of
227 the 23S domain I rRNA (Siarkou et al., 2002). Phenotypic differences have been observed in the
228 inclusion morphology and polypeptide profiles of these two strains, in mouse cross-protection
229 experiments and in antibody cross-reactivity to variable segments of MOMP (Vretou et al., 1996;
230 Vretou et al., 2001). Studies in pregnant mice have demonstrated these strains to be different
231 from other *C. abortus* strains in terms of their ability to colonise the placenta and fetus
232 (Bouakane et al., 2003). Furthermore, mouse protection studies have shown a decreased
233 effectiveness of the live 1B vaccine in terms of organism recovery (Bouakane et al., 2005).
234 However, the differences observed did not prevent inactivated AB7-based vaccines from
235 providing protection against heterologous challenge with LLG (Caro et al., 2003).

236 This study presents the first assessment of the genetic diversity that exists within the 18
237 *pmp*-encoding genes of *C. abortus*. The *pmp*-encoding regions of *C. abortus* were largely
238 homogeneous with the majority of strains exhibiting genotypes similar to reference strain S26/3.
239 However, novel genotypes were identified in all *pmp*-encoding loci. Although the biological
240 significance of this variation in terms of pathogenesis is currently unknown, this sequence
241 heterogeneity should be an important consideration when using *pmps* as the basis for diagnostic
242 test or vaccine development.

243

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248

249 **Conflict of interest statement**

250 The authors declare no conflict of interest.

251

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357 **Figure Legends**

358

359 Figure 1.

360 (A) *C. abortus* RFLP profiles generated following RE digestion of polymorphic
361 membrane protein 16-17 locus PCR products from twenty representative strains; 1, DC1. 2, POS.
362 3, LLG. 4, 8385. 5, S152/3. 6, FAG. 7, S26/3. 8, S26/3. 9, 53. 10, C94/1. 11, Z339. 12, AO-2.
363 13, T28. 14, Syva-1. 15, S26/3. 16, AO-7. 17, T19. 18, AO-4. 19, 532. 20, 15. The RFs used for
364 analysis have been highlighted. The size of the upper and lower RF is indicated at the side of the
365 gel. (B) Evolutionary relationships of *C. abortus* strains at the 16-17 locus. The posterior
366 probability of discrete groupings is indicated by the number at the node.

Table 1. *C. abortus* strains used for PCR-RFLP analysis

Country of origin	Host species	Strain
France	Sheep	AB7
Germany	Cow	03DC34, 03DC36, C11/98, Z339
	Goat	532
	Pig	DC12
	Sheep	53, C1/98, C2/98, C7/98, C9/98, C10/98, C17/98, DC1, DC4, DC7
Greece	Goat	FAG, LLG
	Sheep	POS
Ireland	Sheep	C94/1, C94/2, C95/23, C95/27, VS88/576
Italy	Cow	LV350/93
Namibia	Goat	C21/98
Spain	Goat	Syva-1
	Sheep	AO-2, AO-3, AO-4, AO-7, Zar-2B, Zar-5
Tunisia	Goat	15
UK	Sheep	83/12, 86/30, 90/345, A22, H574, S3/3, S26/3, S82/3, S95/3,
		S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28

Table 2. Primers used for the PCR-amplification of *pmp*-encoding genes.

Region ^a	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp) ^a
BA [<i>pmp1B</i> , <i>pmp2A</i>]	AGAATATCATACGTGCACCGCAGTTAG	TTCACTCGCACCACTGACTC	8329
EH [<i>pmp3E</i> , <i>pmp4E</i> , <i>pmp5E</i> , <i>pmp6H</i>]	AAGATTAACGAGCTTCCTGC	CTTGCTTATGCAGCTCGGGG	10547
HG [<i>pmp7G</i> , <i>pmp8G</i> , <i>pmp9G</i> , <i>pmp10G</i>]	TTAGAATGTTCTTGCAAGCCTGT	TGACGGTTCTACAGGGACC	14514
G [<i>pmp11G</i> , <i>pmp12G</i> , <i>pmp13G</i> , <i>pmp14G</i> , <i>pmp15G</i>]	TTAAAAATGCACCCTGCTG	CCTCTGCACCTCAAAACACA	15145
16-17 ^b [<i>pmp16G</i> , <i>pmp17G</i>]	TGAGCCAAATCCTTCTACAGCAAT	AGTCTTAGCGTCACAGGCCGAG	5613
18D [<i>pmp18D</i>]	TTAGAAGATCAAACGCATACCACAATTC	GTCTACATTTTCTCACTCCGTA	4569

^a Based on *C. abortus* S26/3 genome sequence [NC_004552]

^b Primers have been described previously as S5765 and S2429 (Longbottom et al., 1998)

Table 3. *C. abortus* strains organized by RFLP grouping at each *pmp*-encoding locus.

Locus	RFLP group	Strains	PSRF
BA	1	03DC34, 03DC36, 83/12, 86/30, 90/345, 532, A22, AB7, AO-2, AO-4, AO-7, C1/98, C2/98, C7/98, C9/98, C10/98, C17/98, C21/98, C94/1, C94/2, C95/23, C95/27, DC1, DC4, DC7, FAG, H574, 15, LV350/93, Syva-1, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Zar-2B, Z339	unresolved
	2	LLG, POS	0.59
	3	C11/98, 53	0.75
EH	1	03DC34, 53, 532, 83/12, 86/30, 90/345, A22, AB7, AO-2, AO-4, AO-7, C2/98, C7/98, C9/98, C10/98, C17/98, C21/98, C94/1, C94/2, C95/23, C95/27, DC1, DC4, DC7, DC12, FAG, LV350/93, S3/3, S26/3, S95/3, S124/3A, S152/3, T17, T19, T20, T28, Zar-2B, Z339	unresolved
	2	LLG, POS	0.87
	3	S404/5, S507, S82/3	0.70
HG	1	03DC34, 53, 532, 83/12, 86/30, 90/345, A22, AO-2, AO-7, C2/98, C7/98, C9/98, C10/98, C17/98, C21/98, C94/1, C95/23, C95/27, DC1, DC4, DC7, FAG, H574, LV350/93, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Z339	unresolved
	2	LLG, POS	0.79
	3	AO-4, Zar-2B	0.72
	4	AB7, C94/2	0.56
G	1	53, 532, 83/12, 86/30, 90/345, A22, AB7, AO-7, C7/98, C9/98, C21/98, DC1, DC7, FAG, LV350/93, S3/3, S26/3, S82/3,	unresolved

Locus	RFLP group	Strains	PSRF
		S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Z339	
	2	LLG, POS	0.62
	3	AO-4, Zar-5	0.77
16-17	1	03DC34, 03DC36, 53, 83/12, 86/30, 90/345, A22, AB7, AO-2, AO-4, AO-7, C2/98, C7/98, C10/98, C21/98, C95/23, C95/27, DC1, DC4, DC7, FAG, H574, LV350/93, Syva-1, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Zar-2B, Z339	unresolved
	2	LLG, POS	0.91
	3	C94/1, C94/2	0.83
	4	15, 532	0.51
	5	C1/98, C17/98, C9/98	0.55
18D	1	03DC34, 03DC36, 53, 532, 83/12, 86/30, 90/345, A22, AO-2, AO-3, AO-4, AO-7, C1/98, C2/98, C7/98, C9/98, C10/98, C11/98, C17/98, C21/98, C94/1, C94/2, C95/23, C95/27, DC1, DC4, DC7, H574, 15, LLG, LV350/93, POS, Syva-1, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, VS88/576, Zar-2B, Zar-5, Z339	unresolved
	2	AB7, FAG	0.98

Table 4. Relative evolutionary rates for *pmp*-encoding gene loci

Region	Mean	95% confidence interval, lower limit	95% confidence interval, upper limit
BA	0.425	0.142	0.770
EH	0.928	0.569	1.406
HG	0.874	0.648	1.114
G	1.488	1.105	1.885
16-17	1.468	1.005	1.948
18D	0.135	0.015	0.368

Figure 1

