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1 **Title**

2 Phylogentic analysis of *Anaplasma ovis* strains isolated from sheep and goats using *groEL*
3 and *mps4* genes.

4
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11

12 Running head: *Anaplasma ovis* in Cyprus

13 Keywords: *Anaplasma ovis*, Cyprus, *groEL*, *msp4*

14

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19

20

21 **Abstract**

22 Evidence of *Anaplasma* spp. in goats and sheep in Cyprus has been demonstrated by
23 previous research. Herein, further research was performed for the identification of the exact
24 *Anaplasma* spp. resulting in the identification of *Anaplasma ovis* strains in all samples
25 examined. We used a bioinformatics as well as a molecular approach (study of *groEl* and
26 *msp4* genes) in order to verify the validity of the results. All samples depicted the presence of
27 *A. ovis* regardless of the host (goat or sheep).

28

29 **Introduction**

30 Anaplasmosis is a disease caused by pathogens belonging to the genus of *Anaplasma*
31 (Rickettsiales: *Anaplasmataceae*). *A. marginale*, *A. centrale* and *A. ovis* are obligate
32 intracellular bacteria, specifically located within membrane-attached cytoplasmic vacuoles of
33 vertebrate and invertebrate host cells (Dumler et al., 2001) and they use ruminants as their
34 main host (Kocan et al., 2003; Lew et al., 2003). The pathogens are biologically transmitted
35 through ticks; vertebrates and ticks with persistent bacteremia can be considered as natural
36 pathogen reservoirs (Dumler et al., 2001; Kocan et al., 2003; Rikihisa, 2003). Ovine
37 anaplasmosis is most commonly related with haemolytic anaemia in goats. However, *A. ovis*
38 infections in sheep have also been reported (Friedhoff, 1997; Splitter et al., 1956).

39 The interaction of *Anaplasma* spp. and their hosts is mediated by the major surface proteins
40 (MSPs). Thus, rapid evolution of these genes is likely to occur due to the natural selective
41 pressure exerted by the immune system of the hosts (Brayton et al., 2006; de la Fuente et al.,
42 2005a; Hotopp et al., 2006; Kocan et al., 2004). Former surveys on *A. ovis* revealed a
43 variation of the *msp4* gene which lead to the identification of three different genotypes (de la

44 Fuente et al., 2006; de la Fuente et al., 2002a; de la Fuente et al., 2005c; Yabsley et al.,
45 2005). Moreover, heat shock operon *groESL* has been used for identification of
46 *Anaplasmataceae* (Sumner et al., 1997) as well as partial *groEL* genes, which encode a 60-
47 kDa heat shock protein, GroEL (Park et al., 2005).

48 In a study conducted in Cyprus (Chochlakis et al., 2008) blood samples collected from sheep
49 and goats were tested by PCR for the presence of *Anaplasma* spp. and *A. phagocytophilum*.
50 Of the genotypes revealed, four corresponded to *Anaplasma* spp. In the current study,
51 samples which corresponded to these genotypes were processed by PCR amplification of
52 *groEL* and *msp4* genes, Restriction Fragment Length Polymorphism (RFLP) and sequencing
53 analysis in order to identify the *Anaplasma* spp. to which they corresponded.

54 55 **Materials and methods**

56 **Sampling**

57 Six hundred and eighty nine (689) blood samples (343 from sheep and 346 from goats) were
58 drawn, by trained Veterinarians, from March 2005 – March 2006, from 38 different regions
59 of the 5 prefectures of Cyprus (Nicosia, Limassol, Famagusta, Larnaka, Pafos). Blood
60 samples were tested for the presence of *Anaplasma* DNA using 16S rRNA gene primers.
61 Four *Anaplasma* spp. genotypes were revealed (EU090181, EU090183, EU090184 and
62 EU090185) (Chochlakis et al., 2008).

63 All tests were carried out at the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses
64 and Geographical Medicine (WHO Collaborating Centre) in Crete (Greece).

65

66 **Amplification of *groEL* and *msp4* genes**

67 Primers HSPB (TTA(GA)AA(GATC)CC(GATC)CCCAT(GATC)CC(GATC)CCCATG CC-
68 3') and HSPC (5'-AAATGGCGAATGTTGT(TA)GT(TC)AC-3') (brackets indicate mixed
69 bases) were used to amplify an approximately 1650 bp fragment of the *groEL* gene (Lew *et*
70 *al.*, 2003). Primers msp43 (5'-CCGGATCCTTAGCTGAACAGGAATCTTGC-3') and
71 msp45 (5'-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC-3') were used to amplify
72 an approximately 850 bp fragment of the *msp4* gene (de la Fuente *et al.*, 2007). Negative
73 controls (distilled water) were used every 10 samples to ensure the accuracy of the method.
74 The first sample that was tested positive by PCR and sequence analysis was further used as
75 the positive control. PCR products were electrophoresed on 1% agarose gel and visualized
76 following 30-minute incubation in GelStar Nucleic Acid Gel Stain (Lonza, USA) according
77 to the manufacturer's instructions. Positive PCR products were purified (PCR product
78 purification kit, Qiagen, Germany) and positive PCR products were re-amplified in order to
79 multiply the DNA and gain more appropriate results in sequence analysis.

80

81 **Prediction of RFLP patterns using Bioinformatics tools – RFLP analysis**

82 DNA sequences for *Anaplasma groEL* and *msp4* genes were retrieved from the National
83 Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST>)
84 database. MegAlign module of the *Lasergene* Ver.7.1 software (*DNASTAR* Inc., Madison,
85 WI, USA) and Seqbuilder module of the *Lasergene* Ver.7.1 software (*DNASTAR* Inc.,
86 Madison, WI, USA) were used; *HaeIII* (Fermentas) and *ApoI* (New England Biolabs)
87 restriction enzymes corresponding to *groEL* and *msp4* genes were used (Figure 1) as follows:
88 16µl nuclease-free water, 2µl 10X Buffer (Buffer R for *HaeIII* and NEBuffer 3 for *ApoI*), 1µl

89 of purified PCR product and 0.5µl of the corresponding enzyme where gently mixed,
90 incubated for 1 hour at 37°C, electrophoresed at 1.5% agarose gel and visualised as above.

91

92 **Sequencing analysis**

93 Purified PCR products were directly sequenced with the above described primers using the
94 sequencer CEQ 8000 Beckman Coulter (Bioanalytica – Genotype, Athens). All PCR
95 products were sequenced twice to verify the revealed sequences. The sequences revealed
96 were processed using nucleotide BLASTn, Chromas v1.49 and *Lasergene* Ver.7.1 software.

97

98 **Revealed sequence accession numbers**

99 As regards *groEL*, the accession numbers submitted at GenBank database were FJ460434,
100 FJ460435, FJ460436, FJ460437, FJ460438, FJ460439, FJ460440, FJ460441, FJ460442,
101 while for *msp4* the accession numbers were FJ460444, FJ460445, FJ460446, FJ460447,
102 FJ460448, FJ460449, FJ460450, FJ460451, FJ460452, FJ460453, FJ460454 and FJ460455.

103

104 **Phylogenetic analysis**

105 Clustalx 2.0 was used to align sequences and MEGA software v. 4.0.2 to construct the
106 phylogenetic trees (Figures 2, 3). The Neighbor-Joining (NJ) algorithm (Saitou and Nei,
107 1987) with Kimura 2-parameters correction (Kimura, 1980) were used with bootstrap
108 analysis of 1000 iterations.

109

110 **Results**

111 An algorithm was created (Figure 1) to distinguish the species of *Anaplasma* spp. detected in
112 ruminants can be distinguished. Digesting the *groEL* gene with *HaeIII* reveals a band near
113 900bp narrowing the selection to *A. centrale* and *A. marginale*, where discrimination
114 between is conferred by the absence (*A. centrale*) or presence (*A. marginale*) of a 92bp band.
115 Absence of the band around 900bp denotes that the species is *A. ovis*. Digestion of the *msp4*
116 gene with *ApoI* may reveal a single 849bp band (*A. marginale*) or two bands (765bp and
117 87bp) (*A. centrale*) (537bp and 315bp) (*A. ovis*). Digestion of our *msp4* and *groEL* PCR
118 products with *ApoI* and *HaeIII* restriction enzymes respectively, revealed profiles denoting
119 the presence of *A. ovis* (pattern not shown).

120 In the case of *groEL* we retrieved parts of the genomic sequence the length of which, varied
121 between 698bp and 1649bp. Consequently, comparison of the sequences was performed in
122 the sequence region that could be cross-checked among all sequencing products. In the case
123 of *msp4* we obtained the whole length sequence. Nine *groEL* sequences (4 detected in goats
124 and 5 in sheep) and 12 *msp4* sequences (5 detected in goat and 7 in sheep) were revealed.

125 As regards *msp4*, a 99% identity, among *A. ovis* strain AM5 (EF067341), *A. ovis* strain
126 HungaryV8 (EF190511), *A. ovis* strain Italy20 (AY702923) and *A. ovis* strain Italy147
127 (AY702924) was revealed (Figure 2). In the case of *groEL*, one high similarity (99%)
128 candidate, *A. ovis* isolate OVI (AF441131) was revealed (Figure 3).

129 The *groEL* sequences alignment revealed a high degree of similarity since several of the
130 nucleotide base substitution mutations were silent mutations (Table 1). We found 54
131 mutation sites hosting 45 point mutations and 3 triplet insertions. The mutation level
132 observed in strain goat 9 (FJ460435) was higher than expected and higher compared to the
133 rest of the sequences (Table 1). As regards *msp4* sequences, we detected 53 mutation sites,

134 all of which were point mutations (Table 2). Eleven of these were silent mutations. In most
135 silent mutations the initial amino acids were replaced by others with similar properties on
136 charge or hydrophobicity (e.g. replacement of L by P); in less occasions a non polar amino
137 acid was replaced by a polar amino acid, negatively or positively charged and *vice versa*.
138 Eleven mutation regions were parsimonious in both genes. The percentage of transitions and
139 transversions was almost the same, 45.3% and 54.7% respectively for *mSP4*; 44.2% and
140 55.8% respectively for *groEL*.

141

142 **Discussion**

143 Genetic characterization of *Anaplasma* spp. pathogens in ruminant and non-ruminant hosts
144 can be differentiated by examining the *mSP4* gene and protein sequence as already described
145 (de la Fuente et al., 2007; de la Fuente et al., 2005a; de la Fuente et al., 2005b; de la Fuente et
146 al., 2002b). Phylogenetic studies have used *groEL* genes to distinguish among the species of
147 *Anaplasma* spp. (Lew et al., 2003). In previous surveys it has also been stated that *mSP4*
148 genotypes could exhibit heterogeneity among geographic regions, hosts and perhaps among
149 individuals of the same flock (de la Fuente et al., 2007; Hornok et al., 2007). Similar
150 conclusions have been drawn for the *A. ovis groEL* gene as it has been found to exhibit a
151 much higher heterogeneity compared to *A. marginale* and *A. centrale groEL* genes.
152 Furthermore, past surveys on isolates of *A. marginale* proved that analysis of gene and
153 protein sequences (MSP1a and MSP4) could provide phylogeographic information (de la
154 Fuente et al., 2001).

155 Herein, even though we can comment on the heterogeneity of the sequences, we can not
156 extract any phylogeographic information. The reason could be the relatively small

157 geographic area the island of Cyprus and the often trading of animals among farmers. Several
158 different strains can exist in Cyprus being dispersed throughout the island. This also might be
159 the answer why more similar strains were detected in farms separated by greater distances
160 while more dissimilar ones were found to be present in farms with close proximity (data not
161 shown).

162 Phylogenetic analysis clusters our sequences along with other *A. ovis* strains, clearly
163 distinguishing them from *A. marginale* and *A. centrale*, also supported by the strong
164 bootstrap values both for *msp4* and *groEL* phylogenetic analysis, suggesting that *A. ovis*
165 *groEL* may vary among hosts as has already been supported for *msp4* (de la Fuente et al.,
166 2007).

167 Investigation of individual organisms in a flock in order to determine whether different
168 strains exist in the same flock or even co-exist in single individual could lead to a more
169 sufficient view on the phylogeny of *Anaplasma* spp. Further investigation is of great
170 importance as ovine anaplasmosis although benign, can manifest due to possibly
171 predisposing factors (Friedhoff, 1997) and turn into a persistent subclinical infection (Kieser
172 et al., 1990; Palmer et al., 1998).

173 In the current survey we identified *A. ovis* strains, in sheep and goats of the island of Cyprus,
174 through bioinformatics and sequencing analysis approach of *groEL* and *mps4* genes. We also
175 proposed a model, based on *groEL* and *msp4* genes, for simple and accurate discrimination
176 among the 3 *Anaplasma* spp. In any case, attention should be given in the dispersal of the
177 pathogen through its vectors and the need to recognize the mechanisms of evolution and
178 genetic diversity in this pathogen becomes obvious.

179

180 **Conflict of interest statement:** Authors clearly state that no financial and personal
181 relationships with other people or organisations that could inappropriately influence (bias)
182 their work exist.

183

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188

189 **References**

- 190 Brayton, K.A., Palmer, G.H., Brown, W.C., 2006, Genomic and proteomic approaches to
191 vaccine candidate identification for *Anaplasma marginale*. *Expert Rev Vaccines* 5, 95-101.
- 192 Chochlakis, D., Ioannou, I., Sharif, L., Kokkini, S., Hristophi, N., Dimitriou, T., Tselentis,
193 Y., Psaroulaki, A., 2008, Prevalence of *Anaplasma* sp. in Goats and Sheep in Cyprus. *Vector*
194 *Borne Zoonotic Dis.*
- 195 de la Fuente, J., Atkinson, M.W., Hogg, J.T., Miller, D.S., Naranjo, V., Almazan, C.,
196 Anderson, N., Kocan, K.M., 2006, Genetic characterization of *Anaplasma ovis* strains from
197 bighorn sheep in Montana. *J Wildl Dis* 42, 381-385.
- 198 de la Fuente, J., Atkinson, M.W., Naranjo, V., Fernandez de Mera, I.G., Mangold, A.J.,
199 Keating, K.A., Kocan, K.M., 2007, Sequence analysis of the *msp4* gene of *Anaplasma ovis*
200 strains. *Vet Microbiol* 119, 375-381.
- 201 de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Saliki, J.T., Kocan, K.M., 2002a, Infection
202 of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia

203 *Anaplasma marginale* excludes infection with other genotypes. *Clin Diagn Lab Immunol* 9,
204 658-668.

205 de la Fuente, J., Lew, A., Lutz, H., Meli, M.L., Hofmann-Lehmann, R., Shkap, V., Molad, T.,
206 Mangold, A.J., Almazan, C., Naranjo, V., Gortazar, C., Torina, A., Caracappa, S., Garcia-
207 Perez, A.L., Barral, M., Oporto, B., Ceci, L., Carelli, G., Blouin, E.F., Kocan, K.M., 2005a,
208 Genetic diversity of anaplasma species major surface proteins and implications for
209 anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev* 6, 75-89.

210 de la Fuente, J., Massung, R.F., Wong, S.J., Chu, F.K., Lutz, H., Meli, M., von Loewenich,
211 F.D., Grzeszczuk, A., Torina, A., Caracappa, S., Mangold, A.J., Naranjo, V., Stuen, S.,
212 Kocan, K.M., 2005b, Sequence analysis of the msp4 gene of *Anaplasma phagocytophilum*
213 strains. *J Clin Microbiol* 43, 1309-1317.

214 de la Fuente, J., Torina, A., Caracappa, S., Tumino, G., Furla, R., Almazan, C., Kocan, K.M.,
215 2005c, Serologic and molecular characterization of *Anaplasma* species infection in farm
216 animals and ticks from Sicily. *Vet Parasitol* 133, 357-362.

217 de la Fuente, J., Van Den Bussche, R.A., Garcia-Garcia, J.C., Rodriguez, S.D., Garcia, M.A.,
218 Guglielmone, A.A., Mangold, A.J., Friche Passos, L.M., Barbosa Ribeiro, M.F., Blouin, E.F.,
219 Kocan, K.M., 2002b, Phylogeography of New World isolates of *Anaplasma marginale* based
220 on major surface protein sequences. *Vet Microbiol* 88, 275-285.

221 de la Fuente, J., Van Den Bussche, R.A., Kocan, K.M., 2001, Molecular phylogeny and
222 biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae:
223 Ehrlichieae). *Vet Parasitol* 97, 65-76.

224 Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y.,
225 Rurangirwa, F.R., 2001, Reorganization of genera in the families Rickettsiaceae and

226 Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with
227 Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new
228 species combinations and designation of Ehrlichia equi and 'HGE agent' as subjective
229 synonyms of Ehrlichia phagocytophila. Int J Syst Evol Microbiol 51, 2145-2165.

230 Friedhoff, K.T., 1997, Tick-borne diseases of sheep and goats caused by Babesia, Theileria
231 or Anaplasma spp. Parasitologia 39, 99-109.

232 Hornok, S., Elek, V., de la Fuente, J., Naranjo, V., Farkas, R., Majoros, G., Foldvari, G.,
233 2007, First serological and molecular evidence on the endemicity of Anaplasma ovis and A.
234 marginale in Hungary. Vet Microbiol 122, 316-322.

235 Hotopp, J.C., Lin, M., Madupu, R., Crabtree, J., Angiuoli, S.V., Eisen, J.A., Seshadri, R.,
236 Ren, Q., Wu, M., Utterback, T.R., Smith, S., Lewis, M., Khouri, H., Zhang, C., Niu, H., Lin,
237 Q., Ohashi, N., Zhi, N., Nelson, W., Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sundaram,
238 J., Daugherty, S.C., Davidsen, T., Durkin, A.S., Gwinn, M., Haft, D.H., Selengut, J.D.,
239 Sullivan, S.A., Zafar, N., Zhou, L., Benahmed, F., Forberger, H., Halpin, R., Mulligan, S.,
240 Robinson, J., White, O., Rikihisa, Y., Tettelin, H., 2006, Comparative genomics of emerging
241 human ehrlichiosis agents. PLoS Genet 2, e21.

242 Kieser, S.T., Eriks, I.S., Palmer, G.H., 1990, Cyclic rickettsemia during persistent Anaplasma
243 marginale infection of cattle. Infect Immun 58, 1117-1119.

244 Kimura, M., 1980, A simple method for estimating evolutionary rates of base substitutions
245 through comparative studies of nucleotide sequences. J Mol Evol 16, 111-120.

246 Kocan, K.M., de la Fuente, J., Blouin, E.F., Garcia-Garcia, J.C., 2004, Anaplasma marginale
247 (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a
248 tick-borne rickettsia. Parasitology 129 Suppl, S285-300.

- 249 Kocan, K.M., de la Fuente, J., Guglielmono, A.A., Melendez, R.D., 2003, Antigens and
250 alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 16,
251 698-712.
- 252 Lew, A.E., Gale, K.R., Minchin, C.M., Shkap, V., de Waal, D.T., 2003, Phylogenetic
253 analysis of the erythrocytic *Anaplasma* species based on 16S rDNA and GroEL (HSP60)
254 sequences of *A. marginale*, *A. centrale*, and *A. ovis* and the specific detection of *A. centrale*
255 vaccine strain. *Vet Microbiol* 92, 145-160.
- 256 Palmer, G.H., Abbott, J.R., French, D.M., McElwain, T.F., 1998, Persistence of *Anaplasma*
257 *ovis* infection and conservation of the msp-2 and msp-3 multigene families within the genus
258 *Anaplasma*. *Infect Immun* 66, 6035-6039.
- 259 Park, H.S., Lee, J.H., Jeong, E.J., Park, T.K., Kim, T.Y., Chae, J.S., Park, J.H., Klein, T.A.,
260 Jang, W.J., Park, K.H., Lee, S.H., 2005, Differentiation of Anaplasmataceae through partial
261 groEL gene analysis. *Microbiol Immunol* 49, 655-662.
- 262 Rikihisa, Y., 2003, Mechanisms to create a safe haven by members of the family
263 Anaplasmataceae. *Ann N Y Acad Sci* 990, 548-555.
- 264 Saitou, N., Nei, M., 1987, The neighbor-joining method: a new method for reconstructing
265 phylogenetic trees. *Mol Biol Evol* 4, 406-425.
- 266 Splitter, E.J., Anthony, H.D., Twiehaus, M.J., 1956, *Anaplasma ovis* in the United States;
267 experimental studies with sheep and goats. *Am J Vet Res* 17, 487-491.
- 268 Sumner, J.W., Nicholson, W.L., Massung, R.F., 1997, PCR amplification and comparison of
269 nucleotide sequences from the groESL heat shock operon of *Ehrlichia* species. *J Clin*
270 *Microbiol* 35, 2087-2092.

271 Yabsley, M.J., Davidson, W.R., Stallknecht, D.E., Varela, A.S., Swift, P.K., Devos, J.C., Jr.,
272 Dubay, S.A., 2005, Evidence of tick-borne organisms in mule deer (*Odocoileus hemionus*)
273 from the western United States. *Vector Borne Zoonotic Dis* 5, 351-362.

274

275 **Tables and Figures**

276 **Table 1:** Positions in the *groEL* sequences presenting point mutations or triplet insertions.
277 The number of the corresponding amino acid for each mutation is shown directly below each
278 case. Silent mutations are shown in light green. Reference sequences are in light grey.

279 **Table 2:** Positions in the *msp4* sequences presenting point mutations. The number of the
280 corresponding amino acid for each mutation is shown directly below each case. Silent
281 mutations are shown in light green. Reference sequences are in light grey.

282 **Figure 1:** Schematic representation of the expected patterns using restriction enzyme *HaeIII*
283 on *groEL* gene and *ApoI* on *msp4* gene. An actual 50bp Ladder is shown as a guide.

284 **Figure 2:** Neighbor-joining tree with bootstrap analysis (1000 iterations) showing the
285 clustering of *groEL* sequences. The percentage of replicate trees in which the associated taxa
286 clustered together in the bootstrap test is shown next to the branches.

287
288 **Figure 3:** Neighbor-joining tree with bootstrap analysis (1000 iterations) showing the
289 clustering of *msp4* sequences. The percentage of replicate trees in which the associated taxa
290 clustered together in the bootstrap test is shown next to the branches.

	41bp	44-48bp	68-70bp	76-77bp	205bp	284-287bp	293-296bp	345bp	438bp	513bp	538bp	540bp	571bp	647bp	661bp	671-672bp	689-692bp	725-726bp	729bp
AF441131 <i>A. ovis</i> OVI	C	TAAGG		GC	G	T	C	A	T	T	G	G	G	G	C	TC	TCAA	AT	T
FJ460441 <i>A. ovis</i> goat 76	C	TAAGG		GC	G	T	C	A	T	T	G	G	G	G	T	TG	ATCA	AT	T
FJ460435 <i>A. ovis</i> goat 9	C	TAAGG	GAA	TG	C	CTGG	GCAT	A	G	C	A	C	C	G	C	AC	TCGA	AT	A
FJ460434 <i>A. ovis</i> sheep 8	C	TAAGG		GC	G	T	C	A	T	T	G	G	G	G	C	GC	TCAC	AT	T
FJ460436 <i>A. ovis</i> sheep 37	C	TAAGG		GC	G	T	C	T	T	T	G	G	G	A	C	TC	TCAA	AC	T
FJ460442 <i>A. ovis</i> goat 77	C	TAAGG		GC	G	T	C	A	T	T	G	G	G	G	C	GC	TCCA	TT	T
FJ460439 <i>A. ovis</i> sheep 64	A	TAGAG		GC	G	T	C	A	T	T	G	G	G	G	C	TC	TCAC	TT	T
FJ460737 <i>A. ovis</i> sheep 40	A	TAGAG		GC	G	T	C	A	T	T	G	G	G	G	C	TC	TCAA	AT	T
FJ460440 <i>A. ovis</i> goat 71	A	AGAGA		GC	G	T	C	A	T	T	G	G	G	G	C	CC	TCAC	AT	T
FJ460438 <i>A. ovis</i> sheep 54	A	AGAGA		GC	G	T	C	A	T	T	G	G	G	A	C	TC	TCAA	AT	T
EF520695 <i>A. centrale</i> strain 16	C	TAAGG		GC	G	T	C	C	T	T	G	G	G	G	C	TC	TCAA	AC	C
AF414862 <i>A. marginale</i> Israel	C	TAAGG		GC	G	T	C	C	T	T	G	G	G	G	C	TC	TCAA	AC	C
	14aa	15-16aa	23-24aa	26aa	72aa	95-96aa	98-99aa	115aa	146aa	171aa	180aa	191aa	216aa	221aa	224aa	230-231aa	242aa	243aa	
AF441131 <i>A. ovis</i> OVI	S	IR		A	A			I	I	V	E	E	V	P	F	IN	N	V	
FJ460441 <i>A. ovis</i> goat 76	S	IR		A	A			I	I	V	E	E	V	S	L	NH	N	V	
FJ460435 <i>A. ovis</i> goat 9	S	IR	GK	C	P	PG	GI	I	M	V	N	Q	V	P	Y	ID	N	V	
FJ460434 <i>A. ovis</i> sheep 8	S	IR		A	A			I	I	V	E	E	V	P	C	IT	N	V	
FJ460436 <i>A. ovis</i> sheep 37	S	IR		A	A			I	I	V	E	E	I	P	F	IN	N	V	
FJ460442 <i>A. ovis</i> goat 77	S	IR		A	A			I	I	V	E	E	V	P	C	IH	I	V	
FJ460439 <i>A. ovis</i> sheep 64	Y	IE		A	A			I	I	V	E	E	V	P	F	IT	I	V	
FJ460737 <i>A. ovis</i> sheep 40	Y	IE		A	A			I	I	V	E	E	V	P	F	IN	N	V	
FJ460440 <i>A. ovis</i> goat 71	Y	KR		A	A			I	I	V	E	E	V	P	S	IT	N	V	
FJ460438 <i>A. ovis</i> sheep 54	Y	KR		A	A			I	I	V	E	E	I	P	F	IN	N	V	
EF520695 <i>A. centrale</i> strain 16	S	IR		A	A			I	I	V	E	E	V	P	F	IN	N	V	

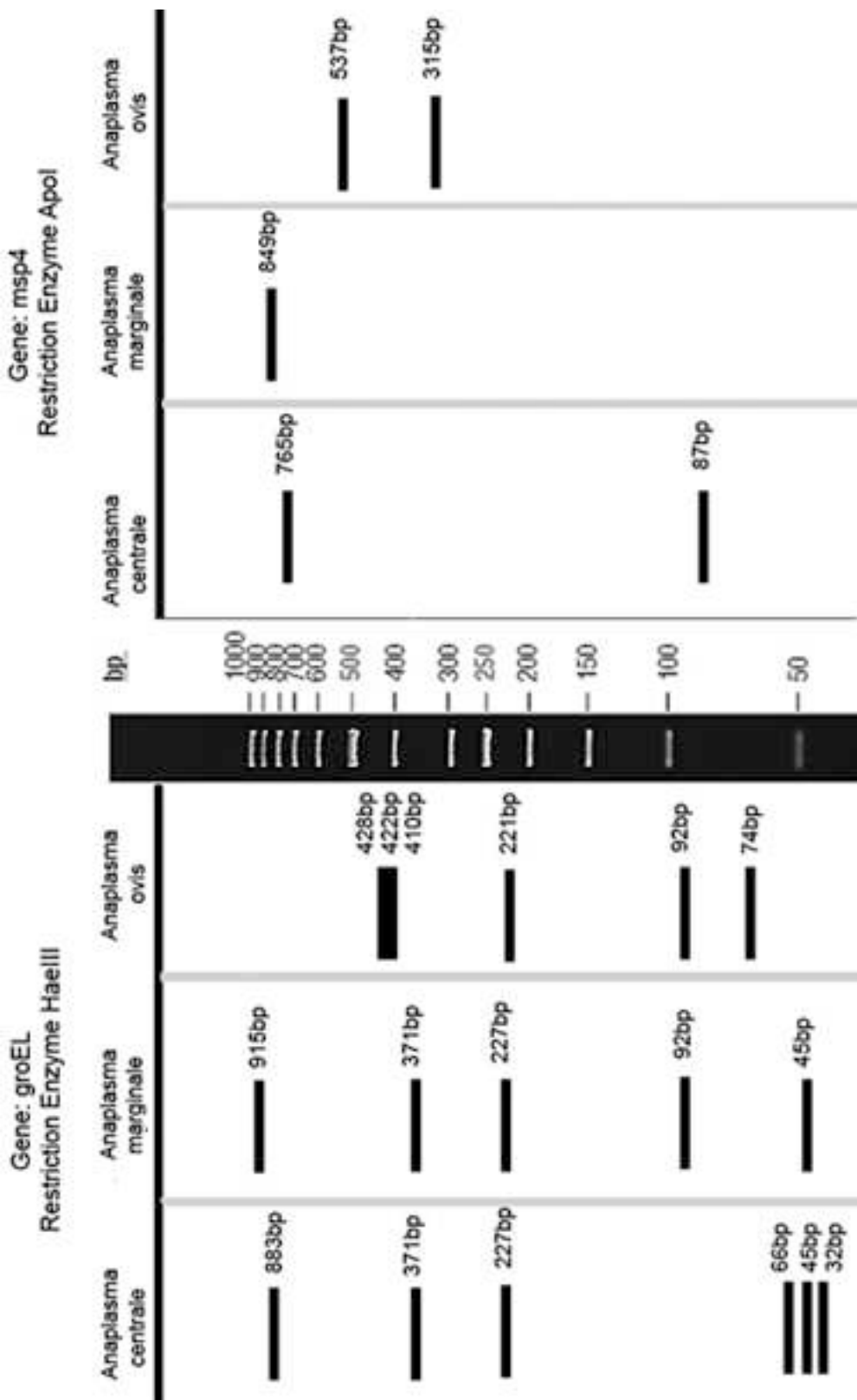
AF414862 <i>A. marginale</i> Israel	S	IR		A	A			I	I	V	E	E	V	P	F	IN	N	V
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Table 1: Positions of the *groEL* sample sequences presenting point mutations or triplet insertions. The number of the corresponding amino acid for each mutation is shown directly below each case. Light green is used to show the amino acids that remained unchanged due to silent mutation. The sequences coloured in light grey correspond to reference sequences for *Anaplasma groEL*.

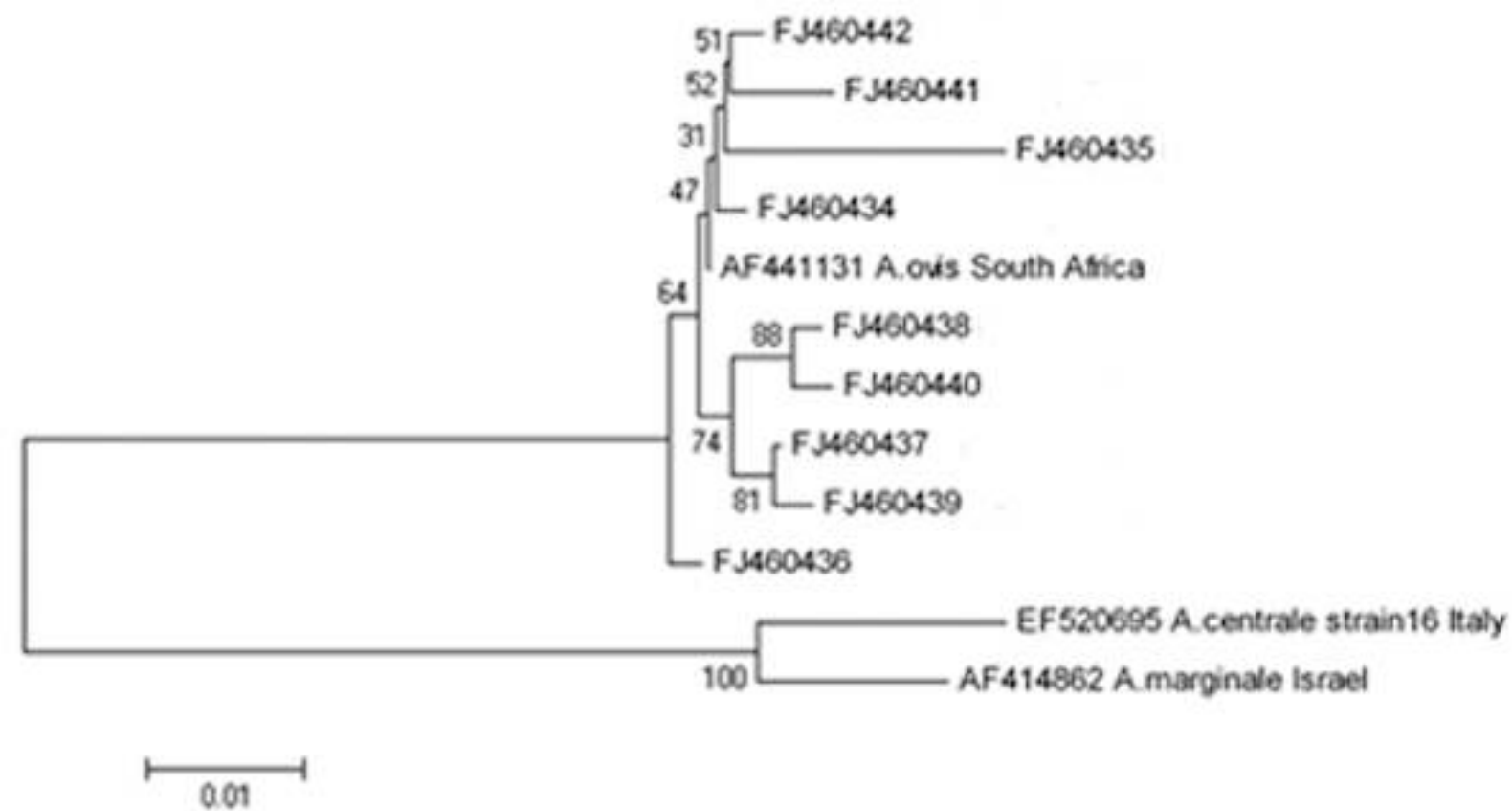
	53bp	75bp	119bp	126bp	470bp	546bp	660bp	695bp	736bp	767bp	770bp	789bp	801bp	817bp	819-824bp	827-828bp
EF067341 <i>A. ovis</i> SpainAM5	C	G	G	G	T	T	T	T	T	A	T	C	A	G	TTTTAA	TT
AY702923 <i>A. ovis</i> Italy20	C	G	G	G	T	T	T	T	T	A	T	C	A	G	TTTTAA	TT
EF190511 <i>A. ovis</i> HungaryV8	C	G	G	G	T	T	T	T	T	A	T	C	A	G	TTTTAA	TT
FJ460454 <i>A. ovis</i> goat 73	C	G	G	G	T	T	T	T	T	A	T	C	A	G	TTTTAA	CT
FJ460447 <i>A. ovis</i> goat 23	C	G	G	G	T	T	T	T	T	C	C	C	A	G	GTTTAA	CT
FJ460448 <i>A. ovis</i> sheep 35	C	G	G	G	T	T	T	C	A	C	T	C	A	G	TTGTGA	CT
FJ460455 <i>A. ovis</i> goat 77	C	G	G	G	T	T	A	T	T	C	T	C	C	G	TGTTGA	CT
FJ460445 <i>A. ovis</i> goat 9	C	G	G	G	T	T	A	T	T	C	T	C	A	G	TTTTGA	CT
FJ460455 <i>A. ovis</i> goat 71	C	G	G	G	T	C	T	T	T	C	T	C	G	G	TTTAAA	CT
AY702924 <i>A. ovis</i> Italy147	C	G	G	G	C	T	T	T	T	A	T	C	A	G	TTTTAA	TT
FJ460452 <i>A. ovis</i> sheep 64	C	G	G	G	C	T	T	T	T	C	T	C	A	G	TTTGAA	CT
FJ460451 <i>A. ovis</i> sheep 40	C	G	G	G	C	T	T	T	T	C	T	C	A	G	TTTGAA	CT
FJ460450 <i>A. ovis</i> sheep 37	A	T	G	G	C	T	T	T	T	A	T	G	G	A	TGTTAC	TT
FJ460449 <i>A. ovis</i> sheep 36	C	T	T	T	C	T	T	T	T	C	T	C	A	G	TTTTAA	CT
FJ460446 <i>A. ovis</i> sheep 10	C	G	G	G	C	T	T	T	T	A	T	C	A	G	TTTTAC	TG
FJ460444 <i>A. ovis</i> sheep 8	C	G	G	G	C	T	A	T	T	A	T	C	A	G	TTTTAA	TT
	18aa	25aa	40aa	42aa	157aa	182aa	220aa	232aa	246aa	256aa	257aa	263aa	267aa	273-276aa		
EF067341 <i>A. ovis</i> SpainAM5	A	G	G	G	V	Y	V	L	Y	K	F	A	A	GFNL		
AY702923 <i>A. ovis</i> Italy20	A	G	G	G	V	Y	V	L	Y	K	F	A	A	GFNL		
EF190511 <i>A. ovis</i> HungaryV8	A	G	G	G	V	Y	V	L	Y	K	F	A	A	GFNL		
FJ460454 <i>A. ovis</i> goat 73	A	G	G	G	V	Y	V	L	Y	K	F	A	A	GFNP		
FJ460447 <i>A. ovis</i> goat 23	A	G	G	G	V	Y	V	L	Y	T	S	A	A	GFNP		
FJ460448 <i>A. ovis</i> sheep 35	A	G	G	G	V	Y	V	S	N	T	F	A	A	GCDP		
FJ460455 <i>A. ovis</i> goat 77	A	G	G	G	V	Y	V	L	Y	T	F	A	A	GVDP		
FJ460445 <i>A. ovis</i> goat 9	A	G	G	G	V	Y	V	L	Y	T	F	A	A	GFDP		
FJ460455 <i>A. ovis</i> goat 71	A	G	G	G	V	Y	V	L	Y	T	F	A	A	GLNP		
AY702924 <i>A. ovis</i> Italy147	A	G	G	G	A	Y	V	L	Y	K	F	A	A	GFNL		

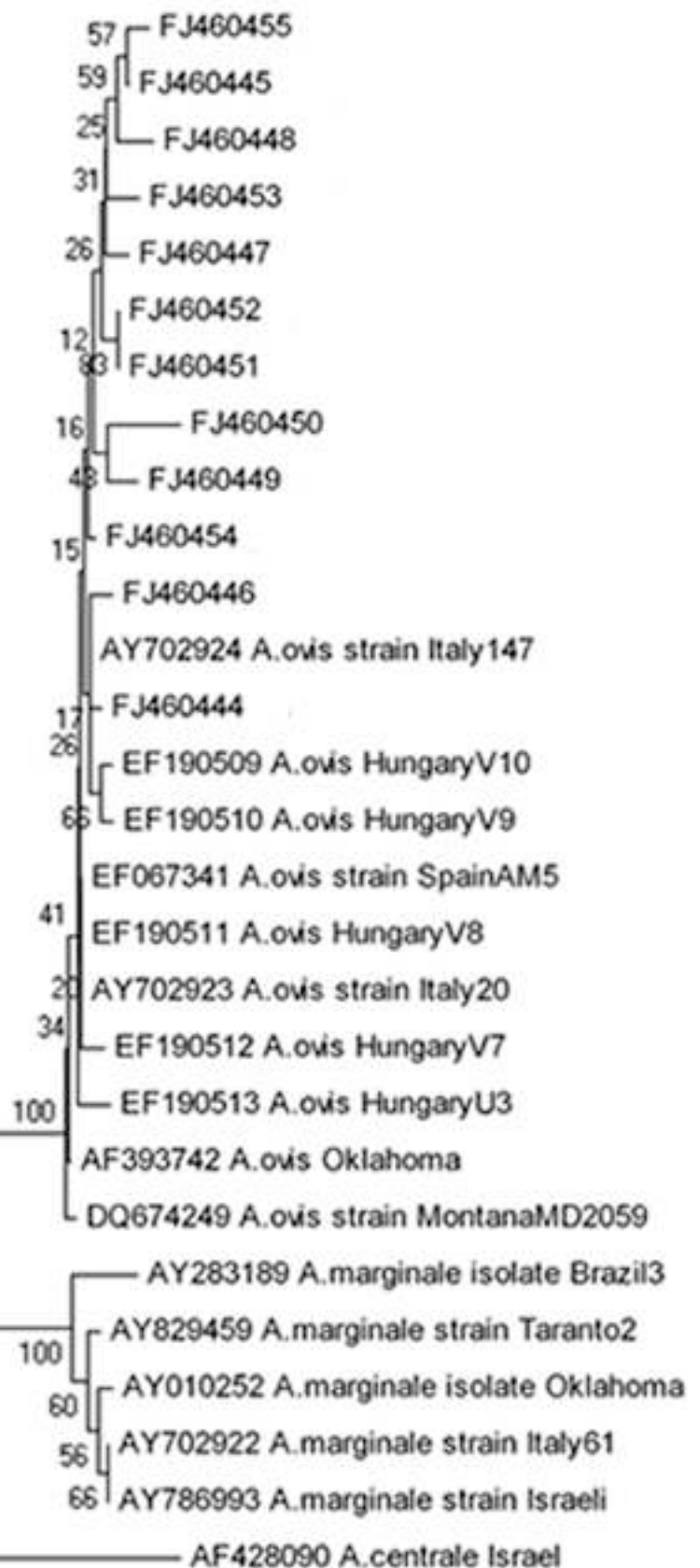
FJ460452 <i>A. ovis</i> sheep 64	A	G	G	G	A	Y	V	L	Y	T	F	A	A	GLNP
FJ460451 <i>A. ovis</i> sheep 40	A	G	G	G	A	Y	V	L	Y	T	F	A	A	GLNP
FJ460450 <i>A. ovis</i> sheep 37	D	G	G	G	A	Y	V	L	Y	K	F	A	A	SVTL
FJ460449 <i>A. ovis</i> sheep 36	A	G	V	G	A	Y	V	L	Y	T	F	A	A	GFNP
FJ460446 <i>A. ovis</i> sheep 10	A	G	G	G	A	Y	V	L	Y	K	F	A	A	GF T L
FJ460444 <i>A. ovis</i> sheep 8	A	G	G	G	A	Y	V	L	Y	K	F	A	A	GFNL

Table 2: Positions in the *msp4* sequences presenting point mutations. The number of the corresponding amino acid for each mutation is shown directly below each case. Light green is used to show amino acids that remained unchanged due to silent mutation. The sequences coloured light grey, correspond to reference sequences.



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