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**Recombination and purifying and balancing selection determine the evolution of
major antigenic protein 1 (*map 1*) family genes in *Ehrlichia ruminantium***

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Running head: Evolution of *map1* family genes of *Ehrlichia ruminantium*

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1. ABSTRACT

Heartwater is an economically important disease of ruminants caused by the tick-borne bacterium *Ehrlichia ruminantium*. The disease is present throughout sub-Saharan Africa as well as on several islands in the Caribbean, where it poses a risk of spreading onto the American mainland. The dominant immune response of infected animals is directed against the variable outer membrane proteins of *E. ruminantium* encoded by polymorphic multigene families. Here, we examined the full-length sequence of the major antigenic protein 1 (*map1*) family genes in multiple *E. ruminantium* isolates from different African countries and the Caribbean, collected at different time points to infer the possible role of recombination breakpoint and natural selection. A high level of recombination was found particularly in *map1* and *map1-2*. Evidence of strong negative purifying selection in *map1* and balancing selection to maintain genetic variation across these samples from geographically distinct countries suggests host–pathogen co-evolution. This co-evolution between the host and pathogen results in balancing selection by maintaining genetic diversity that could be explained by the demographic history of long-term pathogen pressure. This signifies the adaptive role and the molecular evolutionary forces underpinning *E. ruminantium map1* multigene family antigenicity.

74

75 2. INTRODUCTION

76 *Ehrlichia ruminantium* is an obligate intracellular Gram-negative bacterium causing
77 heartwater in wild and domestic ruminants. This pathogen is transmitted by ticks of the genus
78 *Amblyomma* and occurs throughout sub-Saharan Africa and on several islands in the
79 Caribbean, where it poses a threat of spreading to the American mainland (Burridge et al.,
80 2002). The disease has a significant economic impact on livestock production in endemic
81 countries as reported previously (Mukhebi et al., 1999).

82 Outer membrane proteins (OMPs) of Gram-negative bacteria are known to play important
83 roles in interaction with the host (Lin et al., 2002). Multigene families coding for OMPs have
84 been characterized in several bacterial species of the genus *Ehrlichia*, including the outer
85 membrane protein p28 (*omp-1*) in *Ehrlichia chaffeensis* (Ohashi et al., 1998b; Yu et al.,
86 1999), the p30 outer membrane protein (*p30*) in *Ehrlichia canis* (McBride et al., 1999;
87 Ohashi et al., 1998a), and the major antigenic protein 1 (*map1*) in *E. ruminantium* (Sulsona et
88 al., 1999; van Heerden et al., 2004). As the molecules encoded by these genes are recognized
89 by the host immune system (Cheng et al., 2003; Jongejan and Thielemans, 1989; Li et al.,
90 2002; McBride et al., 1999), they have been exploited as potential targets for the development
91 of vaccines and serodiagnostic tests (Crocquet-Valdes et al., 2011; Feburay et al., 2017;
92 McBride et al., 1999; Nyika et al., 2002; Ohashi et al., 1998a; Peter et al., 2001). Several
93 lines of evidence suggest that host immune evasion could result from the differential
94 expression of individual genes within the paralogs (Ge and Rikihisa, 2007; McBride et al.,
95 2003; Ohashi et al., 2001; Reddy and Streck, 2000). Hence, for designing effective vaccines

and diagnostic tools, it is important to understand the diversity and evolutionary mechanisms of these multigene families.

Homologous recombination (HR) is a housekeeping mechanism associated with the maintenance of chromosome integrity and generation of genetic variability. HR was initially defined as the result of the sexual process in prokaryotes as in eukaryotes and was later acknowledged as a major DNA repair process. Both genetic and biochemical studies have revealed the crucial role that HR plays in all organisms in the repair of a variety of DNA damages of exogenous and endogenous origin (Kuzminov, 1999; Michel et al., 2004). Additionally, HR is essential for the bacterial genome by allowing integration of homologous alien DNA arising from transformation or conjugation (Smith, 1991; Lorenz and Wackernagel, 1994). It also helps adaptive mutations and removal of the deleterious mutations hitchhiking with them (Otto and Michalakis, 1998), thus allowing allelic recombination between closely related strains (Feil, 2004). Recombination between homologous segments in genomes leads to chromosomal instability (Hughes, 1999; Rocha, 2004), and among bacteria, the rate of chromosomal rearrangements correlates with the number of repeated sequences in genomes (Rocha, 2003). Further, intrachromosomal HR between large repeated regions is often adaptive, allowing the generation of genotypic diversity in pathogens (Finlay and Falkow, 1997; Mehr and Seifert, 1998; Rocha and Blanchard, 2002).

The *mapI* multigene family of *E. ruminantium* comprises 16 paralogs tandemly arranged in the genome (van Heerden et al., 2004). All the paralogs are maintained in the same order in the genomes of the *E. ruminantium* strains sequenced so far (Collins et al., 2005; Frutos et al., 2006; Nakao et al., 2016). Transcriptional analysis revealed that all the paralogs were

transcriptionally active when cultured in bovine endothelial cells (Bekker et al., 2005; van Heerden et al., 2004), while this was not the case in ticks and tick cell lines (Bekker et al., 2005; Postigo et al., 2007). Differential expression of MAP1-family proteins was also reported using different analytical approaches (Marcelino et al., 2012; Postigo et al., 2008). These findings may support the possible involvement of *map1* paralogs in adaptation to different host environments.

The diversity of *map1* paralogs has been analysed only for a limited number of strains so far (Barbet et al., 2009). The present study extended the comparison by including *E. ruminantium* strains from wide geographic origins to understand the evolutionary mechanisms of these polymorphic genes. The results indicated that recombination and purifying and balancing selection play a significant role in the evolution of *map1* paralogs.

3. MATERIALS AND METHODS

3.1 Bacterial strains

The *E. ruminantium* strains used in this study are listed in Table 1. All *E. ruminantium* strains were grown in bovine aorta endothelium cells and subjected to DNA extraction as described previously (Nakao et al., 2010). We also included two *Amblyomma variegatum* samples which were positive for *E. ruminantium* infection as reported previously (Nakao et al., 2011). Sequences encoding the locus of all 16 *map1* paralogs were either retrieved from the database Welgevonden (Erwe and Erwo), Gardel, Crystal Springs, Kerr Seringe, Pokoase 417 and Sankat 430) or determined by long PCRs followed by Sanger sequencing as described below.

3.2 Long-range PCR and Sanger sequencing of *map1* paralogs

Four long-range PCR assays were developed using primers amplifying approximately 20 kb of the genomic region spanning all 16 *map1* paralogs (Fig. 1). The primer sequences used for long-range PCR assays are listed in Table S1. PCR was carried out using high-fidelity PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced using primers listed in Table S1. The sequencing reactions were conducted using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the products were analysed on an ABI Prism 3130x genetic analyser (Applied Biosystems). The obtained sequences were submitted to the DNA Data Bank of Japan (DDBJ) (<http://www.ddbj.nig.ac.jp>) under accession nos. AB818931 to AB818946.

Downstream analysis

To detect the possible signatures of positive, negative, or neutral selection across *map1* family genes as well as those of HR we carried out the following analysis:

3.4 Sequence alignment and nucleotide diversity

Multiple sequence alignment was performed using MUSCLE implemented in MEGA7 (Kumar et al., 2016). To estimate genetic variability across the gene family, genetic diversity (nucleotide and haplotype diversity and the mean number of nucleotide differences) and their standard deviations were calculated for the entire dataset and for each gene. The number of haplotypes were determined using DNaSP v5 (Librado and Rozas, 2009). To infer the population demographic history and the dynamics of these ehrlichial

pathogens we measured the haplotype mismatch distribution patterns (Rogers and Harpending, 1992) for each family gene. Departures of the observed sum of squares differences (SSD) from the simulated model of expansion were tested with the chi-square test of the goodness of fit statistic and Harpending's raggedness index 'r' (Harpending, 1994) following 1000 coalescent simulations. Analysis of mismatch distribution patterns was supported by two coalescent-based estimators of neutrality; Tajima's D (Tajima, 1989) and Fu's F (Fu, 1997) statistics. The expected value for Tajima's D and Fu's F is zero, and both positive and negative deviations are informative about the distinct demographic and/or selective events. The significance of these two statistics was tested with 1000 coalescent simulations in Arlequin v3.5 (Excoffier and Lischer, 2010).

3.5 Detection of recombination breakpoints

From abundant algorithms and software tools designed to detect and analyse recombination, we used the Genetic Algorithm Recombination Detection (GARD) implemented in the Datamonkey 2.0 modern web application (Weaver et al., 2018) to analyse multiple-sequence alignments for recombination to estimate the number and location of breakpoints and segment-specific phylogenetic trees. The method searches for all possible breakpoints in the sequence alignment, infers phylogenies for each putative non-recombinant fragment, and assesses goodness of fit using Akaike Information Criterion (AIC) (Sugiura, 1978), an information-based criterion derived from a maximum likelihood model fit for each segment augmented by a genetic algorithm (GA) heuristic to quickly explore a large-state space (Kosakovsky Pond et al., 2006).

3.6 Detection sites under positive/negative selection

Single-Likelihood Ancestor Counting (SLAC) implemented in the Datamonkey web-server was executed to identify the sites evolving under the influence of positive, neutral, and/or negative selection. This method depends on a combination of maximum-likelihood (ML) and counting approaches by inferring the nonsynonymous (dN) and synonymous (dS) substitution rates on a per-site basis for a given coding alignment and corresponding phylogeny. The method assumes that the selection pressure for each site is constant along the entire phylogeny. The default significance cut-offs used are $P = 0.1$ for SLAC and posterior probability = 0.9. Additionally, dN and dS were also calculated using MEGA7.

4. RESULTS

1.4 Diversity and population demography

A large number of haplotypes and a high level of haplotype diversity was observed across the dataset (Table 2; Figs. S1 & S2, with *map1*, *map1-4*, *map1-5* and *map1-14* scored 11 haplotypes in each, and the same scored high haplotype diversity = 0.97). The mismatch distribution pairwise differences pattern under sudden or spatial expansion revealed bi- and multimodal shapes with significant deviation of the goodness-of-fit, Sum of Square Deviation, and Harpending's raggedness index (Table S2 & Fig. S3A-O). The positive value of Tajima's D is the result of an excess of intermediate frequency alleles that indicates either population bottlenecks or structure and/or balancing selection, of which we assume the latter (see the section 4.4 and Fig. 2). Negative values of Tajima's D for *map1-4* and *map1-3* designate an excess of low frequency alleles indicating positive selection of these two genes (Fig. 2).

4.2 Homologous recombination

HR was detected in seven genes of the family of *map1*. Among them, *map1* scored the highest recombination breakpoints ($r = 262$) followed by *map1-2* ($r = 208$), and this dropped dramatically to 106, 101, and 100 in *map1-6*, *map1-5*, and *map1-14*, respectively (Fig. 3). The least recombination breakpoints detected were in *map1-10* ($r = 30$) and *map1-1* ($r = 29$). The actual location of the recombination breakpoints is illustrated in Fig. 4, for the four highly recombining genes. For instance, *map1* has shown three locations of breakpoints around 145, 220, and 280, whereas, *map1-2* has shown four locations of breakpoints around 95-100, 330-350, 500, and 590.

4.3 Negative diversifying selection

We detected negative purifying selection in 12 out of 16 genes and no single positive selection was detected (Fig. 5). Again, *map1* scored the highest negative selection with the selection site being 48 compared to the following *map1-2* that only scored 8 selection sites (Fig. 5). Five genes (*map1-1*, *map1-5*, *map1-10*, *map1-12*, and hypothetical gene (HG)) did not show either negative or positive selection, indicating neutral selection. This result was supported by separate analysis of the dN and dS nucleotide substitution that also displayed the same order (Fig. S4).

4.4 Balancing selection

We assumed a balancing selection for the following reasons: first, the excess of dN polymorphisms represents either balancing or purifying selection (Table 2) (Fijarczyk et al., 2016); second, a positive Tajima's D value for all the gene family except two genes, *map1-3* and *map1-4*, that are under directional positive selection (Fig. 2) (Fijarczyk et al., 2016);

third, an excess of polymorphisms and high frequencies of segregating sites, as indicated clearly in Table 2. The purifying selection detected can be explained by the purifying balancing selection as shown in Fig. 5, where *map1* also scored the highest in purifying balancing selection. In contrast, *map1-3* and *map1-4* were found to experience purifying directional selection.

The patterns of genetic variation are shaped by two stochastic events that are the history of coalescent events and the history of mutational events. Under neutrality, mutations are uniformly distributed along the branches of a genealogy, and therefore, the number of mutations occurring on a branch is proportional to its length (see Fig. 6A-D), the time to coalescence is longer in (A), and moderate in (B) or short in (C & D), depending on the time required to reach this balancing selection for A and B. Considering recombination events, different regions of the genome can have distinct gene genealogies.

DISCUSSION

Negative/purifying or background selection plays a substantial role in maintaining the long-term stability of *E. ruminantium* populations across different countries by removing deleterious mutations. Therefore, this type of selection is more prevailing for the success of evolution in optimizing the functions of an organism. Purifying selection ensures that deleterious mutations cannot take over a population and that any improved structures when fixed in a population are maintained as long as they are required. Host-parasite interactions are a renowned example of this type of situation. In this regard, the host immune system evolves to recognize a special structure on the parasite and allows its removal. This in turn

induces negative selection on the current form of the parasite while leading to positive selection of variants that cannot be recognized by the host (Charlesworth et al., 1995).

In this study, a strong purifying selection was illustrated in *map1* gene compared to other family genes, indicating that this gene plays an essential functioning role in the immune system. On the other hand, other members of the gene family demonstrated varied levels from moderate to weak purifying selection, which could be explained as a signature of functional orchestration that results in each family gene playing parts with varying degrees. This negative/purifying selection is the main evolutionary force that has shaped most of the outer membrane gene families and could be explained by the demographic history of longstanding pathogen pressure.

We also detected balancing selection on this *map1* gene family, a type of selection that is known to maintain the genetic variation in immunity genes. Balancing of host–parasite coevolution is well documented in literature (Charlesworth and Charlesworth 2010; Eizaguirre et al., 2012; Phillips et al., 2018). This coevolution between hosts and pathogens results in balancing selection by maintaining the genetic diversity at immunity genes such as the *map1* family. In other words, *E. ruminantium* overcomes the host immune system by maintaining extensive and strong genetic diversity in *map1* gene among the 16 genes in the family, with moderate to weak diversity in other family genes in infected cattle hosts, thus trying to escape recognition by the immune system of the cattle host.

It is well known that balancing selection is expected to operate and maintain high variation in some immune genes through mechanisms of overdominance, negative frequency-dependent selection, or temporally and spatially fluctuating selection. All these are expected to work on *map1* family genes, operating with various degrees in these paralogous

277 genes that play a major role in balancing selection plus negative purifying selection in the
278 evolution of these immunity-related genes.

279 Selection here tends to predominantly maintain *map1* and to some extent *map1-2* and
280 *map1-6* (Fig. 4). The genetic signature of balancing selection is an excess of polymorphic
281 sites at intermediate (balanced) frequencies, relative to expectations under neutrality
282 (Weedall and Conway, 2010). This balancing selection seems to be involved in maintaining
283 diversity at *map1* that coordinates recognition between the self and non-self (Richman and
284 Kohn, 1999), which can be considered as a good immuno-diagnostic or vaccine candidate
285 gene.

286 It has been previously documented that *E. ruminantium map1* variants are not
287 geographically constrained and show no evidence of having evolved under positive
288 selection pressure (Allsopp et al., 2001). Additionally, Hughes and French (2007) reported
289 evidence for HR in the *map1* gene, which supports our current findings. Besides the above,
290 we also demonstrate a greater HR rate at *map1* (Figs. 3 and 5) indicating a higher genomic
291 adaptation in this region. This along with ongoing balanced selection forces that are purged
292 by “purifying” any lower fitness “mutants” produced, prevents them from accumulation. In
293 contrast, wherever recombination is low there is a greater density of selective variants that
294 do not segregate freely, lowering the efficiency of selection and consequently the
295 adaptation rate. It appears that recombination increases proportionally with the intensity of
296 selection at the loci/gene subject to recombination. Many reports revealed a positive
297 correlation between recombination and adaptation. For instance, Levin and Cornejo (2009)
298 simulated mutation, recombination, selection and inter-population competition to explore
299 the conditions under which: i) recombination augments the rates of evolution in bacterial

300 populations and, ii) when the capacity for HR is favoured in competition with non-
301 recombining populations. They demonstrated that under broad conditions, HR occurring at
302 rates in a range estimated for *Escherichia coli*, *Haemophilus influenza*, *Streptococcus*
303 *pneumoniae*, and *Bacillus subtilis* can increase the rate of adaptive evolution in bacterial
304 populations.

305 It appears that recombination increases the rate at which populations adapt to their
306 environment, whereby the capacity for shuffling homologous genes within a population
307 provides an advantage to the recombining strain when competing with populations without
308 this capacity. Commonly, recombination in bacteria is a rare event and not a part of the
309 reproductive process. Nonetheless, recombination is broadly defined to include the
310 acquisition of genes from external sources via horizontal gene transfer (from other
311 populations of bacteria of the same and different species, as well as from eukaryotes and
312 archaea) and plays a central role as a source of variation for adaptive evolution in many
313 bacterial species. In this manner, bacteria can expand their ecological niches by expressing
314 the genes and genetic elements obtained from other bacterial populations of the same and
315 different species, as well as from eukaryotes and archaea. This scenario may be ideal
316 especially in vector-borne bacteria where diverse populations of microbiota and other
317 organisms may be encountered.

318 Recombination in the form of the receipt and incorporation of genes and genetic elements
319 from other strains and species of bacteria (Mazodier and Davies, 1991) as well as archaea
320 and eukaryotes (Nelson et al., 1999; Brown, 2003) plays a prominent role as a source of
321 variation for the adaptive evolution of many bacterial species (Lawrence and Ochman,
322 1998; Ochman et al., 2000; Gal-Mor and Finlay, 2006). However, Bekker et al. (2005)

found that a recombination between two *map1* genes, namely at *map1-3* and *map1-2*, had occurred in one subpopulation with deletion of one entire gene.

As previously noted, the *E. ruminantium map1* multigene family is regulated differently in the host and tick cell environments subject to recombination causing an altered gene arrangement and different transcriptional activities (Bekker et al., 2005). This has also been demonstrated in malaria parasites, where the stage of the parasite and the period of exposure to the immune system determine the pattern of selection (Weedall and Conway, 2010). These observations support our results in which a similar pattern of evolution in the *E. ruminantium map1* gene family is observed across different countries and time points suggesting the coevolution of these parasites in their host and the vector.

CONCLUSION

Here, we identified negative and balanced polymorphism selections in the *map1* multigene family, a selective pressure that has shaped *E. ruminantium* in response to environmental changes over time and space, most noticeably observed in *map1* followed by *map1-2* and to a lesser extent in the other gene family members.

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TABLES

Table 1. *Ehrlichia ruminantium* strains used in this study.

Strain	Geographical origin	Year of isolation	Reference
Crystal Springs	Zimbabwe	1990	(Byrom et al., 1991)
Gardel	Guadelope, Caribbean	1982	(Uilenberg et al., 1985)
Ifé Nigeria	Nigeria	1983	(Ilemobade et al., 1978)
Kerr Serigne	Gambia	2001	(Faburay et al., 2005)
Kwanyanga	South Africa	NR ^a	(Mackenzie and van Rooyen, 1981)
Lutale	Zambia	1986	(Jongejan et al., 1988)
Pokoase 417	Ghana	1996	(Bell-Sakyi et al., 1997)
Sankat 430	Ghana	1996	(Bell-Sakyi et al., 1997)
Um Banein	Sudan	1981	(Jongejan et al., 1984)
Welgevonden (Erwo)	South Africa	1985	(Plessis, 1985)
Welgevonden (Erwe)	South Africa	1985	(Plessis, 1985)
Zeerust	South Africa	1979	(Jongejan et al., 1980)
Uganda P016 ^b	Uganda	2008-2009 ^c	(Nakao et al., 2011)
Uganda T020 ^b	Uganda	2008-2009 ^c	(Nakao et al., 2011)

^aNR, not recorded. ^bTick ID. ^cYear of tick collection.

356 **Table 2: Genetic diversity indices of *map1* family genes.**

Gene	L	N	H	H. D	S	(π)	π (SS)	π (NS)
HG	716	14	7	0.8132	30	0.017(0.009)	27	8
<i>map1</i>	972	14	11	0.9670	117	0.124(0.063)	182	83
<i>map1-1</i>	858	14	9	0.9011	28	0.011(0.006)	28	1
<i>map1+1</i>	849	14	8	0.9451	33	0.022(0.011)	34	6
<i>map1-2</i>	966	14	9	0.9231	89	0.107(0.054)	115	44
<i>map1-3</i>	972	14	8	0.8901	48	0.019(0.010)	57	25
<i>map1-4</i>	906	14	11	0.9560	50	0.016(0.008)	50	14
<i>map1-5</i>	648	14	11	0.9560	57	0.064(0.033)	65	23
<i>map1-6</i>	900	14	9	0.9121	62	0.052(0.027)	74	25
<i>map1-7</i>	922	14	8	0.8242	37	0.017(0.009)	35	10
<i>map1-8</i>	859	14	9	0.8791	29	0.016(0.009)	30	12
<i>map1-9</i>	872	14	10	0.9451	31	0.013(0.007)	30	3
<i>map1-10</i>	781	14	6	0.8352	22	0.012(0.007)	21	9
<i>map1-11</i>	886	14	9	0.9231	26	0.012(0.007)	25	7
<i>map1-12</i>	831	14	8	0.8901	31	0.013(0.007)	31	3
<i>map1-13</i>	895	14	8	0.9011	57	0.031(0.016)	62	11
<i>map1-14</i>	984	14	11	0.9670	31	0.039(0.020)	34	16

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358 L, length (bp). N, number of analysed sequences. H, number of haplotypes. HG,
359 hypothetical gene. S, number of segregating sites. π , pairwise sequence diversity (Jukes
360 and Cantor). SS, number of synonymous substitutions. NS, number of nonsynonymous
361 substitutions.

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574 **FIGURE LEGENDS**

575 **Figure 1. A schematic representation of *map1* family genes.**

576 **Figure 2. Tajima's D neutrality test scenario for *map1* family genes.** Positive and
577 negative values indicate balancing and directional selections, respectively.

578 **Figure 3. The number of homologous recombination points (potential breakpoints) in**
579 ***map1* family genes.** It illustrates that *map1* and *map1-2* are experiencing the highest
580 recombination.

581 **Figure 4. Actual locations of recombination breakpoints in the four highly**
582 **recombining genes.** Three positions of breakpoints in *map1* and *map1-2* were observed at
583 locations 150, 225, 275-280, and 95, 340-350, 505, respectively. Only two positions of the
584 breakpoints in *map1-6* and *map1-14* were observed at locations 490 and 495, and 190 and
585 200, respectively.

586 **Figure 5. Detection of negative/purifying selection in *map1* family genes.** It indicates
587 that *map1* was subjected to the highest amount of purifying selection compared to the
588 following gene *map1-2* and the rest of the gene family.

589 **Figure 6. Genealogies and the effect of balancing selection.** The trees show the effect
590 balancing selection on tree topologies, giving rise to long internal branches proportional to
591 the amount of selection in *map1* (A) and *map1-2* (B). No balancing selection was reflected
592 in the short internal branches as seen in *map1-10* (C) and *map1-12* (D).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

A: *map1*

B: *map1-2*

C: *map1-6*

D: *map1-14*

Fig. 5

Fig. 6