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11. The effect of recombination in *Aeromonas*

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Abstract. Although several approaches have been attempted, the estimation of recombination frequencies in natural populations of bacteria remains challenging. Previous studies have demonstrated a wide variety of situations among bacterial species, ranging from the clonal diversification of *Salmonella* or *Escherichia coli*, which are mainly due to mutation, to the frequent recombination found in *Neisseria gonorrhoeae* or *Helicobacter pylori*. Most of the population studies done with bacterial species suggest that recombination occurs in nature but that it is infrequent compared to mutation. Consequently, bacterial populations consist largely of independent clonal lineages. Our research suggests little or null influence of recombination in the genetic structure of '*Aeromonas hydrophila* Species Complex', despite the presence of some strains with recombinant gene fragments.

Introduction

Bacteria reproduce asexually, giving two identical individuals after their division, with the exception of changes produced by mutation or recombination. Although this reproduction process is not associated with

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recombination, in contrast with eukaryotes, bacteria have acquired three basic mechanisms by which they can incorporate genes from other bacterial species: transformation, conjugation, and transduction. Nevertheless, although bacteria might incorporate foreign genes from other species, their genomes are not simply arbitrary assortments of genes of mixed heritage. Once the bacterial cells acquire genes by means of one of these mechanisms, they have to be incorporated into a replicon; if not, the introduced genes would become diluted in the population or degraded by the restriction endonucleases. Bacterial interchange promotes the acquisition of novel genetic elements, the impact of which has been extensively studied in human and animal pathogens and commensals, where they are often associated with the emergence of new phenotypes [1]. Recombination in bacteria is: always restricted to small DNA fragments, unidirectional, independent of reproduction, and occurs with a relatively low frequency, although genes codifying virulence factors or antibiotic resistance experiment more frequent recombination changes. The incorporation of genes or parts of genes through recombination always results in mosaic genomes that are composed of regions with different evolutionary histories [2]. Homologous recombination is widespread in the genomes of many bacteria, and is usually a consequence of *recA* mediated homology-dependant recombination. When the incorporated fragment replaces an identical DNA sequence its effect cannot be detected, although the process seems to be very frequent when the two bacteria involved in this interchange are closely related. Homologous recombination in this case might play a crucial role in DNA repair [3]. On the contrary, if recombination has a measurable effect on the genome of the recipient, it is considered as an effective recombination event.

The impact of recombination on bacterial phylogenies has been the subject of considerable discussion [4-9]. Recently, with the availability of sequencing techniques and the analytical power of new programmes, the detection of recombination events has increased dramatically. This has led to the questioning of existing phylogenies and the methods used for their construction, such as Maximum Likelihood (ML) and Maximum Parsimony (MP), which assume that the analyzed sequences have the same evolutionary history. The presence of recombination would break this assumption, since in this case sequences would have different underlying phylogenies that are more easily envisaged as a network rather than a tree. Due to the importance of recombination in evolutionary analysis, it is essential to be able to identify whether a given set of sequences has undergone recombination events, define the boundaries of the recombinational units, and evaluate the impact of recombination on our ability to reconstruct evolutionary histories and estimate population genetic parameters.

To investigate genetic exchange in bacteria, large data sources have been used, such as those deposited in the Multi Locus Sequence Typing (MLST) databases at www.mlst.net [10]. Multilocus data allow us to determine the degree of recombination based on the type of population structure. If the population shows a clonal structure (linkage disequilibrium), then recombination is absent and the accumulated genetic changes will be a consequence of mutation. However, in the case of bacteria, this assumption is not always true and the clonal structure is not always broken, even if a certain degree of recombination is present [11, 12].

The increasing availability of whole genome sequences has enabled a more complete study of the recombination process in bacteria. The genes sequenced in a MLST study (usually six or seven) are not always representative of the entire genome, and can give biased results. The analyzed sequences do not correspond to the complete gene, only fragments of about 400-500 bp, so the changes determined cannot be representative of the full gene, particularly in the case of a protein-codifying gene. Unfortunately, full genome sequences that could obviate all these questions are still usually limited to a few isolates of each species and frequently have been chosen for specific reasons (clinical, environmental or industrial, etc.), so are not representative of the entire population.

In this study we will consider the impact of recombination on bacterial phylogenies and the consequences of inaccurate approaches to inferring phylogenetic relationships. Traditionally, recombination in a given set of sequences has been identified by the incongruence of the different gene trees analyzed, the presence of mosaic structures, and variations in the G+C content or the codon bias. Several methods have been developed to test the presence of recombination in a given set of sequences, and to identify the parental and recombinant individuals or the recombinational break-points. Those methods can be classified in different categories: similarity, distance, phylogenetic, compatibility, and nucleotide substitution distribution [13, 14]. The performance of these methods varies depending on the level of recombination, but in general most of them are efficient, and although they can have trouble in detecting recombination when the level of divergence is low, their discriminatory power increases when the level of recombination is high [5]. In addition, methods that use the substitution patterns or incompatibility among sites seem to be more powerful than those based on phylogenetic incongruence. This might be partially explained by the fact that, in general, phylogenetic methods can only detect recombination events that change the topology of the tree, and at high recombination rates there should be many such events [5]. What is important is to increase the chances of detecting recombination while minimizing the detection of false positives, so

the chosen method will depend on the level of divergence among the sequences analysed [15, 16]. In either case, the best option is not to rely on a single method to detect recombination [13, 17].

1. Recombination in bacteria

Although several approaches have been attempted, the estimation of recombination frequencies in natural populations of bacteria remains challenging. One of the parameters commonly used is the determination of the rate of recombination relative to mutation [18], but this is not always easy to calculate, except in the case of the most recent events [4], which might not be representative of the entire history of the population.

The determination of the relative importance of mutation in comparison with recombination is central to bacterial population genetics [18]. Previous studies have demonstrated a wide variety of situations among bacterial species, ranging from the clonal diversification of *Salmonella* [19] or *Escherichia coli* [20], which are mainly due to mutation, to the frequent recombination found in *Neisseria gonorrhoeae* [21] or *Helicobacter pylori* [22]. Most of the population studies done with bacterial species suggest that recombination occurs in nature, and indeed may be highly important in generating variation, but that it is infrequent compared to mutation. Consequently, bacterial populations consist largely of independent clonal lineages.

Comparison of results from analyses performed with different methodologies is problematic; nevertheless, studies using the same methods across different genera have suggested wide variation in recombination rates with value differences of two or three orders of magnitude [23]. In addition, conflicting levels of recombination have been obtained for concrete bacterial species, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* or *Haemophilus influenzae* [24], depending on the sampling and analytical methodologies used. The analyzed isolates have to be sampled carefully in order to be informative about the underlying recombination process. If they are not representative of the whole population, an important bias might be introduced. Unfortunately, in most of the populations studied, particularly in the case of pathogen bacteria, samples are not fully representative, usually with an overrepresentation of virulent strains, which are frequently under higher selective forces than the corresponding non-virulent strains.

Recombination studies using whole genome data have contributed to a better understanding of recombination in bacteria. Several studies have reported differences in the prevalence of recombination at different regions of the same bacterial genome [25], being apparently higher in those genes under

positive selection [25, 26]. Genomic regions encoding proteins with a role in pathogenicity are often under positive selection and frequently exhibit high rates of recombination, even in the case of bacteria in which recombination is relatively rare [28, 29]. A possible explanation for the relative prevalence of recombination in positive selected regions of the bacterial genome is that the only observable recombination is likely to be the one that unites beneficial mutations and removes deleterious ones.

2. The genus *Aeromonas*

The genus *Aeromonas* Stanier 1943 belongs to the family *Aeromonadaceae* within the class *Gammaproteobacteria* [30]. Aeromonads are autochthonous inhabitants of aquatic environments, including chlorinated and polluted waters, although they can also be isolated from a wide variety of environmental and clinical sources. They cause infections in vertebrates and invertebrates, such as frogs, birds, various fish species, and domestic animals. In recent years, some authors have considered *Aeromonas* as an emergent pathogen in humans, producing intestinal and extraintestinal diseases. Aeromonads are facultative anaerobic chemoorganotrophs capable of anaerobic nitrate respiration and dissimilatory metal reduction [30].

Several attempts have been made to generate phylogenies using DNA gene sequences to reconstruct the correct genealogical ties among species in *Aeromonas* [31-33], but the genes chosen for this purpose are not always suitable, and do not necessarily give congruent phylogenies [34, 35]. Recently, two papers presenting MLST schemes for *Aeromonas* have been published [10, 36], and there is an online MLST database for the genus *Aeromonas*, managed by Keith Jolley and curated by Barbara Cardazzo (http://pubmlst.org/aeromonas). All this accumulated data should help to establish a reliable clustering of the *Aeromonas* species and elucidate their exact boundaries.

Finally, the availability of complete genomes of different species is also useful in this task, but unfortunately, in the case of *Aeromonas*, only six genomes have been completed to date, corresponding to: the type strain of *A. hydrophila* ATCC 7966, isolated from a tin of milk [37]; the strain A449 of *A. salmonicida*, a fish pathogen described by Reith *et al.* ([38], Fig. 1); an *A. caviae* strain Ae398 isolate from a stool sample [39]; an *A. veronii* strain B565 isolated from an aquaculture pond sediment [40]; and more recently, an *A. aquariorum* strain AAK1 isolated from blood [41] and the highly melanin-yielding *A. media* strain WS [42]. The information given by the genomes of *A. hydrophila* and *A. salmonicida* indicates that while they are of identical size (4.7Mb) and share multiple housekeeping and virulence genes, *A. salmonicida*

has acquired several mobile genetic elements, and undergone genome rearrangements and loss of genes in the process of adapting to a specific host. The genome of *A. veronii* is smaller (4.3Mb) and contains fewer virulence genes. Similarly, *A. caviae* presents a small genome (4.43Mb), but in contrast to *A. veronii* several putative virulence genes have been identified, as in *A. aquariorum*, which has the biggest genome reported for *Aeromonas* (4.81Mb). The last genome completed corresponds to an *A. media* strain (4.3Mb) in which no virulence genes have been reported.

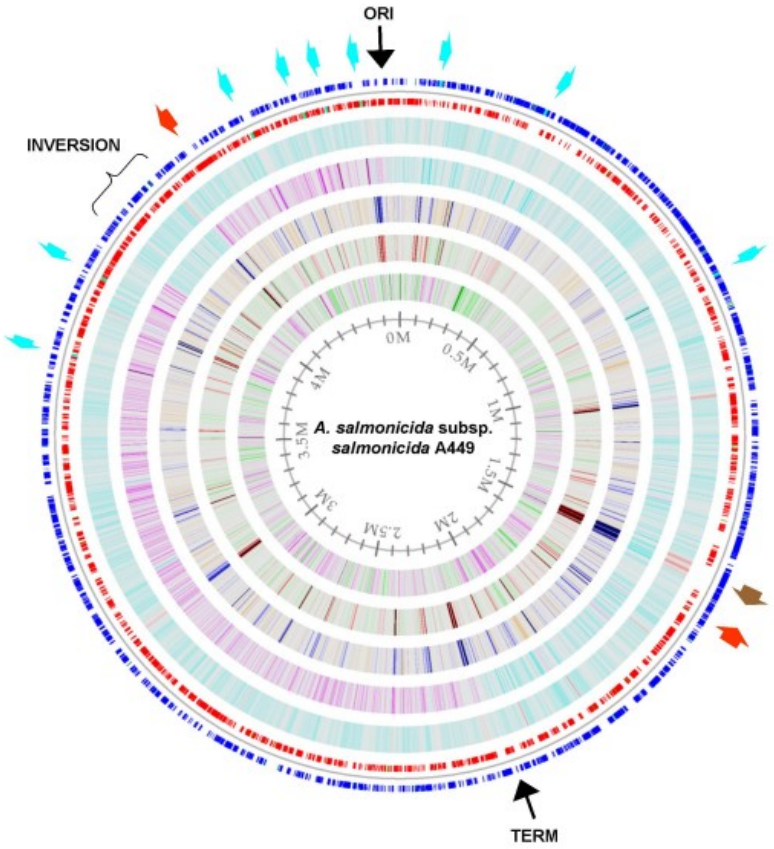

Figure 1. A genome atlas representation of the *A. salmonicida* subsp. *salmonicida* A449 chromosome (ref. [38]).

3. The '*Aeromonas hydrophila* species complex'

An example of the taxonomic complexity of the genus *Aeromonas* is the difficulty in discriminating between the phenotypically and genetically closely related species belonging to the "*Aeromonas hydrophila* species complex" (AHC), which includes: *A. hydrophila*, composed of three subspecies: *A. hydrophila* subsp. *hydrophila, A. hydrophila* subsp. *ranae* and *A. hydrophila* subsp. *dhakensis*, *A. bestiarum*, *A. popoffii*, and *A. salmonicida,*

divided in five subspecies: *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *achromogenes, A. salmonicida* subsp. *pectinolytica*, and *A. salmonicida* subsp. *smithia* [30, 43]. Recently, two additional species have been described in this group, *A. aquariorum* and *A. piscicola* [44, 45]. Members of the AHC were first described as strains producing the enzymes elastase, lecitinase or stapholysin [46]. They are genetically closely related and share multiple phenotypic characteristics, which makes discrimination among the species included in this group extremely difficult [43].

In order to establish the population structure and divergence of the species included in the AHC group, Fusté *et al.* [12] studied a set of representative strains, in which they analyzed the nucleotide sequences (total or partial) of 6 housekeeping genes. The authors concluded, from the linkage disequilibrium analysis and sequence divergence results, that the AHC is composed of four robust groups that basically correspond with the phenotypically described species *A. hydrophila*, *A. bestiarum*, *A. popoffii*, and *A. salmonicida*, in which recombination, if present, does not break their clonal structure.

4. Population structure and recombination in *Aeromonas*

The few references in the bibliography dealing with recombination in *Aeromonas* reach similar conclusions about its low incidence, with the exception of the study by Silver *et al.* [35], which reports a notable effect of recombination in the "*A. veronii* species complex". In this study, the strains were obtained from patients, veterinary samples, and medicinal leeches. The aligned sequences were investigated for evidence of recombination because the maximum-likelihood inferred phylogenies for each gene family showed low bootstrap support for most clades. Appling two tests for recombination, employing a variety of approaches, it was demonstrated that at least for some strains, horizontal gene transfer occurs at a sufficient frequency to blur the signal from vertically inherited genes, despite strains being adapted to distinct niches.

Martino *et al.* [11] analyzed a collection of *Aeromonas* including 23 type and reference strains, and 77 strains isolated from fish, crustaceous and molluscs in a MLST study using 6 housekeeping genes. Based on an eBURST analysis, the authors determined the recombination/mutation (*r/m*) ratio for the entire population and for the three major groups identified. The *r/m* values obtained (ranging between 0.07 and 0.13) suggest a reduced rate of recombination. Analysis with the SplitsTree program revealed that most of the genes, although showing a reticulate network, were not significantly affected by intragenic recombination with the exception of *recA*. When a set

of programmes included in the RDP3 package were applied to the *Aeromonas* sequences, several recombination events were identified supported by at least three of those programmes. Nevertheless, they concluded that, in the case of *Aeromonas*, the impact of recombination may be negligible, based on the very similar topologies of the phylogenetic trees, the low *r/m* rates, and the reduced network structure determined by the split decomposition analysis.

Roger *et al.* [47], in a MLST study that includes isolates from different origins but with a particularly high representation of clinical strains, determined that the standardized I_A (I_A^S) values showed the existence of significant linkage disequilibrium, indicative of a clonal population structure. When using at least four methods of the RDP software, they detected some recombination among the population in all but two of the seven loci analyzed. In addition, the use of split decomposition determined that most of the sequence types (STs) were not affected by recombination, even though more recombination events were found within the clonal complexes, particularly for the STs in the *A. caviae* clade. Differences in branching order were observed in both distance and ML trees when gene and concatenated sequence trees were compared, suggesting the occurrence of recombination. The authors conclude that recombination is present in the genus *Aeromonas*, at least in some strains, but at a relatively low frequency.

Finally, our group determined the genetic population structure of a group of *Aeromonas* corresponding to the AHC [12], which had been previously analyzed by enzyme electrophoresis (MLEE), revealing a clear clonal structure with strong linkage disequilibrium among 15 different protein loci [48]. We used a higher number of AHC isolates including representatives of *A. piscicola* [45] and *A. aquariorum* [44], two more recently described strains grouped within the AHC. The I_A^S values obtained in this study were different from 0 in all cases, indicating the absence of recombination and again, revealing strong linkage disequilibrium when considering both the total population and the different sets of species. This is in spite of the high number of alleles per locus and polymorphic sites and huge genetic diversity.

During the last years, with the availability of the first DNA sequence data of individual genes, evidence of recombination at the molecular level has accumulated for *Aeromonas* in housekeeping genes such as *dnaJ*, *gyrB* and *recA* [11, 35, 47] or structural and accessory genes [35]. In our study we have also determined the presence of potential recombinant fragments in the *recA* and *dnaJ* genes of some strains. However, although these strains cluster separately when the corresponding gene tree is constructed, revealing a possible different origin of the gene fragments, they group together with the other strains of the same species when a concatenated tree is generated. This confirms that recombination is not sufficient to break the genetic cohesion of this group.

5. Intragenic recombination in the *dnaJ* gene of the '*Aeromonas hydrophila* species complex'

We have recently studied the possible existence of recombination in the *dnaJ* gene (Fig. 2) in an AHC group with 90 strains (87% of environmental and 13% of clinical origin). Group I included 29 *A. salmonicida* strains (5 subspecies), Group II 31 strains (22 *A. bestiarum*, 5 *A. popoffii* and 4 *A. piscicola*) and Group III 30 strains (26 *A. hydrophila* and 4 *A. aquariorum*). Gene sequences used were obtained from our previous work [12]. We added a new strain *A. hydrophila* JCM 3968 (GenBank accession numbers: JN711671 (*dnaJ*), JN711562 (*cpn60*), JN711795 (*gyrB*), KC525968 (*mdh*), KC525969 (*recA*) and JN712375 (*rpoD*)). We determined the presence of mosaic structures, different G+C content and codon usage bias in the sequences but none of the results were conclusive.

In our study we also detected incongruences in the phylogenies when the *dnaJ* gene and the concatenated trees were compared (Fig. 3). Five strains, 2 *A. bestiarum* (orange), 1 *A. hydrophila* (blue), and 2 *A. salmonicida* (green), clustered out of the corresponding species group in the *dnaJ* gene tree (Fig. 3a), revealing a possible different origin of the gene fragments. Nevertheless, they grouped together with the other strains of the same species when a concatenated tree was generated (Figs. 3b and 4).

Consequently, we also analyzed our sequences using six recombination detection programmes in the RDP4 package, which significantly detected possible recombination events in one (*A. salmonicida* CECT 5214) out of the five strains, when all strains or the corresponding species subgroups were analyzed (Table 1). The investigation also provided well-supported evidence for recombination in two *A. hydrophila* strains (CECT 4330 and CECT 5734), which clustered among the other *A. hydrophila* isolates in the *dnaJ* and the concatenated trees, although they were separated in a deeper branch. No recombination events were detected among the *A. bestiarum* isolates (Group II).

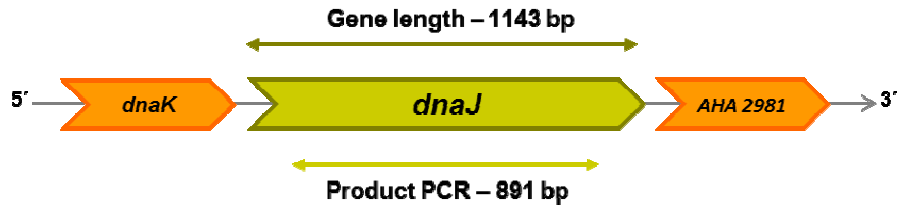

Figure 2. Schematic representation of the *dnaJ* locus and its flanking regions for *Aeromonas*, based on the whole genome sequence of *Aeromonas hydrophila* ATCC 7966^T (GenBank accession number CP000462, [37]). Partial sequences for *dnaJ* (891 bp) were obtained from the GenBank database or determined as previously described [49].

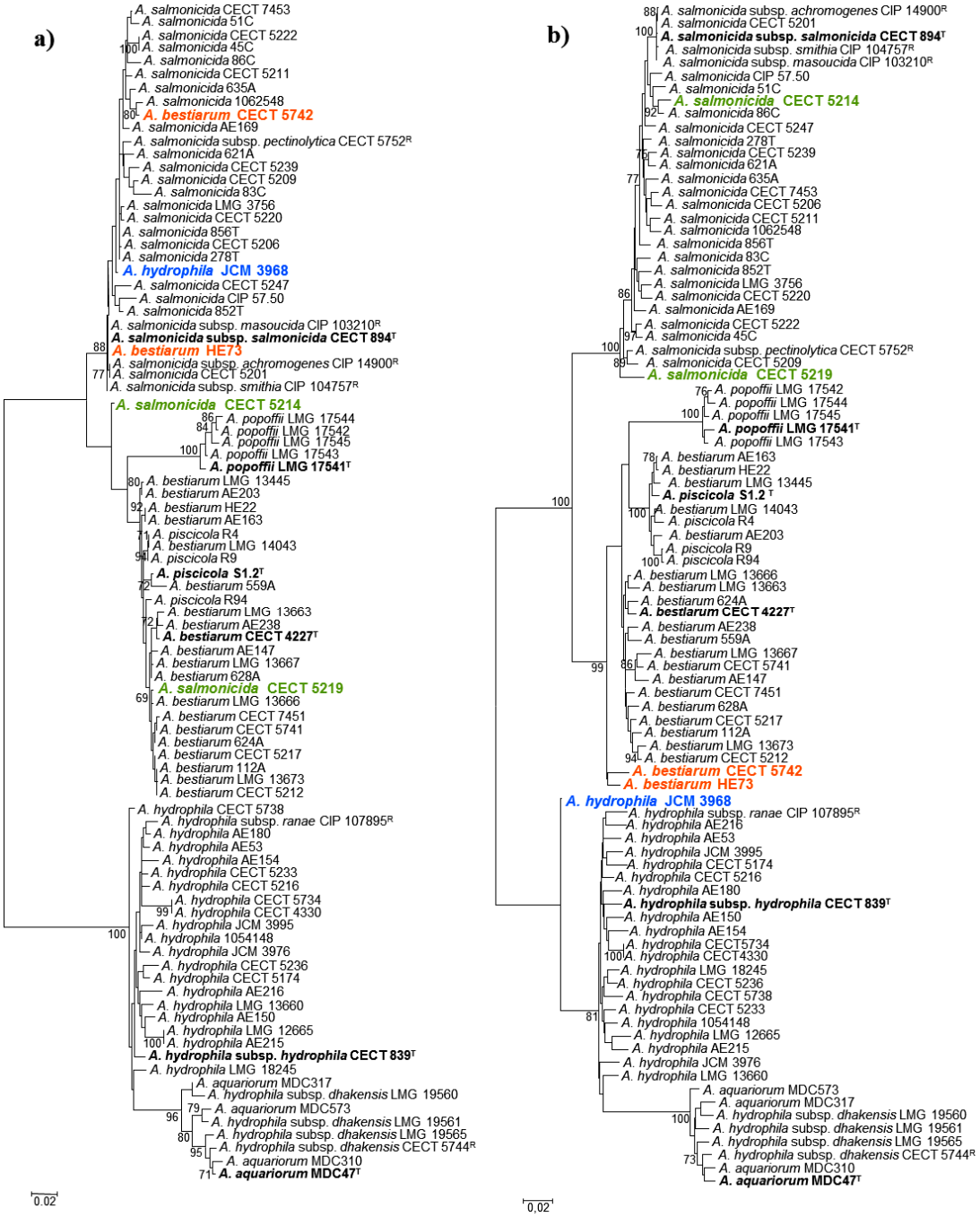

Figure 3. Phylogenies of AHC species inferred from single and concatenated genes: a) Maximum likelihood (ML) tree obtained from *dnaJ* sequences (891 positions) based on the Tamura-3-parameter (T92+G+I) as a model of nucleotide substitution; b) ML tree from concatenated sequences of six genes (5,379 positions) based on the Tamura-Nei model (TN93+G+I). ML trees were constructed using MEGA5 software (http://www.megasoftware.net, [50]). Bootstrap values (≥70%) from 500 replications are shown at the nodes of the tree. The scale bar indicates the number of nucleotide substitutions per site. The type strains of *Aeromonas* species belonging to AHC are indicated in bold. The five strains of the AHC in which we detected incongruences in the *dnaJ* tree are shown in colours.

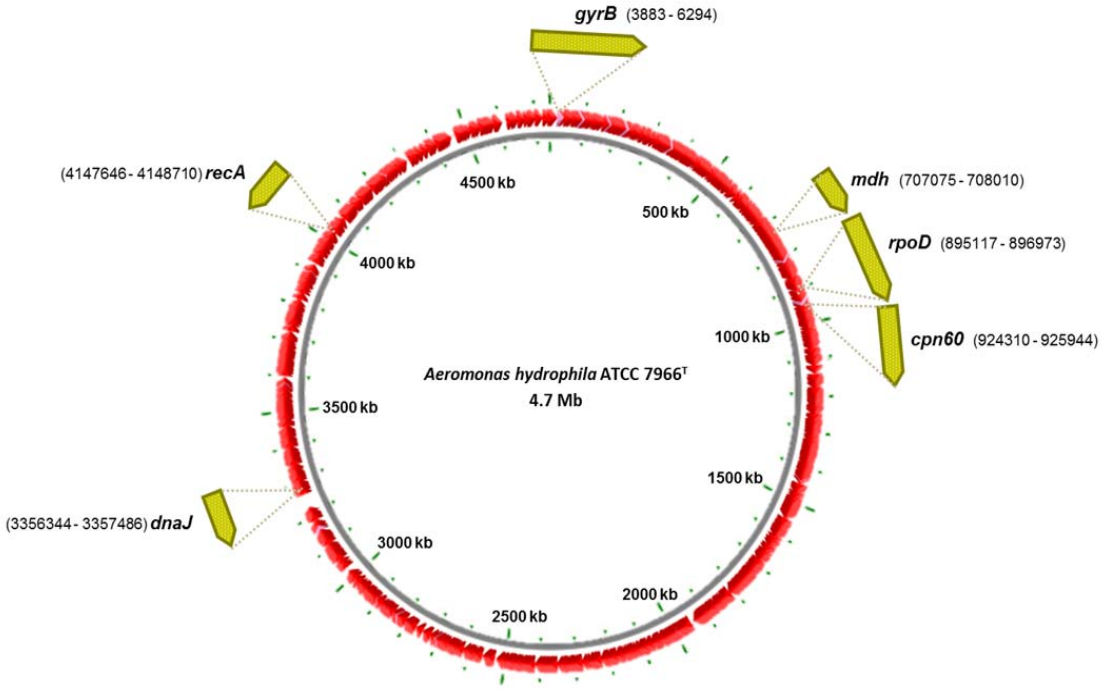

Figure 4. Distribution of six genes sequenced in the circular map of the genome of *A. hydrophila* ATCC 7966^T (GenBank accession number CP000462, [37]). Genes are shown outside the circle and have standard abbreviations. Arrows indicate direction of transcription. Detailed genomic position is listed in parentheses after each gene name.

Table 1. Recombination analysis summary.

	Recombination sequence	Number of methods detecting recombination events with statistical significance	Recombination detection methods						Events
			1	2	3	4	5	6	
***All strains* N=90**									
	A.salmonicida CECT 5214	3	■		■			■	1
Group I *A. salmonicida* **N=29**									
	A.salmonicida CECT 5214	4		■	■	■		■	1 or 2
	A.salmonicida CECT 5219	1				■			1
Group III *A. hydrophila* **N=30**									
	A.hydrophila CECT 4330	2				■		■	1
	A.hydrophila CECT 5734	2				■		■	1

Automated screening for recombination from multiple alignment of *dnaJ* sequences was performed using programme RDP4 (http://darwin.uvigo.es/rdp/rdp.html, [51]), which used six recombination detection programmes: RDP (1), GENECONV (2), BootScan (3), MaxChi (4), Chimaera (5) and 3Seq (6), with their default parameters. Sequences statistically supported by at least two recombination detection programmes (*P*-value <0.05) were considered as possible recombinants.

6. Conclusions

Developments in gene sequence analysis have greatly enhanced the study of recombination in bacterial populations. Gene-wide approaches to mapping bacterial diversity, which have already proved effective for gaining insight into bacterial evolution, have the potential to reveal the phenotypic basis of genetic diversity in *Aeromonas*, and to investigate the dynamics of this complex bacterial community. The objective of the work described in this chapter has been to evaluate the importance of the presence of recombination events and their influence on phylogenies, as it has been frequently postulated that in bacterial populations, horizontal gene transfer (HGT) is so common that it precludes the existence of biological species. Our research suggests little or null influence of recombination in the genetic structure of AHC species, despite the existence of some strains with recombinant gene fragments.

Assuming that the cohesion of major phylogenetic groups within the prokaryotes is due to vertical transmission and common ancestry rather than preferential HGT, it is possible to construct robust phylogenies reflecting the evolutionary history of bacteria, using a sufficient number of orthologous housekeeping genes (concatenated trees). In these phylogenies, bacterial species are delineable as 'classical Darwinian' evolutionary lineages [52-55].

The foregoing consideration does not exclude the existence of horizontal gene transfer, which in fact occurs, and has important evolutionary consequences, but it is doubtful that HGT is the essence of modern genome phylogeny [53]. Moreover, as demonstrated in *Salmonella, Streptococcus,* and *Bacillus*, homologous recombination decays exponentially with sequence divergence; in other words, a sequence divergence between two strains of 10% suppresses the recombination rate between them by a factor of about 100 [56, 57]. We are currently pursuing recombination studies in other genes of this *Aeromonas* group.

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