1 INTRODUCTION

Palaeomicrobiology aims at analyzing ancient microorganisms, especially pathogens obtained from the remains of infected hosts (Donoghue and Spigelman, 2006; Drancourt and Raoult, 2005). Aside of an historical interest in characterizing precisely past infectious diseases (Drancourt, 2012), understanding the evolutionary genome, either a descendant or an outgroup, is likely to predict derived syntenic features as ancestral in some cases (Rissman et al., 2009), which is a problem for genomes such as the Y. pestis genomes that contains many repeats and are highly rearranged (Darling et al., 2008). There exists only one scaffolding method that allows to compare with several related genomes while using a phylogenetic tree (Husemann and Stoye, 2010), but it is not designed to scaffold an ancient genome. We address this specific

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problem here and describe a phylogenetic approach to scaffold ancient bacterial contigs that adapts existing methods initially designed to predict ancestral genome features from the comparison of extant genomes.

The design of predictive methods to reconstruct ancestral genomic features is a relatively ancient field of computational genomics, dating back to methods such as Fitch’s algorithm for reconstructing ancestral genomic sequences (Fitch, 1971). Advances in computational paleogenomics include improved methods for reconstructing ancestral genome sequence (Blanchette et al., 2004; Diallo et al., 2010; Liberles, 2007), gene content (Cohen et al., 2010; Csuroś, 2010; Szöllösi et al., 2012) and gene order. The latter ones have been used for reconstructing ancestral genomes organization of bacteria (Fremez et al., 2007; Wang et al., 2006), animals (Alkeşyev and Pevzner, 2009; Chauve and Tannier, 2008; Ma et al., 2006; Muffato et al., 2010; Ouangraoua et al., 2011; Putnam et al., 2007), plants (Murat et al., 2010; Sankoff et al., 2009), yeasts (Bertrand et al., 2010; Gordon et al., 2009) or protists (Ma et al., 2008). Recent developments provide exact and fast algorithms that handle repeats as well as diverse types of genome rearrangements and chromosome structures (Berard et al., 2012; Chauve et al., 2013; Jones et al., 2012; Manuch et al., 2012).

We describe here how to adapt and integrate some of these methods to process ancient bacterial contigs. We apply our method to the Black Death agent genome, using the genomes of eleven closely related descendants and outgroups from the Y.pestis and *Yersinia pseudotuberculosis* clades, whose phylogeny is given in Bos et al., (2011): we correct, order and orient the ancient contigs of the of the medieval Black Death agent chromosome into a single scaffold and estimate the inter-contigs DNA sequences, and we describe a preliminary analysis of this reconstructed ancestral genome.

## 2 METHODS AND ALGORITHMS

We are given a set of contig sequences for an ancestral genome *A*, together with a set of related extant sequenced genomes, descendants and outgroups of *A*, organized into a phylogenetic tree *T*. Our scaffolding method FPSAC relates to a generic scheme for reconstructing ancestral genome organization (Chauve and Tannier, 2008; Ma et al., 2006; Jones et al., 2012), and is composed of four phases:

1. **Computing homologous families.** A homologous family is composed of at least one contig segment (ancestral marker) and several non-overlapping extant genomes segments (extant markers), that pairwise align, with high similarity, along their whole length. Each homologous family is assigned a multiplicity bounding the number of occurrences (copy number) of ancestral marker(s) from this family in the ancestral genome *A*.

2. **Computing putative ancestral adjacencies.** An ancestral adjacency is composed of two ancestral markers that are believed to have been contiguous in *A*. We predict them using a Dollo parsimony principle that takes advantage of the internal position of *A* in the considered phylogenetic tree. All adjacencies are weighted according to their phylogenetic conservation, defining a weighted adjacency graph.

3. **Scaffolding from ancestral adjacencies.** If the set of all ancestral adjacencies is not compatible with a multichromosomal circular chromosomal structure that respects the multiplicity constraints of homologous families, we compute a maximum weight subset of adjacencies that is compatible with such a circular chromosomal structure. Next, as adjacencies alone can define several contig orders, due to repeated ancestral markers forming tangles in the adjacency graph, conserved intervals spanning repeats are used to clear the ambiguities, in a way similar to the use of mate-pairs to scaffold extant genomes.

4. **Estimating inter-markers gap lengths and sequences.** For each ancestral adjacency, the length of the ancestral gap between the two involved markers is estimated from the length of the gap between the corresponding extant adjacencies (extant gaps). The sequences of the extant gaps whose length agrees with the estimated ancestral gap length are aligned into a multiple sequence alignment that is used to reconstruct a putative ancestral gap sequence.

### 2.1 Computing homologous markers families

We map the ancient contigs onto the extant genomes. Every significant hit (defined here by a length of at least 100 nt with 95% of identity) indicates two homologous sequences, one located on a contig and one located on an extant genome. Owing to rearrangements and repeats, some contigs do not align over their whole length to every extant genome, indicating potential evolutionary breakpoints. To detect families of homologous segments, we apply an iterative segmentation procedure, which produces contig and extant genome segments such that (1) contig segments align over their whole length to extant genomes segments and (2) pairs of extant genome segments do not overlap (i.e. either they have the same coordinates, or they are completely disjoint).

From a set of pairwise contigs/genomes alignments, we cut the contigs and the corresponding extant genome segments if either (1) or (2) is not satisfied. Assume first that (1) is violated: there is a segment [a, b] from a contig of length *ℓ* that aligns to an extant genome, with *a > 1* or *b < ℓ* or both. We assume that *a > 1*; the other case is treated symmetrically. The contig is cut into two segments, with coordinates *[1, a − 1]* and *[a, ℓ]* and the corresponding genome segments are cut accordingly. All others alignments of segments from this contig overlapping coordinate *a* are also cut into two subsegments at this position in the same way as previously mentioned. We iteratively apply this procedure until (1) is verified for all pairwise alignments, thus defining a new set of pairwise contigs/genomes alignments. Next, assume that (2) is violated: two different contigs have segments aligning to two overlapping regions of an extant genome, say *[a, c]* and *[b, d]*, with *a < b < c < d*. In this case, the two contigs are cut into two segments so that the four resulting segments align to genome segments with coordinates *[a, b]*, *[b, c]* (for two of them) and *[c, d]* (see Fig. 1). After iteratively applying this procedure until (2) is satisfied, it is possible that (1) is violated again. To make the procedure converge, we remove short alignments (below the length threshold used to define significant hits) and repeat the
two procedures until (1) and (2) are both satisfied. Then all aligned sequences naturally cluster into sets of highly similar ancient and extant sequences forming homologous families.

2.2 Multiplicity of homologous families

Next, we assign to each homologous family a multiplicity that is the expected number of occurrences of the ancestral marker of the family in the ancestral genome. The multiplicity of a family is computed from the number of occurrences of the extant markers in the extant genomes (the family profile) to minimize the number of evolutionary gain/loss along the branches of the considered phylogenetic tree. It is computed by a linear time dynamic programming algorithm [see Csurös (2010) for example].

2.3 Computing ancestral adjacencies

To account for the orientation of markers in predicted ancestral syntenic features (adjacencies and intervals), we decompose each marker (ancestral or extant) into two marker extremities, its head and its tail, a standard approach in genome rearrangement studies (Chauve et al., 2010).

Adjacencies are then defined in terms of marker extremities instead of markers, and are computed following a Dollo parsimony principle described in Chauve and Tannier (2008): two ancestral marker extremities form an ancestral adjacency if they are contiguous (no other marker is between them in the chromosome) in at least two extant genomes whose evolutionary path in T contains A.

Adjacencies are weighted according to their patterns of phylogenetic conservation as described in Ma et al. (2006) [see also Chauve and Tannier (2008)]. The weighted adjacency graph is defined as follows: its vertices are the markers extremities and its edges are the weighted adjacencies.

2.4 Computing ancestral scaffolds

An ancestral scaffold is a linear or circular order of ancestral markers. The set of ancestral adjacencies might not translate into an unambiguous set of ancestral scaffolds for two reasons: (1) there might not exist a set of circular or linear markers orders that contain all adjacencies and respect the multiplicity of each marker, and (2) even if ancestral adjacencies can be organized in ancestral scaffolds, several sets of scaffolds can exist because of marker multiplicities (Fig. 2).

To address point (1), we compute a maximum weight subset of ancestral adjacencies such that every marker extremity belongs to a number of adjacencies that is at most the multiplicity of the marker family (Wittler et al., 2011; Manuch et al., 2012); for an ancestral marker of multiplicity \( m \), each of its extremities can belong to at most \( m \) ancestral adjacencies. Such a selected subset of ancestral adjacencies, that is computed in polynomial time Manuch et al. (2012), is compatible with an order of the markers into a set of linear and/or circular scaffolds which respects the copy number constraint given by the ancestral marker multiplicities.

It is important to note that, although bacterial genomes can be composed of several circular molecules (chromosomes and plasmids), the algorithm we use does not control the resulting chromosomal structure (in terms of the number of scaffolds and of their linearity/circularity). The problem of computing a maximum weight subset of adjacencies that can be realized into a constrained chromosomal structure is NP-hard, as it includes the Maximum Weight Path Cover Problem (Ma et al., 2008). Relaxing the constraints on the chromosomal structure leads to a tractable problem (Manuch et al., 2012); moreover, if the resulting adjacencies can be realized into a set of linear segments, then this defines an optimal solution to the Maximum Weight Path Cover Problem, and so, an optimal set of scaffolds.

To address point (2), we rely on conserved intervals that span markers with multiplicity \( >1 \) (see Fig. 2 for an illustration of this principle). More precisely, we define a repeat cluster as a maximal connected subgraph of the adjacency graph induced by extremities of ancestral markers with multiplicity \( >1 \). A repeat spanning interval of \( R \) in a given genome \( G \) is a sequence of markers in \( G \) of the form \( a \ x_1 \ldots x_k \ b \) such that the multiplicity of \( a \) and \( b \) is 1 and the \( x_i \)'s all belong to the repeat cluster \( R \). A repeat spanning interval is conserved if it appears, up to a complete reversal, in two genomes whose evolutionary path in \( T \) contains \( A \). Identifying all conserved repeat spanning intervals can be done in time linear in the total size of all repeat clusters. Next, repeat spanning intervals are weighted using the same method as ancestral adjacencies, and for each repeat cluster \( R \), we greedily select repeat spanning intervals that are both compatible with the adjacencies selected during the previous step, which contain markers of \( R \), and satisfy the multiplicity constraints of the markers of \( R \) (Chauve et al., 2013).

Provided all repeats are spanned by enough conserved intervals, this results into an unambiguous scaffolding that includes all ancestral markers, including repeated ones. Otherwise, this means that the evolutionary signal present in the considered extant genomes is not sufficient to resolve repeats in the ancestral genome, in which case, adjacencies composed of two repeats that...
do not belong to a repeat spanning interval are discarded, resulting in a more fragmented, but unambiguous, set of scaffolds.

2.5 Estimating inter-contig gaps lengths and sequences

An ancestral gap in an ancestral scaffold is the sequence located between two consecutive ancestral markers (say $X$ and $Y$). For each ancestral gap, we consider the extant genomes in which occurrences of $X$ and $Y$ are consecutive (no extant marker is between them) and in the same respective orientations as in the ancestor, thus defining an extant gap $X – Y$. We define a conserved extant gap as an extant gap whose length is equal in two extant genomes whose evolutionary path in $T$ contains $A$, following a Dollo criterion. The lengths of conserved extant gaps $X – Y$ define a length interval for the ancestral gap $X – Y$. If there is no conserved extant gap, the ancestral gap length interval is defined by the minimum and maximum extant gap lengths between $X$ and $Y$. We align all sequences of extant gaps between markers $X$ and $Y$ whose length is in this interval into a multiple sequence alignment. A parsimonious estimation of each ancestral gap sequence is computed from the corresponding alignment of extant gap sequences using the classical Fitch algorithm (Fitch, 1971).

3 RESULTS

We describe here the result of our method FPSAC applied to the dataset described in Bos et al. (2011), followed by a preliminary analysis of the resulting scaffolded chromosome.

3.1 Data

The input data are the 2134 larger assembled contigs (500 nt and above) described in Bos et al. (2011), and the DNA sequences of the fully assembled chromosomes of four $Y.pseudotuberculosis$ genomes and seven $Y.pestis$ genomes, of which five are believed to descend from the $Y.pestis$ strain that was involved in the Black Death pandemic (Fig. 3).

3.2 Contig segmentation and homologous families

The sequences of the 2134 contigs were mapped to the full genome sequences of the 11 selected extant genomes using Megablast (Zhang et al., 2000) with default parameters. As already noted by Bos et al. (2011), 29 contigs did not map on the $Yersinia$ genomes, leaving 2105 ancestral contigs to analyze. The segmentation step resulted in 2616 homologous families. Almost all families have multiplicity 1, but 21 of them have multiplicity greater than 1, and among them, 20 have multiplicity 2 or 3, which indicates that most repeated parts of the genomes were not present in the larger contigs. We removed the last family, which corresponded to the 5S ribosomal protein family, because of its combined short length (133 nt) and high multiplicity (8). The amount of DNA encoded by the ancestral markers, when multiplicity is accounted for, is 3846616 nt of ancestral DNA, whereas the initial contigs encode 4013159 nt.

3.3 Comparative scaffolding

We detected 2634 putative ancestral adjacencies. Only 6 adjacencies of these 2634 putative ancestral adjacencies needed to be discarded to obtain a maximum weight subset of adjacencies compatible with a set of linear/circular scaffolds. There were 29 conserved repeat spanning intervals, and 2 of them needed to be discarded to extract a maximum weight subset that defined an unambiguous set of three large linear scaffolds, in which all contigs are represented.

There are six possibilities for joining these three scaffolds into one circular scaffold. Extant adjacencies between markers located at the scaffolds extremities were computed and defined an order and orientation for the three scaffolds: two adjacencies between scaffold extremities were supported by all outgroup species, whereas no adjacency between scaffold extremities was supported by ingroup species, and the last adjacency was supported by one outgroup ($Y.pestis$ Microtus) and involved a marker absent from all $Y.pseudotuberculosis$ genomes.

3.4 Gap lengths and sequences

Out of 2636 ancestral gaps only 22 did not have a length interval supported according to the Dollo criterion. In most other cases, length intervals were narrow: 2561 of the gaps (out of 2636) have a length interval whose bounds differ by at most 10 nt.

Next for each ancestral gap, we aligned all extant gaps whose lengths fell in the ancestral gap length interval, using Muscle (Edgar, 2004) (version 3.8.31), and constructed an ancestral sequence from each alignment using Fitch’s algorithm (Fitch, 1971). This resulted into a single sequence containing alternating sequenced ancestral contig segments and estimated ancestral gap sequences, illustrated in Figure 4.
found that 99.47% of the initial contigs appear in the reconstructed sequence with at least 95% of identity over 95% of their length, and that 98.66% of the gaps between consecutive contigs were reconstructed with the exact length in the reconstructed sequence. Regarding chimeric contigs, 99.14% of them were detected as chimeric. These high accuracy numbers are consistent with previous simulations on the reconstruction of ancestral gene orders from randomly rearranged extant genomes (Ma et al., 2006), although here we can also observe a high accuracy in the reconstructed gap lengths, which was not considered in previous simulations.

3.6 Analysis of the reconstructed ancestor
The pipeline described previously resulted in an ancestral genome sequence of length 4.6 Mb showing that roughly 775 kb were added to the ancestral marker sequences by the gap sequences estimation step.

In the resulting scaffold, each occurrence of an ancestral marker corresponds to one or several segments of the initial contigs. The ordering of these segments is mostly compatible with the initial contigs. We found only one chimeric contig (see Fig. 5), split into two non-adjacent markers in the ancestral genome organization. Also four contig segments were found to be duplicated: a large part (>500 nt) of each is probably present in more than one occurrence in the ancestral genome, whereas the initial assembly predicted only one occurrence. Finally, 63 contigs have a sequence that is found, up to small variations, inside another contig, whereas their number of extant occurrences suggest they have multiplicity 1, so we believe they are redundant. An alternative explanation is that they are derived mutations of the ancient genome, which, in such a case, would not be ancestral to current strains.

Regarding the six discarded adjacencies, two of them point toward a possible large-scale inversion. Both, the selected adjacencies and intervals, as well as the discarded ones, have similar phylogenetic support. So this alternative structure cannot be ruled out as non-ancestral, which raises the question of the possible coexistence of different genome architectures among the Y.pestis infecting the host individual whose remains were used for sequencing.

We also took advantage of the availability of a full chromosome sequence for the main chromosome of the Black Death agent to analyze its structure and evolution at the whole-genome scale. We first analyzed insertion sequence (IS) elements that have been suspected to be involved into the high rearrangement rate of Y.pestis genomes (Chain et al., 2004). We mapped extant IS to the reconstructed ancestral chromosome (see Supplementary Material). This resulted in 92 ancestral gaps and markers containing IS. We confirmed this comparative annotation with an automatic annotation of the reconstructed chromosome sequence. We could also observe that a large proportion of these IS (at least 58) were already present in the last common ancestor of all Y.pestis strains, whereas they are almost completely absent from the genomes of the considered Y.pseudotuberculosis, thus providing more evidence that the Y.pestis speciation from its Y.pseudotuberculosis ancestor was characterized by a burst of IS insertion (Chain et al., 2004).
We also analyzed the genome rearrangements between the reconstructed ancestral sequence and the extant genome sequences by sampling inversion scenarios between the ancestral genome and the extant genomes using the software DCJ2HP (Miklós and Tannier, 2010). There are 8–9 inversions between the *Y.pseudotuberculosis* strains and the medieval genome, and 9–22 inversions when compared with the (evolutionarily closer) *Y.pestis* strains, showing a clear acceleration of evolutionary rearrangement following the Black Death *Y.pestis* divergence (see Supplementary Material). As noticed by Darling *et al.* (2008), we can also observe that inversion breakpoints are not randomly distributed and used (Fig. 4): highly used ones are concentrated in one-third of the chromosome, around its probable replication origin. The positions of the inversion breakpoints are also highly correlated with IS, as remarked earlier (Deng *et al.*, 2002): 76 of the 118 mapped breakpoints are close (<1000 nt distant) to some predicted IS, whereas this number drops to 39 for uniformly sampled random coordinates ($P$-value <10^-3). Rearrangements are numerous in all *Y.pestis* branches, strongly suggesting that they could be driven by the IS.

### 4 DISCUSSION

#### 4.1 Contig segmentation and marker multiplicities

Aligning contigs to extant genomes and using these alignments to segment contigs might at first seem counterintuitive, as it increases the fragmentation of the initial assembly. However, it allows us to take advantage of the available fully assembled extant genomes to identify potential chimeric contigs and to extract potential repeated sequences from the contigs, which would have been collapsed into a single contig, a well-documented issue with assembling from short reads (Treangen and Salzberg, 2012). Our approach follows a recent suggestion by Roy *et al.* (2012) to rely on shorter contigs of higher quality (here in terms of mapping to related genomes). This phase benefited from the high sequence conservation in the *Y.pestis* clade that allowed us to rely on high similarity pairwise alignments as input of the segmentation phase. Less conserved data would likely require more involved methods to compute a segmentation into non-overlapping homologous families (Angiuoli and Salzberg, 2011; Minkin *et al.*, 2013).

Finally, the possibility to infer the multiplicity of contig segments from the alignment on extant genomes, using comparative genomics methods designed to study the evolution of gene families, offers an elegant alternative, specific to aDNA assembly, however, to current copy number estimation methods that rely on the depth of coverage, which can be uneven when sequencing highly fragmented aDNA.

#### 4.2 Estimating ancestral gap sequences

The key idea is that conserved adjacencies are also likely to define conserved gaps. In the data processed, we can observe that for most ancestral gaps, a strict Dollo parsimony criterion identifies conserved gaps. Moreover, again benefiting from the high sequence conservation of the *Y.pestis* genomes, we could estimate most of the ancestral gap sequences from the multiple alignments of the corresponding extant gaps using a standard ancestral character reconstruction method. If greater sequence variation was observed, more powerful methods designed to infer ancestral DNA from a multiple alignment would be appropriate (Blanchette *et al.*, 2004; Diallo *et al.*, 2010; Liberles, 2007). In a future work, we aim to use the reconstructed gap sequences as a template to exactly assemble these gaps from the sequenced reads. However, optimally mapping aDNA reads onto extant DNA requires specific protocols that have recently been developed for eukaryotic aDNA, but still needs to be established for bacterial aDNA (Schubert *et al.*, 2012).

#### 4.3 Scaffolding and comparative genomics

The FPSAC method follows principles similar to most existing scaffolding methods designed for extant genomes. It relies on extracting a genome structure from a graph (the adjacency graph), whose vertices are sequence elements and edges indicate connectivity between pairs of vertices. In most scaffolding algorithms, edges of this graph are defined by mate-pair reads, whereas we rely on adjacencies and intervals that are conserved under a Dollo parsimony criterion. The main difference we can observe is the low number of tangles in the graph we obtained compared with the usual large number observed in graphs based on mate-pairs, in part because of the absence of repeated sequences in the analyzed contigs. It is interesting to observe that, despite the fact that *Y.pestis* genomes are highly rearranged, FPSAC was able to capture a clear signal regarding the organization of markers along the ancestral chromosome. Also important is the use of recently developed polynomial time exact algorithms to extract a consistent set of adjacencies while accounting for the multiplicity of repeated segments (Manuch *et al.*, 2012) and to assess the compatibility of repeat spanning intervals with a given adjacency graph (Chauve *et al.*, 2013).

#### 4.4 Applicability to other datasets

We applied FPSAC to a dataset with specific characteristics. The assembled ancestral contigs were obtained using a library-enrichment approach and are assumed to belong to the genome of an internal node of a phylogeny, whose leaves are sequenced and assembled. Moreover, the clade of interest contains high sequence conservation and highly rearranged genomes. We address the impact of these different points below and discuss the applicability of FPSAC to a wider range of datasets.

In the case where descendants of the ancient genome of interest are not available, either due to lineage extinction or because they have not been sequenced, other comparative methods can be used, that do not rely on a Dollo parsimony principle.
(Husemann and Stoye, 2010). So the only important requirement to use FPSAC is the availability of the genome sequences of at least two related genomes whose evolutionary path contains the ancestor of interest. From there, the performances of the scaffolding obtained with FPSAC will depend both on the level of sequence conservation of both sequence identity and synteny in the considered related genomes. Diverged extant genomes might result in difficulty to obtain homologous families, which inadequately span the initial set of ancient contigs, as well as computing their multiplicities. High rearrangement rates might result in wrong adjacencies because of convergent evolution. Useful indicators to assess the results obtained with FPSAC are thus both the coverage of contigs and extant genomes by ancestral markers and in the number of discarded adjacencies during the scaffolding phase.

The method we describe can be applied as is even if some of the chosen closely related extant genomes are not fully assembled. The impact of unassembled extant genomes is likely to be more fragmented scaffolding than what we observed on the Black Death agent dataset because of undetected ancestral adjacencies or repeat spanning intervals.

Finally, if the initial ancient contigs originate from a mixture of microbial backgrounds, for example if they result from de novo assembly of reads obtained through shotgun sequencing, then FPSAC can be used to assemble contigs subsets that have been identified, through comparison with extant genomes sequences, as belonging to well identified clades and satisfy the requirements described previously. However, even this initial step is currently a challenge, for example because of repeated sequences belonging, up to variations, to several genomes (Pell et al., 2012). The problem of applying a method such as FPSAC to a whole set of contigs originating from a mixture of genomes, that is, for scaffolding an ancient metagenome, is an important research avenue.

5 CONCLUSION

Technological sequencing advances can now provide sequences from whole ancient bacterial genomes, which promises to be an invaluable source of knowledge for understanding pathogen evolution. However, assembling ancient bacterial genomes poses specific issues, in particular because of high fragmentation in numerous contigs. In parallel, computational paleogenomics by comparative methods has grown tremendously, and computational methods can now provide ancestral genome sequences accounting for substitutions or small indels, ancestral gene content or ancestral genome organizations, at the level of full chromosomes, but, until now, were never combined to scaffold and estimate the sequence of an ancient bacterial chromosome. In the present work, we described a general method to combine both sequencing and computational reconstruction, and illustrated its potential on a real dataset.

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