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Corynebacterium pseudotuberculosis may be under anagenesis and biovar Equi forms biovar Ovis: a phylogenetic inference from sequence and structural analysis

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Abstract

Background: *Corynebacterium pseudotuberculosis* can be classified into two biovars or *biovars* based on their nitrate-reducing ability. Strains isolated from sheep and goats show negative nitrate reduction and are termed biovar Ovis, while strains from horse and cattle exhibit positive nitrate reduction and are called biovar Equi. However, molecular evidence has not been established so far to understand this difference, specifically if these *C. pseudotuberculosis* strains are under an evolutionary process.

Results: The ERIC 1 + 2 Minimum-spanning tree from 367 strains of *C. pseudotuberculosis* showed that the great majority of biovar Ovis strains clustered together, but separately from biovar Equi strains that also clustered amongst themselves. Using evolutionarily conserved genes (*rpoB*, *gapA*, *fusA*, and *rsmE*) and their corresponding amino acid sequences, we analyzed the phylogenetic relationship among eighteen strains of *C. pseudotuberculosis* belonging to both biovars Ovis and Equi. Additionally, conserved point mutation based on structural variation analysis was also carried out to elucidate the genotype-phenotype correlations and speciation. We observed that the biovars are different at the molecular phylogenetic level and a probable anagenesis is occurring slowly within the species *C. pseudotuberculosis*.

Conclusions: Taken together the results suggest that biovar Equi is forming the biovar Ovis. However, additional analyses using other genes and other bacterial strains are required to further support our anagenesis hypothesis in *C. pseudotuberculosis*.

Keywords: *Corynebacterium pseudotuberculosis*, Evolution, Molecular phylogeny, Structural biology

Background

The genus *Corynebacterium* belongs to the bacterial phylum *Actinobacteria*, also known as *Actinomycetes*. This phylum comprises *Mycobacterium*, *Nocardia* and *Rhodococcus* genera, which together form a supra-generic group known by their initials as CMNR [1–3]. These organisms share some common features, such as:

- (i) A specific well-organized cell wall mainly characterized by the presence of vast components of peptidoglycan, mycolic acid, and arabinogalactan [4–7];
- (ii) high G + C content (47 %–74 %) [5];
- (iii) Gram-positive [8].

Within the genus *Corynebacterium*, the species *Corynebacterium pseudotuberculosis* is reported to be a facultative intracellular pathogen in mammals [9, 10]. The pathologies associated with *C. pseudotuberculosis* are of great importance to veterinary medicine because this bacterium is considered the main etiologic agent of

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caseous lymphadenitis (CLA). CLA is characterized by abscess formation in the thorax and abdomen, or in major lymph nodes, causing dermonecrosis and finally resulting in hypertrophy in the affected region [9, 11]. CLA is mostly found in small ruminants (mainly sheep and goats), but other mammals, such as cattle, pigs, deer, sheep, horses, camels and even human beings, although with very rare incidences, can be affected [12, 13]. Curiously, infection by *C. pseudotuberculosis* may cause other diseases, such as ulcerative lymphangitis (UL), a pathology of lymphatic vessels of the lower extremities, particularly hind legs, which is most frequent in horses [14, 15].

In order to understand differences in clinical presentation by infection of *C. pseudotuberculosis*, some studies have proposed to classify this microorganism from genetic, morphological and biochemical points of view [16–18]. Especially based on its ability to breakdown nitrate [19, 20], *C. pseudotuberculosis* was classified into two biovars, Ovis and Equi. Strains isolated from sheep and goats, which are usually negative in nitrate reductase activity, were classified as biovar Ovis; whereas the strains isolated from horse and cattle, which are usually positive in the nitrate reduction test, were classified as biovar Equi.

Additionally, studies have attempted to define these two biovars (Ovis and Equi) using restriction endonucleases (*EcoRV* and *PstI*) on chromosomal DNA or focusing on nitrate reduction determination methods [21]. Discrimination of both isolates was also possible using other methodologies, such as restriction fragment length polymorphism analysis of 16S ribosomal DNA [19, 20, 22] and pulsed-field gel electrophoresis (PFGE) in combination with biochemical analysis [23]. Other studies have investigated the possible evolutionary divergences of the genus *Corynebacterium* using 16S rRNA sequences [24–26], the preferred genetic tool used to characterize organisms taxonomically [27]. Although 16S rRNA (*rmsE*) gene sequencing is highly useful with regards to bacterial classification, published data has proven analysis of the partial nucleotide sequences of the RNA polymerase β -subunit gene (*rpoB*) is more accurate for *Corynebacterium* species [18, 28].

There is phenotypic evidence (nitrate test) and genotypic evidence (Enterobacterial repetitive intergenic consensus sequence-based - ERIC-PCR and Single-nucleotide polymorphism (SNP) analysis [29, 30]) showing differences between the two biovars. However, our goal in this paper is to investigate the evolutionary differences between biovars Equi and Ovis of *C. pseudotuberculosis*. Thus, in this study, we performed ERIC 1 + 2 PCR and evolutionary analysis, using maximum likelihood method in combination with gene and protein structural analysis, of genes *rsmE*, a vital component of the ribosome, and *rpoB*, a region of strong influence of RNA polymerase activity, to find a kinship and phylogenetic distances. To

study the molecular divergences between biovar Ovis and Equi of *C. pseudotuberculosis*, we also considered other genes like *gapA*, which has been used as a target in taxonomic comparisons of bacteria, taking into account the functions that this gene infers in possible differences in the metabolism of carbohydrates, glycolysis and cell survival [31, 32], and *fusA*, that has been used for phylogenetic analysis and taxonomic classification of bacterial species of the genus *Pantoea*, a pathogen to humans and plants [33].

Methods

ERIC 1 + 2 PCR Minimum-spanning tree

The minimum-spanning tree (MST) was generated using ERIC1 + 2-PCR data from 367 *C. pseudotuberculosis* strains, including 226 biovar Ovis field strains [17, 29, 34], 139 biovar Equi field strains (34 strains with published data [29] and 105 strains isolated from equines in USA – unpublished data), type strain ATCC 19410^T and vaccine strain 1002. The *C. pseudotuberculosis* ATCC 19410^T type strain and 1002 vaccine strain were genotyped by ERIC1 + 2-PCR one time in each of the four different assays gathered in the present study ([17, 29, 34] and unpublished data). The MST was built using UPGMA, to calculate the distance matrix, and Prim's algorithm associated with the priority rule and permutation resampling [35, 36]. The MST presented is the top scoring tree, i.e., the tree with the highest overall reliability score.

Dataset

Eighteen *C. pseudotuberculosis* strains with available genome sequences (Additional file 1) had their sequences of genes/proteins *rpoB*, *gapA*, *fusA*, and *rsmE* (Additional file 2) submitted to phylogenetic and structural analyses. ERIC1 + 2-PCR results were available for 13 of those 18 strains. All genome sequences were available from the NCBI database [37, 38]. Protein functional information was from UniProt database annotation [39].

Alignment and phylogenetic analysis

We have used Clustal-X [40] for multiple sequence alignment (MSA) and Jalview [41] to visualize and edit the MSA, create phylogenetic trees, explore molecular structures and annotation. The analyses on the transition and transversion mutations were done using the software MEGA 6 [42]. Average Nucleotide Identity (ANI) [43] was employed to evaluate relatedness among strains in substitution of the labour-intensive DNA-DNA hybridization (DDH) technique.

In order to create the phylogenetic trees, we first obtained an evolutionary model adapted to the MSA. Therefore, we used Adaptive Server Evolution (<http://www.datamonkey.org/>) portal to define one evolutionary

model. The outcome of these tests indicate the TN93 model [44]. Seaview [45] was then used to construct the tree based on the model previously presented by the Adaptive Server Evolution portal. The tree was created using the maximum likelihood method performed by PHYML [46], which is available in Seaview. Branch support consistencies were evaluated using the nonparametric bootstrap test [47] with 250 replicates and the approximate likelihood ratio test (ALRT) [48]. The viewing and editing of the tree was carried out using the Figtree tool [49, 50] that enabled us to either characterize different gene groups based on the bootstrap values calculation or to represent the evolutionary time scale. The multiple sequence alignments contain all four genes used in this work.

Statistical analysis

The timetree was generated using the RelTime method [51]. Divergence times for all branching points in the user-supplied topology were calculated using the Maximum Likelihood method based on the General Time Reversible model [52]. Bars around each node represent 95 % confidence intervals which were computed using the method described by Tamura et al. [42]. The estimated log likelihood value of the topology shown is -34964.1082. Similar evolutionary rates were merged between ancestors and descendants so that many clocks were identified in the topology. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 29.1188 % sites). The tree is drawn to scale, with branch lengths proportional to the relative number of substitutions per site. Also, the statistic Tajima's D [53] was used in order to compare the average number of pairwise differences with the number of segregating sites. The Tajima's D statistical can be understood below:

$$E[\pi] = \theta = E \left[\frac{S}{\sum_{i=1}^{n-1} \frac{1}{i}} \right] = 4N\mu$$

Where S means the number of segregations sites, n the number of samples and i is the index of summations. Follows the Tajima's D statistical test, there are factors that can change the expected values of S and π . The crux of Tajima's D test statistic is the difference in the expectations for these two variables (which can be positive or negative). Finally, D is calculated by considering the differences between the two estimates of the population genetics parameter θ . The D value is obtained by dividing these differences, that is called d by the square root of its variance $\sqrt{\hat{V}(d)}$.

$$D = \frac{d}{\sqrt{\hat{V}(d)}}$$

The alignment with all 4 genes from two *C. pseudotuberculosis* strains, vaccine strain 1002 from biovar Ovis and strain E19 from biovar Equi, and one *Arcanobacterium haemolyticum* strain, AH 20595 employed as an external group, were used for this statistical analysis. P -value less than 0.05 were used to reject the null hypothesis of equal rates between lineages.

Estimation of the pattern of nucleotide substitution

In order to observe the probability of transition ($G \leftrightarrow A$) and transversion ($A \leftrightarrow C$) substitutions, the MSA, of all 4 genes from biovars Equi and Ovis, was taken into account, with the aim to determine the types of molecular changes that were occurring. The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution method was used with the gamma model that corrects for multiple hits, taking into account the rate substitution differences between nucleotides and the inequality of nucleotide frequencies [54]. The analysis involved 19 (external group inside) nucleotide sequences. The transition/transversion ratio (R), that is the number of these replacement, was calculated by $R = [A * G * k_1 + T * C * k_2] / [(A + G) * (T + C)]$ with rate ratios of k_1 evaluating purines and rate ratios of k_2 evaluating pyrimidines. Codon positions included were 1st + 2nd + 3rd + Non-coding. All positions containing gaps and missing data were eliminated. There were a total of 6542 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [42].

Structural analysis

For structural analysis, the gene sequences were translated to amino acid sequences using the Transeq program: <http://www.ebi.ac.uk/Tools/emboss/>. After the amino acid alignment, we constructed 3-D models of the proteins and interpreted the molecular differences and consequences. Comparative molecular modeling of proteins was performed with the software Modeller [55]. Additional file 3 presents the details of the template structures using information from NCBI [52] and PDB (Protein Data Bank) [56]. Twenty models were built for each of the templates using MODELLER and one model for each template. PyMOL V.1.5.0.4 was used to visualize three-dimensional protein structures (Schrödinger, LLC). All homology models were evaluated using several different model evaluation tools such as PROCHECK [55], evaluating the stereochemistry quality, Discrete Optimized Protein Energy (DOPE) score [56], a statistical potential able to provide a energetic validation and RMSD obtained from a structural alignment with a protein with similar function,

to provide an functional validation. The latter was carried out in the software PyMOL. For all analysis we selected amino acids that are closer than 4 angstroms (Å) from the variant amino acid.

*We want to inform that this work does not use any participants, children, parent or guardian.

Results

C. pseudotuberculosis biovar Ovis and biovar Equi strains clustered separately by ERIC 1 + 2 PCR

The ERIC 1 + 2 Minimum-spanning tree from 367 strains (365 field strains, type strain ATCC 19410^T and vaccine strain 1002) of *C. pseudotuberculosis* showed that the great majority of biovar Ovis strains clustered together, but separately from biovar Equi strains that also clustered amongst themselves (Fig. 1-a) (Additional file 4). The same was observed when the minimum-spanning tree was constructed only from strains with complete genome sequences (Fig. 1-b) (Additional file 4). Moreover, analysis of all *C. pseudotuberculosis* strains depicted the existence of five major clonal complexes; three that clustered around biovar Ovis and two around biovar Equi strains.

The clonal complex labelled 1 was almost totally composed of *C. pseudotuberculosis* strains from Minas Gerais State, Brazil, whereas clonal complex 2 contained strains from the states of Minas Gerais, Pernambuco and São Paulo, Brazil. The last clonal complex related to *C. pseudotuberculosis* biovar Ovis strains (3) showed the most diverse composition, including strains from Argentina, Australia, Brazil (Minas Gerais, Bahia, and São Paulo), Egypt, Israel, Scotland and USA. *C. pseudotuberculosis* biovar Equiclinal complexes were mainly composed of strains from Egypt (4) and USA (5).

The repeatability observed for the *C. pseudotuberculosis* ATCC 19410^T and 1002 vaccine strain in ERIC1 + 2-PCR was 84 % considering the four different experiments conducted [17, 29, 34] and also the unpublished data from 105 strains.

Statistical analysis and molecular phylogeny from *C. pseudotuberculosis* Ovis and Equi biovars

The phylogenetic tree was carried out to understand the taxonomic distribution of biovars observed from ERIC-PCR. This result showed a small fragmentation of branches among the 18 *C. pseudotuberculosis* strains. For the analysis we added an external group using the gene sequences from *Arcanobacterium haemolyticum* (AH 20595) that are homologous to the four *C. pseudotuberculosis* genes analyzed. This can be visualized in the tree by a red edge (Fig. 2). The fragmented edges that belong to biovar Equi are highlighted in green and black (only strain 162), while the ones that belongs to biovar Ovis are highlighted in orange. The bootstrap value

appears along the tree as well as in the legend. The result for ANI was 98 % (Additional file 5). The results of the timetree (Additional files 6 and 7) showed a difference among *C. pseudotuberculosis* strains of biovars Equi and Ovis, taking into account the number of genetic distances to a fraction of the time. Biovar Equi strains trees have different branch length and taxonomic separation. On the other hand, biovar Ovis strain trees present more similar genetic distances between them, without differences in branch lengths. In addition, the results obtained in Tajima's D, shown in Table 1, point to the existence of differences among average numbers of pairwise differences with the number of segregating sites. These data can be supported in regards to a Value of Tajima's D < 0, which was in our results -2.07.

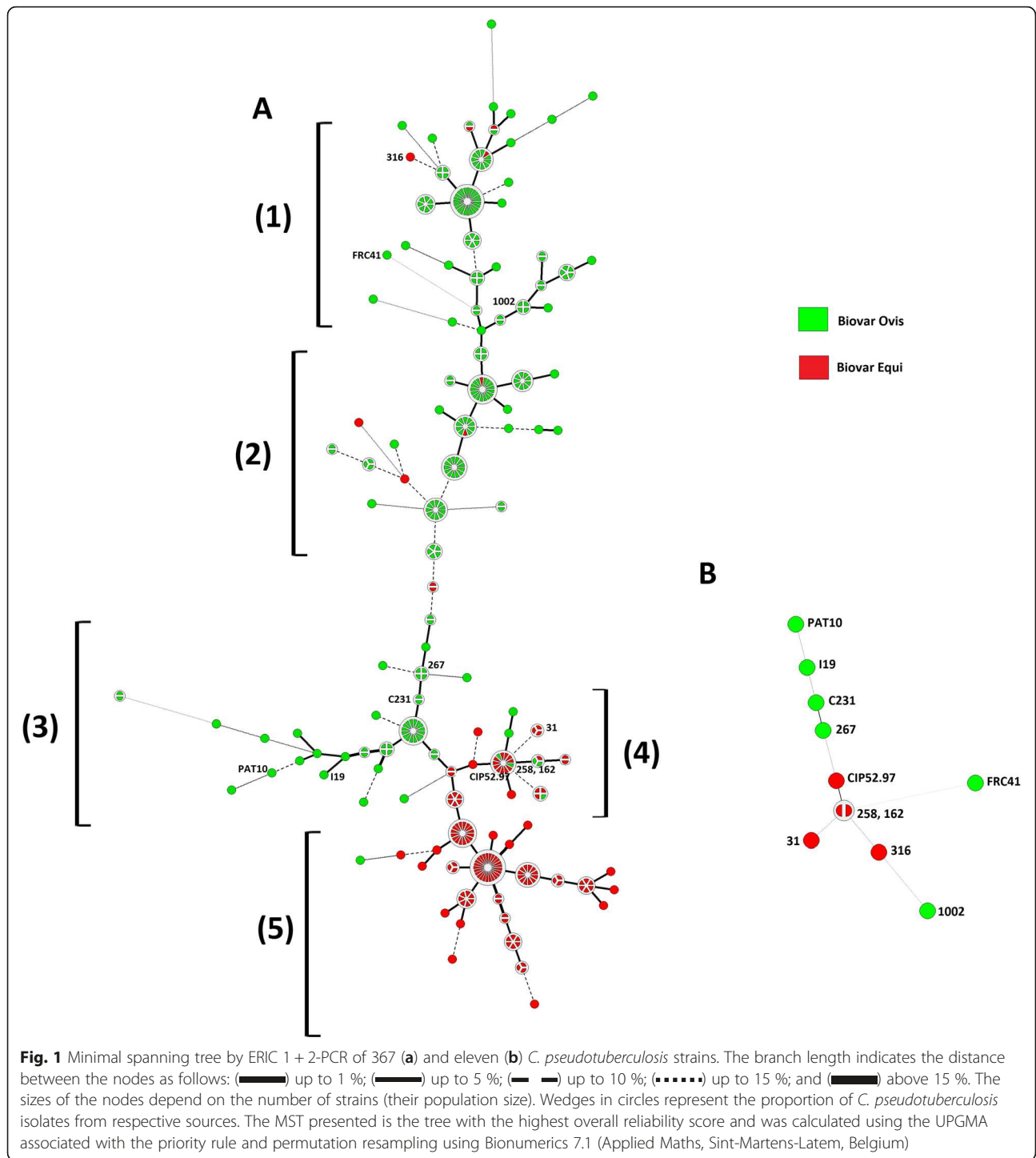
Statistical differences and transition and transversion point mutations detected in the multiple sequence alignment from biovars Ovis and Equi

The statistical results of transitional and transversion replacement has pointed to the existence of specific points mutations for both biovars. For example, the Additional file 8 shows one of the arrows that for biovar Equi there is the presence of guanine instead of adenine, a nitrogenous base found only in biovar Ovis. Table 2 shows the distribution of the nucleotide variation in the complete alignment. We can observe that the percentage of transition mutations is higher than that of the transversion mutations. The nucleotide frequencies are 21.51 % (A), 22.95 % (T/U), 27.59 % (C), and 27.95 % (G). The transition/transversion rate ratios are $k_1 = 13.99$ (purines) and $k_2 = 3.89$ (pyrimidines). The overall transition/transversion bias is $R = 4.35$.

Specific amino acid variation among biovar Ovis and Equi

Next, we evaluated the location of these point mutations on protein structure and whether the physicochemical characteristics of the mutated residues differ between the two biovars. We considered the protein sequences from the four genes used in this study, and amino acid differences were evaluated based on a sequence alignment with proteins (Additional file 9) possessing the same function and known. After the alignment, the position of each mutation in the structure was analyzed in comparison to the active site of each protein. In general, mutations were located in the surface and distant from the active site region (Additional file 10).

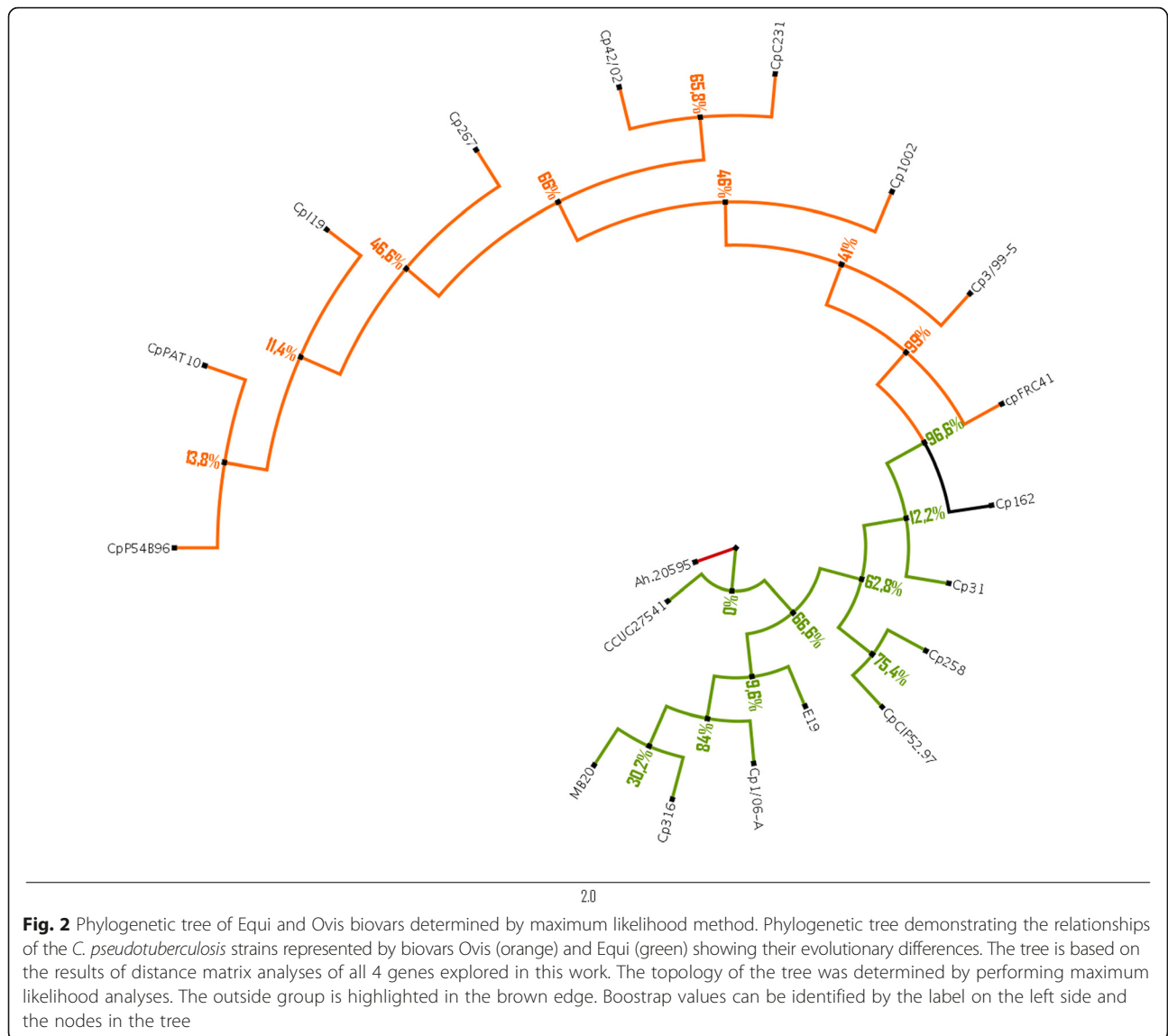
For *fusA* protein observed two residue modifications (Val177Ile and Asp371Glu) when comparing the two biovars. In both cases the amino acids within each pair have the same physicochemical characteristics: both valine and isoleucine are nonpolar and hydrophobic, and aspartate and glutamate are both polar and negatively charged. Therefore, both mutations are conservative and



the mutations most likely don't affect the function of this protein (Additional file 11).

For *gapA* protein, we observed that Asparagine (Asn) was changed to Aspartic acid (Asp) at position 97 when in both the biovars. Both are polar amino acids, however they differ in physicochemical terms, since Asn is neutral and contains a hydrogen bond donor, while Asp is

usually negatively charged at neutral pH. Additionally, at position 207 another amino acid change was observed, from threonine (Thr) to isoleucin (Ile). In this case, the amino acids differ in size (Ile has a bigger side chain) and polarity (while Ile is nonpolar, Thr is a neutral polar amino acid). Interestingly, *C. pseudotuberculosis* strain 162 that belongs to biovar Equi does not show the same



changes when compared to the other strains from the same biovar.

Comparative analysis of the 16S ribosomal RNA methyltransferase (*rsmE*) from *C. pseudotuberculosis* biovars Equi and Ovis showed a His18Arg variation at the N-terminal region and a Thr132Ala variation, at the C-terminal region. Both Histidine (His) and Arginine (Arg) located at position 18 have polar side chains and may be positively charged at neutral pH, however Arg has a much higher pKa. At position 132, the amino acids Threonine (Thr) and Alanine (Ala) differ functionally as Thr is a neutral polar amino acid and Ala is nonpolar (Additional file 10). The Table 3 shows all variations for these proteins.

Finally, for the beta subunit of RNA polymerase (*rpoB*) only one residue difference, Ala979Thr, was observed when comparing biovars Equi and Ovis. In this case there is a change in polarity for these two amino acids (Additional file

10). Curiously *C. pseudotuberculosis* strains 1002, 3/99, and FRC41, belonging to biovar Ovis, shared the same amino acids when compared to biovar Equi strains (Table 4).

Discussion

We earlier suggested from data of 102 *C. pseudotuberculosis* strains that *C. pseudotuberculosis* biovar Ovis and Equi exhibited different ERIC1 + 2-PCR clustering pattern (Dorneles et al., [29]). The present data from 373 strains also showed a clear difference in clustering pattern between *C. pseudotuberculosis* biovar Ovis and Equi, with a few exceptions (Fig. 1-a). However, as the present MST results were based on a representative number of genotyped strains (139 *C. pseudotuberculosis* biovars Equi field strains, 226 *C. pseudotuberculosis* biovar Ovis field strains, from twelve countries and isolated from eight different hosts), it allows us to make more

Table 1 Results from Tajima’s neutrality test

<i>m</i>	<i>S</i>	p_s	Θ	π	<i>D</i>
19	841	0.52	0.15	0.07	-2.07

The analysis involved 19 amino acid sequences. The coding data was translated assuming a genetic code table. All positions containing gaps and missing data were eliminated. There were a total of 1599 positions in the final dataset. Evolutionary analyses were conducted in MEGA6
 Abbreviations: *m* = number of sequences, *n* = total number of sites, *S* = Number of segregating sites, $p_s = S/n$, $\Theta = p_s/a_1$, π = nucleotide diversity, and *D* is the Tajima test statistic [52]

reliable inferences. The five largest clonal complexes were observed in the MST, three were mainly related to *C. pseudotuberculosis* biovar Ovis strains (1, 2, and 3) and two with *C. pseudotuberculosis* biovar Equi strains (4 and 5), from which other clonally related isolated groups emerge. The distinct clustering pattern of *C. pseudotuberculosis* biovar Ovis and Equi strains might reflect the number of genes specific to each biovar [57].

In order to understand the clustering formation, we have used algorithms and techniques to construct qualitative phylogenetic trees to explain the best possible evolutionary relationship between biovars Ovis and Equi from *C. pseudotuberculosis* strains. The difference in the biovars can be clearly observed from the ERIC 1 + 2-PCR MST and phylogenetic tree (Figs. 1 and 2). However, it was not possible to determine if the two biovars belongs to different species based on the results obtained after the phylogenetic tree and average nucleotide identity (ANI) analyses. Although there are some genetic and biochemical differences between them, it was not clear whether the branches, observed at phylogenetic tree, have distinct groups of organisms. Based on our data, we hypothesized that biological speciation may be occurring, for instance, anagenesis, which is a process of progressive evolution of species involving changes in the gene frequency of a population [58].

Based on the anagenesis mode of speciation, we have hypothesized that speciation is occurring within the *C. pseudotuberculosis* at a molecular level. The genes *rmsE*, *rpoB*, *fusA*, and *gapA* that we used are strong candidates for phylogenetic analysis because they are involved in many important cellular processes, such as maintenance of cellular integrity, cell survival and several metabolic

Table 2 Maximum composite likelihood estimate of the pattern of nucleotide substitution

	A	T	C	G
A	-	2.11	2.53	35.92
T	1.98	-	9.86	2.57
C	1.98	8.2	-	2.57
G	27.65	2.11	2.53	-

Each entry shows the probability of substitution (*r*) from one base (row) to another base (column) [54]. For simplicity, the sum of *r* values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics

Table 3 Distribution of amino acids from proteins in biovars. The amino acid variants positions present physicochemical differences, and are observed to be specific to a biovar type. Some variations exhibit an increase or decrease in the number of interactions between amino acids that influence the stability of the protein

Protein	Position	Amino acid	Biovar
<i>fusA</i>	177	Valine (V)	Equi
		Isoleucine (I)	Ovis
	371	Glutamine (E)	Equi
<i>gapA</i>	97	Aspartic acid (D)	Ovis
		Asparagine (N)	Equi
	207	Aspartic acid (D)	Ovis
		Threonine (T)	Equi
<i>rmsE</i>	18	Isoleucine (I)	Ovis
		Histidine (H)	Ovis
	132	Arginine (R)	Equi
		Threonine (T)	Ovis
		Alanine (A)	Equi

reactions [31–33]. According to Dorela et al. [8], *rpoB* is a relevant gene used to explore evolutionary routes serving as a tool to search for new species, differently from 16S rRNA gene sequencing data, that presents resolution problems at genus and/or species level. In our study, it was observed that the 16S sequence may be better in characterizing the molecular differences (mainly in structural analysis) between the two biovars, when compared to the *rpoB* gene. Our multiple sequence alignments analysis showed that all the genes have transition and transversion point mutations, which is defining and separating *C. pseudotuberculosis* biovar Equi from Ovis. In addition, it was observed from the timetree analysis that differences between the two *C. pseudotuberculosis*

Table 4 Distribution of amino acids from protein *rpoB* in biovars. The position 979 has variation in amino acids: Alanine for some strains as 1002, 106, 3/99, 31, 258, 52.97, 162 and FRC41; while the same position is Threonine for other biovars as 42/02, 267, 231, 119, P54B96 and PAT10

Alanine (A) ⁹⁷⁹		Threonine (T) ⁹⁷⁹	
Strain	Biovar	Strain	Biovar
1002	Ovis	42/02	Ovis
106	Equi	267	
3/99	Ovis	231	
31	Equi	119	
258	Equi	P54B96	
52.97	Equi	PAT10	
162	Equi		
FRC41	Ovis		

biovars were due to time of acquired mutations. It is possible to observe that the strains of biovar Equi are more prone to have mutations while those of *C. pseudotuberculosis* biovar Ovis were more stable. It is not possible, even with these findings, to state that the two groups constitute different species. However, taking into account the time slice versus genetic differences, *C. pseudotuberculosis* strains belonging to biovar Equi are under a constant mutational process, regarding the lengths of the branches. Considering the analyses of *C. pseudotuberculosis* strain 162, that have phylogenetic features approaching biovar Ovis, we interpret that some changes are being stabilized giving rise to strains of biovar Ovis. This event may indicate that a biological speciation may be occurring slowly in *C. pseudotuberculosis*. Our hypothesis is that an anagenesis process is happening from *C. pseudotuberculosis* biovar Equi to *C. pseudotuberculosis* biovar Ovis. Such anagenesis is observed when a sufficient number of mutations are fixed in a population, which makes the emergence of a new phenotype possible in the future. Based on our data, we dismissed the possibility of a cladogenesis event because we did not observe the transformation of an organism into two others, but rather the possible genesis of one organism into another. Therefore, it is highly likely that the mutations described in this study strengthen the idea of anagenesis.

Also, the results from Tajima's D statistical test give us the biological interpretation that the proportion of mutations that alter codons for amino acids is higher than expected in both biovars and the population is evolving as per mutation-drift equilibrium. Furthermore, also is possible be interpreted rare alleles present at low frequencies in both biovars and population expansion after a recent bottleneck. Our structural analysis showed that none of the described mutations occurred within these sites or in positions previously described as critical for activity. Therefore, probably they do not affect protein function, but they are useful to indicate phylogenetic distance. For example, it was observed that *C. pseudotuberculosis* strain 162, which belongs to biovar Equi, is phylogenetically closer to biovar Ovis strains than to biovar Equi strains, as observed in the phylogenetic tree (Fig. 2). Probably this phylogenetic rapprochement is due to *C. pseudotuberculosis* strain 162 present Ovis specific mutations, although it is included in Equi biovar based on the nitrate test. Regarding this observation we hypothesize that biovar Ovis is being originated from biovar Equi, as mentioned before, as *C. pseudotuberculosis* 162 strain shares point mutations from both biovars.

Taking into account the reports discussed in the literature, many differences within the genomes of some *C. pseudotuberculosis* strains were reported [8, 29, 57]. Recently, Almeida et al. [30] (unpublished data) found a 99 % concordance in detection by PCR of gene *narG*,

responsible for nitrate reduction, and nitrate reduction test, suggesting that *C. pseudotuberculosis* biovars Equi could be identified by the presence of gene *narG*, whereas biovar Ovis does not present it. Guimarães et al. [34] showed a typing method based on PCR (ERIC-PCR), which proved to be a good method to discriminate genetic differences among *C. pseudotuberculosis* strains. Using this method it was possible to observe that *C. pseudotuberculosis* biovar Ovis and biovar Equi strains clustered separately [29]. The differences in the clustering pattern of *C. pseudotuberculosis* biovar Ovis and biovar Equi strains could reflect the number of specific genes in each biovar [57]. This fact is evident from the complete genome analyses of 18 *C. pseudotuberculosis* strains; among the total 1504 genes, it was shown that *C. pseudotuberculosis* biovar Ovis contains 314 orthologous genes that are shared by all strains from this biovar but are absent from one or more strains of *C. pseudotuberculosis* biovar Equi [57]. Furthermore, *C. pseudotuberculosis* biovar Equi strains have 95 core genes that are absent from one or more strains of *C. pseudotuberculosis* biovar Ovis [57]. Soares et al. [57], working with pathogenicity islands (PAI), found differences in some genes related to *pilus* formation in *C. pseudotuberculosis* biovar Equi and biovar Ovis strains. *Pilus* gene clusters are acquired normally in block through horizontal gene transfer and are composed of a specific sortase gene and the major, base and tip pilin genes. Genetic variation was found in genome analyses between the two *C. pseudotuberculosis* biovars (Almeida et al., [30]). *C. pseudotuberculosis* biovar Ovis strain VD57 had its genome analysed for the presence of SNP's, and the average variation found when it was compared to *C. pseudotuberculosis* biovar Ovis strains was 823 nucleotides, whereas when compared to the *C. pseudotuberculosis* biovar Equi strains that number increased to 25285.3 SNP's.

Regarding the anagenesis theory, we suggest that *C. pseudotuberculosis* biovar Equi is forming, after some genomic changes, *C. pseudotuberculosis* biovar Ovis. Hence, we postulated that *C. pseudotuberculosis* biovar Equi strains could be evolutionarily older compared to *C. pseudotuberculosis* biovar Ovis strains.

Conclusions

C. pseudotuberculosis biovar Equi and biovar Ovis contain different molecular characteristics, although they belong to the same species. The formation of a new species is an event that happens very slowly in nature. It is possible to interpret that a speciation process is occurring from the anagenesis event. With regard to the statistical data, analysis of sequence and structure of proteins addressed in this study, we conclude that *C. pseudotuberculosis* biovar Ovis is being formed from *C. pseudotuberculosis* biovar Equi through anagenesis.

Additional files

Additional file 1: Information about the strains of *C. pseudotuberculosis* in this work. In total, 18 strains were used of which nine strains were Equi and nine strains were Ovis as tabulated below. (PDF 19 kb)

Additional file 2: Information about the genes of *C. pseudotuberculosis*. All four genes were used in this work. These were united in the same alignment for phylogenetic analysis. (PDF 7 kb)

Additional file 3: Ratio templates used for molecular modeling. ϵ Resolution of the structure was determined by experimental methods of electron microscopy. (PDF 35 kb)

Additional file 4: Bionumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) (BMP 1582 kb)

Additional file 5: Average Nucleotide Identity (ANI) from biovar ovis and biovar equi. Typically, the ANI values between genomes of the same species are above 95 %. Our results show the value 98.76 %, which we expected due to the results of phylogeny. One-way ANI 1: 98.64 % (SD: 1.15 %), from 67439 fragments. One-way ANI 2: 98.69 % (SD: 1.20 %), from 101821 fragments. Two-way ANI: 98.76 % (SD: 0.92 %), from 34975 fragments. (PNG 435 kb)

Additional file 6: Molecular clock analysis by Maximum Likelihood method. Relationship between molecular divergence and time. The analysis involved 19 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 6514 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. (PDF 55 kb)

Additional file 7: Results from comparative test with molecular clocks using the Maximum Likelihood method and without molecular clock of strains. (PDF 8 kb)

Additional file 8: Fragment of multiple sequence alignment. The figure shows transition (arrows in blue) and transversion (red arrow) point mutations observed in nucleotide sequences from both biovars Equi and Ovis. (TIF 164 kb)

Additional file 9: Fragment of multiple sequence alignment of protein. The figure shows the substitutions of amino acids, in which most of the cases the changes modify the physicochemical characteristics. (TIF 216 kb)

Additional file 10: Representation of the structure of *gapA*, *rsmE* and *rpoB* by molecular modeling. The molecular differences between the amino acid from biovars Equi and Ovis induce an increase in the number of chemical bonds between amino acids that are close to the variant residue. (TIF 4028 kb)

Additional file 11: Structure of the *fusA* protein in the biovars Equi and Ovis analyzed by molecular modeling [55]. Expansion of 4 angstroms (Å) from the variant amino acid where it is possible to identify clusters of neighboring residues interacting among themselves. (A) Variation between V \leftrightarrow I, (B) Variation between D \leftrightarrow E. (TIF 2499 kb)

Abbreviations

CLA, caseous lymphadenitis; DOPE, discrete optimized protein energy; ERIC-PCR, enterobacterial repetitive intergenic consensus sequence-based; MSA, multiple sequence alignment; MST, minimum-spanning tree; NCBI, National Center Biotechnology Institute; PDB, Protein Data Bank; PFGE, pulsed-field gel electrophoresis; UL, ulcerative lymphangitis; Uniprot, Universal Protein Resource

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The phylogenetic tree was deposited in

TreeBASE under the URL <http://purl.org/phylo/treebase/phylovs/study/TB2:S19338>.

Authors' contributions

AO: Analysis and interpretation from this work, PT: support in modeling and phylogenetic processes, MA, SJ, ST, DB, PG: support with writing the paper, SA: support in obtaining sequences, DB, HF and RF: supports analysis, EMSD, DJH, MBH, APL: discussion on the ERIC PCR method, AS, HF and VA: Research support. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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