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# Abstract

*Mixta* is a recently described genus within the bacterial family *Erwiniaceae* that is closely related to *Pantoea*, *Erwinia*, and *Tatumella*. Depending on the genes and method used for phylogenetic analysis, the genus with which *Mixta* shares the most recent ancestor differs. This study aimed to determine the cause behind these contentious results, and identified genes that may have resulted from horizontal gene transfer. Ten complete or partial genomes – two *Mixta* species, six species from other *Erwiniaceae* genera, and two outgroups – were retrieved from NCBI and annotated. Homologous genes were extracted yielding a dataset of 799 genes. The genes were aligned With the ClustalW algorithm and MEGA-CC was used for model testing and computing phylogenetic trees for each gene for both nucleotide and amino acid sequences. Distance matrices were then extracted from these trees. Nucleotide and amino acid identity analyses were also done in R programming language. *Pantoea* was the closest relative to the *Mixta* species in most of the analyses; however, results were not consistent across analyses. Some genes were also found to be more similar to other, non-*Pantoea* species. Diligence must be given to the selection of genes for phylogenetic analysis and to the method chosen to prevent xenologous signal from distorting the true relationships. Furthermore, future research should consider that different phylogenetic analyses may give different results.

# Introduction

The bacterial family Erwiniaceae includes diverse Gram-negative [1, 2] organisms including the genera *Mixta*, *Pantoea*, *Erwinia*, *Tatumella*, *Buchnera*, *Wigglesworthia*, *Phaseolibacter*, *Kalamiella* (LPSN, <http://www.bacterio.net/>). Three of these, *Mixta*, *Pantoea*, and *Erwinia*, have been the focus of numerous studies given their relevance to plant and human pathogens. For example, the genus *Erwinia* contains plant pathogens (e.g. E. amylovora) [3–5], epiphytes (e.g. E. tasmaniensis) [6, 7], species that may contribute to plant disease [8], and others that are insect-associated [9–11]. Similarly, *Pantoea*, a genus reported by Gavini et al.[12], includes species that inhabit a variety of environments [12–14], including human and plant pathogens [2, 15–20] as well as species associated with plant growth [21, 22], insects [23], and fungi [24, 25]. *Mixta*, originally *Pantoea*, is a novel genus first suggested by Palmer et al. [26] and then later proposed by the same researchers in 2018 [27]. Two species, M. calida and M. gaviniae (previously species of *Pantoea*), were originally found in infant formula and its production environment [13]. Fritz et al. [28] demonstrated that some strains of M. calida were linked with post-surgical meningitis in humans. Currently, there are only five *Mixta* species validly published (LPSN, <http://www.bacterio.net/>).

Understanding the phylogeny of bacterial species and genes allows researchers to investigate the evolutionary history of the species, their relatedness, and the individual genes that allow the species to survive under various conditions [26, 29–33]. The most common methods in phylogenetic analysis are those including 16S ribosomal RNA (rRNA) sequences [34], multi-locus sequence analysis (MLSA) with several housekeeping genes [34–36], and ribosomal MLSA with 53 structural ribosomal proteins [26, 29, 37]. However, the phylogenetic trees generated from these methods may not be very robust [34, 36, 38] because of the lower phylogenetic signal from the highly conserved housekeeping genes [34, 35, 39–42]. This is particularly seen within Erwiniaceae wherein most of our understanding of the phylogeny and interrelationships are based on the 16S rRNA genes [43–47] because of its ubiquity. However, it has insufficient resolution at the species level [36] and the genera *Pantoea*, *Erwinia*, *Tatumella*, and *Mixta* are often not monophyletic [48]. Another issue that may arise with MLSA is unknowingly including paralogues (i.e. genes originating from an intragenomic duplication event) or xenologues (i.e. genes originating from horizontal gene transfer) [26, 49].

The DNA topoisomerase (gyrase) subunit (gyrB), DNA-directed RNA polymerase subunit (rpoB), ATP synthase subunit (atpD), and initiation translation factor (infB) have been shown to be more reliable than 16S rRNA gene sequences for determining intra- and intergeneric relationships [50–53] and a robust method for the Erwiniaceae family [54]. The genera within Erwiniaceae are monophyletic when concatenated partial gyrB, rpoB, atpD, and infB gene sequences are used [38, 55, 56].

Nevertheless, despite efforts to find a suitable method for identifying the species tree for Erwiniaceae genera, *Mixta*’s position with relation to *Pantoea*, *Erwinia*, and *Tatumella* vastly differs between studies [2, 26, 27, 47, 48, 57–59]. Not only do different gene combinations provide different results, but different algorithms and models for alignments and phylogenetic trees also contribute to the contentious results [45, 60]. For example, Prakash et al. [2], Palmer et al. [26], Rezzonico et al. [48], and Gueule et al. [59] all used gyrB, rpoB, atpD, and infB nucleotide gene sequences in their phylogenetic analyses. Prakash et al. conducted a neighbour-joining statistical method while the others used a maximum-likelihood (ML) method. Three of the studies – Rezzonico et al., Prakash et al., and Gueule et al. – placed the *Mixta* species within the *Pantoea* genera whereas the analysis in Palmer et al. placed *Mixta* outside of the other three genera. Palmer et al. [27], Rezzonico et al. [48], and Brady et al. [58] also ran analyses with the four MLSA genes as amino acid sequences. These three studies show contentious results as Rezzonico et al. found that *Mixta* is related closest with *Tatumella*, Palmer et al. found that *Mixta* is related closest with *Pantoea*, and Brady et al. found *Mixta* within the *Pantoea* genus. In a separate study conducted by Palmer et al. [26], two ML trees were computed, one with 1039 core amino acid sequence and the other with 52 ribosomal MLSA nucleotide genes. Both ML trees contained species from *Mixta*, *Pantoea*, *Erwinia*, and *Tatumella*; however, the analysis with the core genes placed *Pantoea* and *Tatumella* as sister taxa followed by two *Mixta* species whereas the analysis with ribosomal MLSA genes placed *Pantoea* and *Tatumella* as sister taxa followed by *Erwinia* and then by the *Mixta* species.

There are two possible causes behind contentious results in phylogenetic analyses. One is the use of different methods, including the use of a different phylogenetic tree model from another study despite both studies conducting maximum likelihood trees and the same sequence type. Additionally, there is no common phylogenetic analysis model between nucleotide and amino acid sequences; therefore, evolutionary history is estimated differently. The other reason may be horizontal gene transfer (HGT) events within the *Erwiniaceae* or from a non-closely-related species. HGT events occur frequently within bacteria and often the genes provide the bacteria the ability to adapt to new environments and/or survive selective pressures.

The objective of this paper was to determine the cause behind the contentious phylogenetic results for the *Mixta* species. To accomplish this, homologous genes were extracted from ten complete or partial genomes - two for each of *Mixta*, *Pantoea*, *Erwinia*, and *Tatumella*, and two outgroups - that were retrieved from public databases. In this study, both the nucleotide and amino acid sequences for the same genes were separately analyzed. Model testing was done for each gene in the dataset and phylogenetic trees were computed using the recommended model. Distance matrices were exported from the phylogenetic trees and these were used to determine which species were most closely related to *Mixta* for each gene. Nucleotide and amino acid identity analyses were also conducted for each gene in order to compare the results between evolutionary history and sequence similarity. Phylogenetic trees were then done with either one gene or multiple genes to confirm results.

# Materials and Methods

## Genomes and Gene Sets

Publicly available full genomes were retrieved from NCBI comprising of ten type strains from *Mixta*, *Pantoea*, *Erwinia*, *Tatumella*, *Enterobacter*, and *Pseudomonas* (Table 1). *Enterobacter cloacae* is in the same order of *Enterobacterales* as *Mixta*, *Pantoea*, *Erwinia*, and *Tatumella* whereas *Pseudomonas syringiae* is in the same class of *Gammaproteobacteria*. The genomes were annotated with Prokka v1.14.1 [61] using default parameters and homologous genes were extracted using GET\_HOMOLOGUES software package [62] with the bidirectional best-hit search algorithm using default parameters. GET\_HOMOLOGUES extracted 954 homologous genes between the ten species.

Table 1. Genomes used in this study.

|  |  |  |  |
| --- | --- | --- | --- |
| Species | Strain | GenBank.Accession.No. | Level |
| Mixta calida | DSM\_22759 | GCA\_002953215.1 | complete genome |
| Mixta gaviniae | DSM\_22758 | GCA\_002953195.1 | complete genome |
| Pantoea agglomerans | NBRC\_102470 | GCA\_001598475.1 | contigs |
| Pantoea septica | LMG\_5345 | GCA\_002095575.1 | contigs |
| Erwinia amylovora | CFBP\_1232 | GCA\_000367625.2 | contigs |
| Erwinia tasmaniensis | ET1/99 | GCA\_000026185.1 | complete genome |
| Tatumella ptyseos | NCTC\_11468 | GCA\_900478715.1 | complete genome |
| Tatumella saanichensis | NML\_06-3099 | GCA\_000439375.1 | contigs |
| Enterobacter cloacae subsp cloacae | ATCC\_13047 | GCA\_000025565.1 | complete genome |
| Pseudomonas syringae pv syringae | ICMP\_3023 | GCA\_001401075.1 | scaffold |

In R [63], two filter functions were written to pass through gene files of the nucleotide sequences. Since both nucleotide and amino acid sequences were analyzed, the filters were only applied to the nucleotide sequences. A list of the passed nucleotide gene files was used to isolate the corresponding amino acid gene files to be used in later analyses.

The first filter passed through gene files that contained ten, single copy sequences. A minimum relative length was set where all sequences in a gene file had to be at least 90% the length of the longest sequence in the same file. For example, if the longest sequence in a file is 1000 bp, then the rest of the sequences had to be at least 900 bp. Of 954 genes, 38 genes were excluded for having at least extra sequence and an additional 175 genes were excluded because at least one sequence did not meet the length requirements.

However, there may be an evolutionary reason why at least one of the sequences did not meet the length requirements. The second filter took the 175 gene files that were denied by the first filter one at a time and split the sequences into two groups; the first group contained the gene sequences that were over the 90% length cutoff and the second contained the rest that were shorter than the cutoff length. The first requirement for this filter was that all sequences in the file were longer than 80% of the length of the longest genes in the file. The second requirement was that both representative of a genus were in the same group, either above or below the 90% cutoff. The two outgroup species were not required to be in the same group since they are not from the same genus. An additional 58 genes were added to the working dataset, resulting in 799 genes. A list of the 799 gene names was used to grab the corresponding amino acid files.

## Gene Alignment

Gene alignments were in R using a ClustalW algorithm. The gene fasta files were read into R using the readDNAStringSet() function (package: Biostrings) [64]. Alignments were done using the msaClustalW() function (package: msa) [65] using default parameters and setting maximum number of iterations to 100. The aligned functions were then converted into a readable format using msaConvert() (package: msa)[65] and written into a new fasta file using the write.fasta() function (package: seqinr) [66]

## Distance Matrices

After alignments, model testing was done for each gene through MEGA-CC [67] for both nucleotide and amino acid sequences. In a command line terminal, MEGA-CC is called along with a text file listing the file pathways to all 799 genes and a MAO file detailing the analysis preferences. Default parameters were used for model testing. This resulted in a comma-separated values (CSV) file for each gene. These CSV files were read into R and the model for phylogenetic analysis was extracted based on lowest Bayesian Information Criterion (BIC). A list of the unique models and the number of genes that required each model are given in Table 2 for nucleotide sequences and in Table 3 for amino acid sequences.

Table 2. The number of genes that required each phylogenetic tree model according to model testing and the lowest BIC for nucleotide sequences.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Model | # | % | Model | # | % | Model | # | % |
| GTR\_G | 89 | 11.14 | K2 | 1 | 0.13 | T92\_G | 313 | 39.17 |
| GTR\_G\_I | 42 | 5.26 | K2\_G | 94 | 11.76 | T92\_G\_I | 33 | 4.13 |
| HKY\_G | 22 | 2.75 | K2\_G\_I | 1 | 0.13 | TN93\_G | 149 | 18.65 |
| HKY\_G\_I | 1 | 0.13 | K2\_I | 2 | 0.25 | TN93\_G\_I | 52 | 6.51 |

Table 3. The number of genes that required each phylogenetic tree model according to model testing and the lowest BIC for amino acid sequences.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Model | # | % | Model | # | % | Model | # | % |
| cpREV | 3 | 0.38 | JTT\_G\_I | 2 | 0.25 | mtREV24\_G\_I | 1 | 0.13 |
| cpREV\_G | 6 | 0.75 | JTT\_I | 1 | 0.13 | rtREV\_G | 3 | 0.38 |
| Dayhoff | 2 | 0.25 | LG | 19 | 2.38 | WAG | 1 | 0.13 |
| Dayhoff\_G | 11 | 1.38 | LG\_G | 563 | 70.46 | WAG\_G | 118 | 14.77 |
| Dayhoff\_I | 2 | 0.25 | LG\_G\_F | 27 | 3.38 | WAG\_G\_F | 2 | 0.25 |
| JTT | 1 | 0.13 | LG\_G\_I | 3 | 0.38 | WAG\_G\_I | 1 | 0.13 |
| JTT\_G | 25 | 3.13 | LG\_I | 5 | 0.63 | WAG\_I | 1 | 0.13 |
| JTT\_G\_F | 1 | 0.13 | mtREV24 | 1 | 0.13 | NA | – | – |

For each unique model, a text file was written listing the gene file pathways of each gene that required that model. In a command line terminal, MEGA-CC [67] was called with a text file listing gene file pathways for a certain model and the corresponding MAO file. This was done for each unique model. These MAO files specified the use of a maximum likelihood statistical method, 500 bootstrap replications, the model, and rate patterns to create a phylogenetic tree for each gene. If a model required a Gamma distribution, the number of discrete Gamma categories was set to 5, which is default.

Distance matrices were extracted from each of the phylogenetic trees and saved as Excel files. These files were read into R and the genetic distances between both of the *Mixta* species and all of the sequences were extracted for further analysis in R. Tables were created in R Markdown using the kable() function (package: knitr) [68] and figures were created using the ggplot() function (package: ggplot2) [69].

# Results and Discussion

After the two filters, 799 of of 954 homologous genes remained. The genes included those that encoded rRNA sequences, enzymes, transporters, and housekeeping genes and were scattered across the *M. calida* and *M. gaviniae* genomes.

## Closest Relative Based on Genetic Distance

Genetic distance gives a numeric estimate of evolutionary change between species. The species that has the shortest genetic distance to a species of interest is considered to be closest relative. *P. septica* was the closest relative to 66.3% and 66.5% of the *M. calida* and *M. gaviniae* genes, respectively, for the nucleotide sequences (Figure 1). In comparison, only 42.7% and 42.8% of *M. calida* and *M. gaviniae* genes, respectively, are closely related to *P. septica* for the amino acid sequences (Figure 1), but this is still the majority. These results suggest that *P. septica* is the closest relative to *Mixta* followed by *P. agglomerans*. For the amino acid sequences, instead of the *Mixta* genes being more closely related to *P. septica*, they are more closely related to the other species in this study (Figure 1). For example, the percentage of genes related to *P. agglomerans* increased from 13.9% for both of the *Mixta* species to 22.3 and 22.4% to *M. calida* and *M. gaviniae*, respectively. This may be due to a loss of phylogenetic signal when using amino acid sequences as a result of codon degeneracy and having a third of available sites.

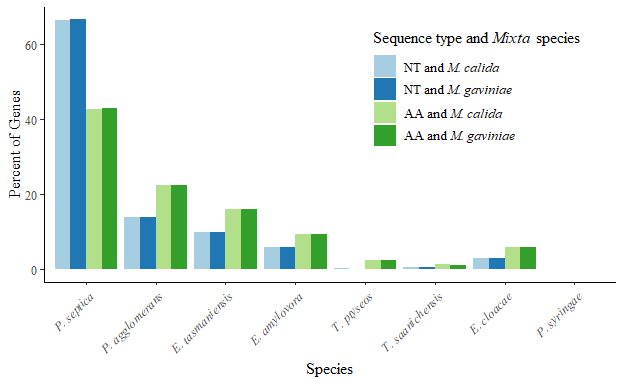


Figure 1. The percentage of genes (out of 799) that were most closely related to any of the eight non-*Mixta* species using genetic distances. The species with the shortest genetic distance from both of the *Mixta* species is considered to be the closest relative. Blue bars are the nucleotide sequences of the genes with light blue bars representing the percentage of *M. calida* genes and dark blue bars representing *M. gaviniae*. Green bars are the corresponding amino acid sequences with light green representing *M. calida* genes and dark blue bars representing *M. gaviniae*. Approximately 66% of *M. calida* and *M. gaviniae* genes were most closely related to *P. septica* when using nucleotide sequences in comparison to only 43% when using amino acid sequences.

Unexpectedly, the number of *Mixta* genes that are more closely related increased from 2.9% to 6.0% when using the amino acid sequences instead of the nucleotide sequences (Figure 1). This, along with the other differences in relatedness for *Mixta* genes between nucleotide and amino acid sequences, suggests that the sequence type used in the analysis will give different results, even when using the same genes.

If genetic distance infers the same closest relative for a *Mixta* gene for both nucleotide and amino acid analyses, then these results may be more reliable. Thus, for genes where the closest relative is an *Erwinia*, *Tatumella*, or *Enterobacter* species both nucletodie and amino acid analyses, they may have resulted from a horizontal gene transfer (HGT) event rather than vertical evolution from a *Pantoea* species. No *Mixta* genes were found to be closely related to *P. syringiae* for either the nucleotide or amino acid sequences (Figure 1). This confirms that *P. syringiae* is a suitable outgroup for this analysis and suggests that any HGT events may be restricted to the order *Enterobacterales*.

Due to limitations in model options in MEGAX [70] for genetic distance estimations, only available models were used to infer genetic distance to determine if model choice may alter results for the closest relative. For genetic distance estimation in MEGAX, GTR (General Time Reversible) and HKY (Hasegawa-Kishino-Yano) models are not available. Additionally, MEGAX does not have the option to add the invariant sites (+I) parameter in genetic distance estimation. As a result, the best model recommended by model testing and lowest BIC value could not always be used (Table 4). For many of the genes, either the Tamura-Nei with a Gamma parameter (TN93\_G) or the Tamura 3-parameter with a Gamma parameter (T92\_G) had to be used instead.

Table 4. The number of genes requiring each model when using phylogentic tree analysis vs genetic distance analysis in MEGAX for nucleotide sequences.

|  |  |  |
| --- | --- | --- |
| Model | # of genes requiring each model | # of genes requiring each available model |
| GTR\_G | 89 | NA |
| GTR\_G\_I | 42 | NA |
| HKY\_G | 22 | NA |
| HKY\_G\_I | 1 | NA |
| K2 | 1 | 3 |
| K2\_G | 94 | 98 |
| K2\_G\_I | 1 | NA |
| K2\_I | 2 | NA |
| T92\_G | 313 | 398 |
| T92\_G\_I | 33 | NA |
| TN93\_G | 149 | 300 |
| TN93\_G\_I | 52 | NA |

To assess whether there were any positional effects of the genes and spatial patterns of the closest relatives, circular plots were generated using the *Mixta* gene IDs for nucleotide sequences (Figure 2) and amino acid sequences (Figure 3). The gene IDs give the approximate location of each gene relative to the *Mixta* genome. The length and colour of a bar represents the species that was the closest relative to the *Mixta* gene at that position. Since the closest relatives were the same both *Mixta* species except for 1 and 2 for nucleotide and amino acid sequences, respectively, only the results for *M. calida* are shown. The differing gene

Since the closest relatives for all but one gene (ttuB where M. calida is with T. ptyseos and M. gaviniae is with P. septica) for both *Mixta* species for the nucleotide sequences, only *M. calida* is shown Amino acids: only two (ttuB where M. calida is with T. saanichensis and M. gaviniae is with P\_septica and secY where M. calida is with E. tasmaniensis and M. gaviniae is with P. agglomerans)

To assess whether there were any positional effects of the genes, circular plots were generated using gene IDs for M. calida and M. gaviniae and the closest relatives (Figures 1 and 2). The genes showing greatest identity (lowest distance) to P. septica are distributed across the genome for both M. calida and M. gaviniae (Figures 1 and 2), further suggesting Mixta and Pantoea species have a recent common ancestor. If many genes were horizontally transferred in a single event, then large blocks of genes from a single species would be expected. However, the Erwinia lines (green) are fairly even throughout the genome and the outgroup lines (red and orange) are separated with genes in between. This suggests that Mixta underwent many HGT after speciation from Pantoea.

Positional effects of all of the species and range of the distances were also evaluated (Figures 3 and 4). For most genes, the genetic distance for each of the eight species ranged from 0 to 1.5 with only a few greater than 2. The cluster of genes around ID 425 in both M. calida and M. gaviniae have a very small range and so are highly conserved among all of the species. These genes were annotated as being ribosomal genes. Furthermore, Pantoea species (blue dots) are typically the closest relative to Mixta, followed by Erwinia (green). The Tatumella species (purple and pink) and the outgroup species (red and orange) vary as to which are the furthest relative to Mixta.

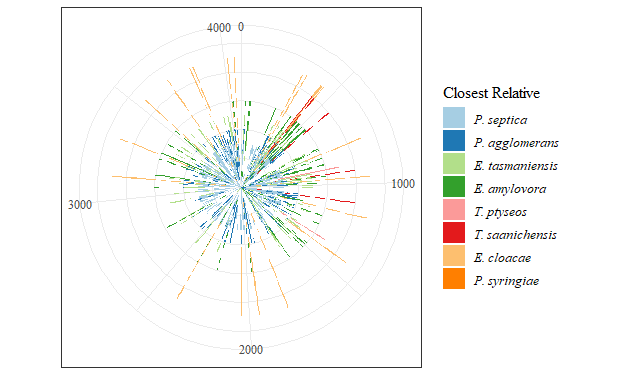
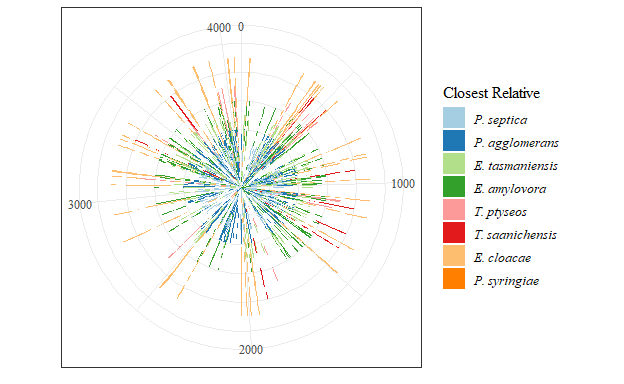


Figure 2.

 # Conclusion

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# Appendix