UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

Genomic diversity, transmission dynamics and drug resistance of Mycobacterium tuberculosis in El Oro province (Ecuador).

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga.

Autor:

León Ordóñez Kerly Gisselle

Tutor:

Dr. Ballaz García Santiago Jesús

Urcuquí, julio 2020

Urcuquí, 26 de mayo de 2020

SECRETARÍA GENERAL
(Vicerrectorado Académico/Cancillería)
ESCUELA DE CIENCIAS BIOLÓGICAS E INGENIERÍA
CARRERA DE BIOLOGÍA
ACTA DE DEFENSA No. UITEY-BIO-2020-00019-AD

A los 26 días del mes de mayo de 2020, a las 15:00 horas, de manera virtual mediante videoconferencia, y ante el Tribunal Calificador, integrado por los docentes:

Presidente Tribunal de Defensa	Dr. GUDIÑO GOMEZJURADO, MARCO ESTEBAN , Ph.D.
Miembro No Tutor	Dr. ALVAREZ BOTAS, FRANCISCO JAVIER , Ph.D.
Tutor	Dr. BALLAZ GARCIA, SANTIAGO JESUS , Ph.D.

El(la) señor(ita) estudiante **LEON ORDOÑEZ, KERLY GISSELLE**, con cédula de identidad No. **0705329548**, de la **ESCUELA DE CIENCIAS BIOLÓGICAS E INGENIERÍA**, de la Carrera de **BIOLOGÍA**, aprobada por el Consejo de Educación Superior (CES), mediante Resolución **RPC-SO-37-No.438-2014**, realiza a través de videoconferencia, la sustentación de su trabajo de titulación denominado: **Genomic diversity, transmission dynamics and drug resistance of Mycobacterium tuberculosis in El Oro province (Ecuador)**, previa a la obtención del título de **BIÓLOGO/A.**

El citado trabajo de titulación, fue debidamente aprobado por el(los) docente(s):

Tutor	Dr. BALLAZ GARCIA, SANTIAGO JESUS , Ph.D.

Y recibió las observaciones de los otros miembros del Tribunal Calificador, las mismas que han sido incorporadas por el(la) estudiante.

Previamente cumplidos los requisitos legales y reglamentarios, el trabajo de titulación fue sustentado por el(la) estudiante y examinado por los miembros del Tribunal Calificador. Escuchada la sustentación del trabajo de titulación a través de videoconferencia, que integró la exposición de el(la) estudiante sobre el contenido de la misma y las preguntas formuladas por los miembros del Tribunal, se califica la sustentación del trabajo de titulación con las siguientes calificaciones:

Tipo	Docente	Calificación
Tutor	Dr. BALLAZ GARCIA, SANTIAGO JESUS , Ph.D.	9.9
Presidente Tribunal De Defensa	Dr. GUDIÑO GOMEZJURADO, MARCO ESTEBAN , Ph.D.	10.0
Miembro Tribunal De Defensa	Dr. ALVAREZ BOTAS, FRANCISCO JAVIER , Ph.D.	9.5

Lo que da un promedio de: **9.8 (Nueve punto Ocho)**, sobre 10 (diez), equivalente a: **APROBADO**

Para constancia de lo actuado, firman los miembros del Tribunal Calificador, el/la estudiante y el/la secretario ad-hoc.

LEON ORDOÑEZ, KERLY GISSELLE
Estudiante

Firmado electrónicamente por: MARCO ESTEBAN GUDIÑO GOMEZJURADO

Dr. GUDIÑO GOMEZJURADO, MARCO ESTEBAN , Ph.D.
Presidente Tribunal de Defensa

SANTIAGO JESUS BALLAZ GARCIA

Firmado digitalmente por SANTIAGO JESUS BALLAZ GARCIA
Fecha: 2020.07.11 11:29:54 -05'00'

Dr. BALLAZ GARCIA, SANTIAGO JESUS , Ph.D.
Tutor

FRANCISCO JAVIER ALVAREZ BOTAS

Digitally signed by FRANCISCO JAVIER ALVAREZ BOTAS
Date: 2020.07.10 18:31:35 -05'00'

Dr. ALVAREZ BOTAS, FRANCISCO JAVIER , Ph.D.
Miembro No Tutor

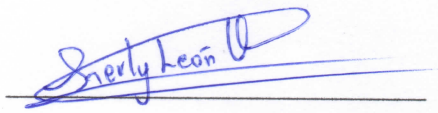
Firmado electrónicamente por:
KARLA ESTEFANIA ALARCON FELIX

ALARCON FELIX, KARLA ESTEFANIA
Secretario Ad-hoc

AUTORÍA

Yo, **Kerly Gisselle León Ordóñez**, con cédula de identidad **0705329548**, declaro que las ideas, juicios, valoraciones, interpretaciones, consultas bibliográficas, definiciones y conceptualizaciones expuestas en el presente trabajo; así cómo, los procedimientos y herramientas utilizadas en la investigación, son de absoluta responsabilidad de el/la autora (a) del trabajo de integración curricular. Así mismo, me acojo a los reglamentos internos de la Universidad de Investigación de Tecnología Experimental Yachay.

Urcuquí, julio 2020.



Kerly Gisselle León Ordóñez

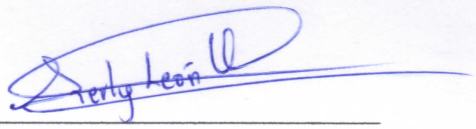
CI: 0705329548

AUTORIZACIÓN DE PUBLICACIÓN

Yo, **Kerly Gisselle León Ordóñez,** con cédula de identidad **0705329548**, cedo a la Universidad de Investigación de Tecnología Experimental Yachay, los derechos de publicación de la presente obra, sin que deba haber un reconocimiento económico por este concepto. Declaro además que el texto del presente trabajo de titulación no podrá ser cedido a ninguna empresa editorial para su publicación u otros fines, sin contar previamente con la autorización escrita de la Universidad.

Asimismo, autorizo a la Universidad que realice la digitalización y publicación de este trabajo de integración curricular en el repositorio virtual, de conformidad a lo dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior

Urcuquí, julio 2020.



Kerly Gisselle León Ordóñez

CI: 0705329548

DEDICATION

To my mother, who is my support to face the challenges that life imposes on me, and who gives me strength and optimism to continue in this process of self-improvement.

Kerly

ACKNOWLEDGMENT

To Yachay Tech University and its professors who have guided me along this path of self-improvement. To my thesis tutor who has given me advice and guidance throughout the entire research process. Thank you all for your unwavering support.

Kerly

Abstract

Tuberculosis (TB) is one of the most important infectious diseases worldwide. Understanding the transmission of MTB is essential to analyze and propose control strategies, and to monitor drug resistance spread. Ecuador is a middle burden country for tuberculosis with 7,200 new cases per year and 490 deaths associated with TB per year. In this research, MTB isolates circulating in the province of El Oro, on the border of Ecuador and Peru were studied to address its potential transnational transmission. Seventy-one strains collected between the 2012 and 2016 years were genotyped by the 24-loci mycobacterial interspersed repetitive units (MIRU-VNTR) and compared to strains from Ecuador and Peru, respectively. Besides, this research included a study of the prevalence of drug resistance of MTB strains in the province of El Oro. The analysis of the results of the genotyping method (24-loci MIRU-VNTR) allows establishing the presence of genotypically related MTB strains. These strains will form clonal complexes and clusters. The comparative analysis with Ecuador showed twenty clonal complexes (CC) comprising four CC between strains of El Oro and Ecuador, two CC with strains of El Oro, and fourteen CC with strains of Ecuador. While the analysis with Peru showed twenty-two clonal complexes that comprise three CC with strains of El Oro and nineteen CC with strains of Peru, without finding any clustering between El Oro and Peru. Genotyping by MIRU-VNTR (24-loci) revealed one (1.41%) lineage 2 - Beijing family, twenty (28.17%) lineages 4 - LAM family, eight (11.27%) lineages 4 - X family, one (1.41%) lineage 4 - S family, nine (12.68%) lineages 4 - Haarlem family and thirteen (18.31%) lineages 4 - T family. Drug resistance was detected in 42 (59.15%) isolates, and all were found to have primary resistance; among these, twelve (16.90%) were resistant to isoniazid, nine (12.68%) to rifampin, eight (11.27%) to streptomycin, two (2.82%) to ethambutol, two (2.82 %) to pyrazinamide, and nine (12.68%) multiple drug resistant (isoniazid and rifampin). In summary, the results showed that there are no clusters between the strains of the border region (El Oro) and Peru, indicating that there is no recent active transmission with Peru compared to Ecuador.

Keywords: *Mycobacterium tuberculosis*, genotyping, lineages

Resumen

La tuberculosis (TB) es una de las enfermedades infecciosas más importantes a nivel mundial. Comprender la transmisión de MTB es esencial para analizar y proponer estrategias de control, y para controlar la propagación de resistencia a los medicamentos. Ecuador es un país de carga media para la tuberculosis con 7200 nuevos casos por año y 490 muertes asociadas a la TB por año. En esta investigación, los aislados de MTB que circulan en la provincia de El Oro, en la frontera de Ecuador y Perú, fueron estudiados para abordar su posible transmisión transnacional. Setenta y una cepas recolectadas entre los años 2012 y 2016 fueron genotipadas por las unidades repetitivas intercaladas micobacterianas de 24 loci (MIRU-VNTR) y se compararon con las cepas de Ecuador y Perú, respectivamente. Además, esta investigación incluyó un estudio de la prevalencia de resistencia a los medicamentos de las cepas MTB de la provincia de El Oro. El análisis de los resultados del método de genotipificación (24-loci MIRU-VNTR) permite establecer la presencia de cepas MTB relacionadas genotípicamente. Estas cepas formarán complejos clonales y clusters. El análisis comparativo con Ecuador mostró veinte complejos clonales (CCs) que comprenden cuatro CCs entre cepas de El Oro y Ecuador, dos CCs con cepas de El Oro, y catorce CCs con cepas de Ecuador. Mientras que el análisis con Perú mostró veintidós complejos clonales que comprenden tres CCs con cepas de El Oro y diecinueve CCs con cepas de Perú, sin encontrar ningún agrupamiento entre El Oro y Perú. La genotipificación por MIRU-VNTR (24 loci) reveló un (1,41 %) linaje 2 - familia Beijing, veinte (28,17 %) linajes 4 - familia LAM, ocho (11,27 %) linajes 4- familia X, un (1,41 %) linaje 4 - familia S, nueve (12,68 %) linajes 4 - familia Haarlem y trece (18,31 %) linajes 4 - familia T. La resistencia a drogas fue detectada en 42 (59,15 %) aislados, y se encontró que todos tenían resistencia primaria; entre estos; doce (16,90 %) fueron resistentes a isoniacida, nueve (12,68 %) a rifampicina, ocho (11,27%) a estreptomicina, dos (2,82 %) a etambutol, dos (2,82 %) a pirazinamida, y nueve (12,68 %) resistentes a múltiples fármacos (isoniacida y rifampicina). En resumen, los resultados mostraron que no hay grupos entre las cepas de la región fronteriza (El Oro) y Perú, lo que indica que no hay una transmisión activa reciente con Perú en comparación con Ecuador.

Palabras clave: *Mycobacterium tuberculosis,* genotipificación, linajes

SUMMARY

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Tuberculosis disease

Tuberculosis (TB) is an airborne disease caused by organisms of the *Mycobacterium tuberculosis* complex (MTBC) that can invade the lungs or any other organ. The route of entry *of M. tuberculosis* is via the respiratory tract where it is ingested by alveolar macrophages through phagocytosis. The pathogen blocks phagosome fusion with the lysosome, ensuring its survival. Due to this mechanism, the pathogen can break the phagosomal membrane producing the release of bacterial products as genetic material inside the macrophage. After infecting the alveolar macrophages, *M. tuberculosis* infects the lung interstitium and the parenchyma where the pathogen increases the amount of cells which produces a multicellular response called a granuloma. The granuloma is involved in the control and persistence of the pathogen, which acts as a bacterial prison with the potential to grow, replicate within and infect the rest of the body. If granuloma only restricts the infection without inducing tissue pathology, the person has latent TB infection and could be a candidate for preventive treatment. But this state can change to an active TB infection. Thus, the patients with *M. tuberculosis* infection are classified as patients with latent TB infection or active TB disease (Bermejo et al., 2007) (Pai et al., 2016).

Latent TB infection is an asymptomatic and non-transmissible state in which the patient develops granulomas where the bacteria are isolated. Active TB is a transmissible state where the patient shows symptoms like cough, fever, and weight loss (Pai et al., 2016). The treatments are based on 4 drugs: isoniazid, rifampicin, pyrazinamide and ethambutol. However, *M. tuberculosis* can develop drug resistance through genetic mutations, which changes the target site or produce a defective enzyme that converts a pro-drug into an active drug. Resistance to first-line drugs (isoniazid and rifampicin) against TB is known as multidrug-resistant TB (MDR-TB), and resistance to first-line drugs and any fluoroquinolone or second-line drugs (kanamycin, capreomycin or amikacin) is known as extensively drug-resistant TB (XDR-TB) (Jasmer et al., 2002) (Cohen et al., 2011).

According to the World Health Organization (WHO), TB disease is one of the top 10 causes of death in the world. In 2017, TB disease reached an estimated 10 million infected people worldwide (5.8 million men, 3.2 million women, and 1 million children). A challenge to a

successful treatment is the drug-resistant TB. Around 558,000 people have developed resistance to rifampicin (RR-TB), the first-line drug, and of these, 82% were MDR-TB. The major risk factors to TB are HIV infection, undernutrition, indoor air pollution, and smoking. India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh, South Africa, and 22 other countries are present in WHO's list of the 30 highest TB burden countries, although the hardness of national epidemics differs among countries.

1.2 Tuberculosis epidemiology in Ecuador and Peru.

In South America, the Pan American Health Organization (PAHO) reported a TB incidence rate of 46.2 per 100,000 population of an estimated total of 282,000 cases in the American region. During 2017, it was estimated that 87% of these cases occurred in 10 countries. Among them, Peru was found in second place with 13% of the estimated cases and Ecuador in tenth place with 3%. On the one hand, Ecuador with a population of 17 million had an incidence of TB (including HIV) of 7,200 cases with a rate of 43 per 100,000 population and the mortality rate of 2.9 per 100,000 population. Patients with multidrug-resistance, mainly towards rifampicin (first-line drug), TB showed an incidence rate of 3.7 per 100,000 population. Also, the provinces of Ecuador with the highest incidence of TB were Guayas, El Oro and Los Ríos. In Ecuador, prevention strategies have focused on the identification of people with respiratory symptoms (duration of expectoration equal to or greater than 15 days), which are examined clinically and bacteriologically. The molecular biology method used for the diagnosis of TB was Rapid Xpert / MTB / RIF Detection, which identifies *Mycobacterium tuberculosis* and detects resistance to rifampicin (Ministerio de Salud Pública, 2017).

On the other hand, Peru with a population of 32 million had an incidence of TB (including HIV) of 37,000 cases with a rate of 116 per 100,000 population and the mortality rate of 6.8 per 100,000 population (World Health Organization, 2018) (Ministerio de Salud-MINSA, 2017). Patients with multidrug-resistance TB show an incidence rate of 9.4 per 100,000 population, being this resistance against isoniazid and rifampicin. But more serious is the presence of the extensively drug-resistant TB, which is a reason of serious concern in the control of tuberculosis in Peru. In addition, epidemiological studies also showed an uncontrolled epidemic of MDR / XDR TB mainly in the cities of Lima and Callao (Del

Castillo et al., 2009). In Peru, tuberculosis is a challenge for public health due to the high incidence of cases, the presence of resistant strains, the association of tuberculosis with infection of the human immunodeficiency virus (HIV), and its presence in all social strata. To face this challenge, Peru has been performing emergency plans to control sensitive tuberculosis, studies about the genetic diversity of *M. tuberculosis*, and the performance of molecular epidemiology surveys to monitor this disease (Bonilla Asalde, 2008).

Ecuador and Peru are neighboring countries with a significant difference concerning the epidemiological situation of the disease. Peru has a higher incidence of tuberculosis compared to Ecuador and a high number of studies related to epidemiology. In Ecuador, a research conducted by Garzón-Chavez et al. (in press) analyzed the genetic diversity of MTBC nationwide and found that there are similar genetic patterns between strains of Ecuador and Colombia, but not between Ecuadorian and Peruvian strains. Therefore, a study of a border region of Ecuador with Peru would allow to analyze the possibility of transmission among these two border countries, which is carried out in this current study.

1.3 Molecular epidemiology of Tuberculosis

The studies about genetic diversity, transmission dynamics and drug resistance of *M. tuberculosis* are essential to the epidemiological control of the disease, which is a challenge in public health. Molecular epidemiology is centered on the application of a set of molecular methods and tools to epidemiological research. It is then defined as the science that studies the causes of the emergence, spread, maintenance and decrease of diseases in populations through the use of molecular techniques (Coll & García de Viedma, 2018). These techniques are used to compare nucleic acid sequences of two or more isolates. In the case of *M. tuberculosis*, related MTB isolates come from the clonal expansion of a single precursor and have a level of similarity between genotypes and phenotypes of greater than found in unrelated isolates (Burgos et al., 2004).

Mycobacterium tuberculosis has a genome with repetitive sequences that allow comparison of the genetic traces of different isolates, thus establishing putative differences among strains. By differentiating isolated strains, it has been possible to obtain new information about the epidemiology of the disease, recent reactivation, and transmission in areas with different prevalence, risk factors such as the frequency of exogenous reinfection (finding areas where

it show some frequency), molecular identification and genetic tracking (García-Pachón & Rodríguez, 2005). Thus, the methods of conventional epidemiology together with molecular epidemiology have allowed a better understanding of epidemic outbreaks, chains, and transmission dynamics, or other characteristics of infectious processes such as immunity and resistance to treatment (Coll & García de Viedma, 2018). Also, it allows knowing the strains that predominate in our community, which strains require a greater surveillance, how the cases attributable to recent transmission vary over time and to determine why they are transmitted (García-Pachón & Rodríguez, 2005) (Burgos et al., 2004) (Barnes & Cave, 2003) (Soolingen, 2001).

One of the best known molecular methods in molecular epidemiology is *Mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR)* standardized and described by Supply (2005). In the next paragraph, the method is briefly explained.

Variable Number Tandem Repeats (VNTRs) are sites inside the genome where there are short sequences of repeat units (namely "tandem repeats"). In the case of *M. tuberculosis*, the tandem repeats are located in intergenic regions dispersed (loci) within its genome, whereby VNTRs are used as markers to genotyping. Based on this, the technique performed a simple PCR amplification of the VNTRs making use of specific oligonucleotides (primers) for flanking regions of the VNTRs, and the determination of the size of the fragments obtained, after electrophoresis. The size indicates the quantity of the amplified VNTR copies. Thanks to that the length (50 to 100 bp) of the tandem repeats is known, the size can be associated with a digit to each VNTR locus (see appendix 1). Each digit reflects the number of tandem repeats present in each locus. For example, if we studied seven loci this generated a numerical code or allele profile composed by seven digits and each digit represent the number of tandem repeats at one of the seven VNTR loci (Frothingham & Meeker-O'Connel, 1998) (Mazars et al., 2000). Nowadays, a set of 12, 15 and 24 loci have been studied, which allow to generate an allele profile that can be used to differentiate each strain according to the variability that it presents in the number of tandem repeats in each amplified locus. From now onward and for clarity, the repeat units will be named as "Mycobacterial Interspersed Repetitive Units" (or MIRUs), each locus as "MIRU-VNTR loci", and the allele profile as "MIRU pattern" (Mathema et al., 2006) (Supply, 2005) (Supply et al., 2000) (Supply et al., 2001)

(Nikolayevskyy et al., 2016). Finally, the MIRU pattern can be analyzed with tools such as MIRU-VNTRplus software, APPLIED MATHS and others.

In the case of MIRU-VNTRplus software (www.miruvntrplus.org), the genotyping data (namely "MIRU pattern") of MTB strains are analyzed alone or study in contrast with a reference database of strains forming the major MTBC lineages. Based on this, the software has two main functions: phylogenetic lineage identification by using reference databases, and analysis and visualization of genotyping data. To perform these functions, the MIRU pattern is analyzed by two genetic distance measures that are$(\delta\mu)^2$, and DSW (stepwise weighted distance) which are based on the stepwise mutation model. This model considers that slipped-strand mispairing accounts for most of the allelic variation in the tandem repeats, whereby it assumes that the distance among strains increases with the difference in the number of tandems repeats in a given locus (Weniger et al., 2010).
In the identification of the phylogenetic lineage, user strains (input data) compared with the reference strains that represent the families of the main MTBC lineages. In order to do so, we will consider a reference value of 0.17, which is called the distance threshold. If the genetic distance among these two strains (user and reference) is less than 0.17, the user strain will be identified with the family to which the reference strain belongs (Weniger et al., 2010).
In the analysis and visualization of genotyping data, the software allows us to create two graphical representations of the relationship between strains: dendrogram and minimum spanning tree (MST). On the one hand, the dendrogram is calculated by the neighbor-joining algorithm, which is based on genetic distance measures. On the other hand, the MST is calculated using locus variant counts. In the MST, the presence of more or less close relationships among the strains is found by taking into account the difference of number of tandem repeats in the locus analyzed for each strain. This difference can be symbolized by the dashed lines and solid lines that connect the strains. Dashed lines represent a low relationship due to more than two loci differences, and solid lines represent a high relationship due to only one or two loci differences. A high relationship among the strains is represented by clonal complexes (CC) and clusters. CCs form when there are one or two different loci, and clusters form when there are no different loci (same MIRU pattern). Because CCs and clusters can mean a high genetic relationship between strains, which suggests possible transmission. This possible transmission is suggested when there are some

MTB strains with similar or identical MIRU patterns and when there are different patterns it is considered to be due to an infection acquired at a different time (Weniger et al., 2010).

1.4 Molecular epidemiology of Tuberculosis in South America

The application of molecular epidemiology has helped to understand the evolution and distribution of the pathogen. Also, it offers a greater accuracy, processing speed and analysis of samples at a lower cost than traditional methods. This has allowed the establishment of phylogenetic studies that reveal the genetic diversity of MTB, which can be classified into 6 lineages (Lineage 1: Indo-Oceanic, lineage 2: East Asian, lineage 3: East African/Indian, lineage 4: Euro-American, lineage 5: West African 1, and lineage 6: West African 2) of main strains. The techniques used are based on standard epidemiological typing such as IS6110 RFLP, spoligotyping, and MIRU-VNTR, which are used to classify strains (Woodman et al., 2019). Some countries in Latin America described the genetic diversity of *M. tuberculosis* to analyze the dynamics of transmission and establish epidemiological control. This has been done through molecular studies based on samples from patients with TB (Gonzalo et al., 2011). Peru, Argentina, Mexico, and Brazil have reported that their predominant *M. tuberculosis* strains have evolved from the Euro-American lineage. Furthermore, there is a genetic distribution that varies between and within countries in Latin America as a result of migratory patterns (Woodman et al., 2019). That is the cases of the mycobacterial Manila and Beijing families in Mexico and in Peru respectively, as a result of historical links with Asia (Woodman et al., 2019) (Flores-López et al., 2017).

Among the countries with the highest TB rates in Latin America is Peru; this country has conducted studies based on molecular epidemiology to establish controls for the presence of new mycobacterial strains and their transmission. The molecular methods for the epidemiological analysis of TB by Peru were the Restriction Fragment Length Polymorphism (or RFLP), spoligotyping, MIRU-VNTR, single nucleotide polymorphisms, and DNA fingerprinting analysis. By doing this, Peru has established the presence of three main families: Haarlem, Latin American Mediterranean (LAM) and Beijing (Barletta et al., 2013) (Iwamoto et al., 2012) (Cáceres et al., 2014). In Peru, it has been possible to improve in the diagnosis without clinical suspicion, the study of epidemic outbreaks, the study of latent TB

(distinguish endogenous reactivation from exogenous reinfection), the determination of risk factors for TB (such as those infected with HIV / AIDS), and the study of the geographic distribution of clones of *M. tuberculosis*, as well as the exploration of phylogenetic associations with drug resistance (MDR TB and XDR) (Sheen et al., 2013).

Ecuador is another country that has a middle burden TB, where the strategy to control tuberculosis disease is based on the detection, diagnosis, treatment, as well as informative programs about the disease. This depends on the surveillance of positive cases done by the Dirección Nacional de Vigilancia Epidemiológica, which helps control the infected population and regulates the infection (Ministerio de Salud Pública, 2017). Unfortunately, Ecuador has limited information about the genetic diversity of *M. tuberculosis* with only four research works using the molecular epidemiology approach to the genotype of the TB strains, the analysis of their association with drug resistance, and the study of transmission dynamics. For this reason, it has been possible to detect the presence of the Beijing, LAM, Ghana, Cameroon, S, X, Delhi, and Haarlem families, which are associated with MDR-TB (Zurita et al., 2019) (Jiménez et al., 2017) (Garzón et al., 2019) (Garzón-Chavez et al., in press). This demonstrates the need for further molecular studies to establish the presence of new strains or the association of the current strains with drug resistance, and the surveillance of the insertion of a new strain or possible transmission with border countries like Peru (Jiménez et al., 2017).

2. PROBLEM STATEMENT

In Ecuador, the epidemiological control of tuberculosis focuses on detection, diagnosis, and treatment. Thus, the research centers of the Ministerio de Salud Publica are limited to the analysis of the positive cases to confirm the diagnosis and perform resistance tests that allow meeting a control strategy (Ministerio de Salud Pública, 2017). In this context, molecular studies are a necessary tool to know the prevalence of specific *M. tuberculosis* strains in the country and possible transmission patterns (microorganisms in closely related or divergent groups) inside and outside of the country. In this way, this would help us enhance our understanding of the genetic variability of *M. tuberculosis*, the epidemiological links among populations, and the risk factors that otherwise would be nearly impossible to identify with the use of more traditional epidemiological methods (Perdigão et al., 2017). Therefore, it is

necessary to conduct a study of the genetic diversity of *M. tuberculosis* and its possible patterns of transmission through the use of genotyping methods, which are a powerful tool that can complement the current strategies used for the epidemiological control of tuberculosis.

3. OBJECTIVES

3.1 *General objective*

- To analyze the genomic diversity, the transmission patterns linked to the pathogen, and prevalence of drug resistance TB in samples of patients from El Oro province.

3.2 *Specific objectives*

- To determine the presence of new strains due to patterns of human migration on the border regions associated with possible transmission of *M. tuberculosis*.
- To apply molecular methods for epidemiological tuberculosis controls in Ecuador.

4. MATERIALS AND METHODS

The present work was carried out mainly in the Research Laboratory of the Universidad de las Américas (UDLA). The samples and drug susceptibility test used in this research were obtained from the Instituto Nacional de Salud Pública e Investigación Leopoldo Izquieta Pérez (INSPI).

4.1 *Study population and data collection*

El Oro province is located in the south of the country on the border with Peru. Its population is 600,659 people and half inhabit are in the city of Machala (Instituto Nacional de Estadística y Censo, 2010). This province has a constant migration due to the commercial activity between Ecuador and Peru (ALADI, 2013). This fact allows the analysis of the genetic diversity of *M. tuberculosis*, possible transmission and insertion of new strains in Ecuador. In this context, sputum samples were obtained from patients with TB from El Oro Province between 2012 and 2016, which were collected and stored by the INSPI institute. In addition, the information about resistance test, year and sociodemographic data (city and sex) was also retrieved and recorded (Table 1 and 2).

Table 1. Distribution of *Mycobacterium tuberculosis* isolates across El Oro Province

City	***Number of isolates***	***Average (% of total)***
El Cambio	1	1.4
Machala	67	94.4
Piñas	2	2.8
Santa Rosa	1	1.4
TOTAL	71	100

Table 2. Socio-demographic data of patients with TB

Sex	***Number of patients***	***Average (% of total)***
Female (F)	18	25.4
Male (M)	48	67.6
No information available	5	7.0
TOTAL	71	100

4.2 *MTBC isolates*

In this research, we used 71 MTB isolates from patients having pulmonary and extrapulmonary TB. Samples were collected in the years: 2012 (n = 35), 2013 (n = 9), 2014 (n = 8), 2015 (n = 16), 2016 (n = 2), and no information (n = 1). Also, antibiotic resistance tests were done against rifampicin, isoniazid, pyrazinamide, ethambutol, and streptomycin (anti-TB drug sensitivity tests or PSD).

4.3 *Drug susceptibility testing*

A first-line drug susceptibility test was performed based on the multiple proportions method described by Canetti et. al (1963), which uses a Löwenstein-Jensen (L-J) medium with minimum inhibitory concentrations of 40 ug / mL for rifampicin, 0.2 ug / mL for isoniazid, 4 ug / mL for ethambutol and 200 ug / mL for pyrazinamide.

4.4 *DNA isolation*

The samples of *M. tuberculosis* isolates were grown in a Löwenstein-Jensen medium (L-J) and resuspended in 500 ul of TE buffer (10mM Tris and 1mM EDTA, pH 8.0). Subsequently, it was boiled at 95 °C for 45 min, and then centrifuged at 10,000 g for 5 min. Finally, the supernatant was stored at -80 °C without DNA purification until it was used for genotyping or typing.

4.5 Molecular procedures

4.5.1 *Reagents*

GoTaq Master Mixes was purchased from Promega. Primers forward and reverse were acquired from eurofins. RT-PCR Grade Water, UltraPure Agarose, GeneRuler 50 bp DNA Ladder, and SYBR Safe DNA Gel Stain were purchased from Thermo Fisher Scientific.

4.5.2 *Molecular typing method (MIRU-VNTR)*

MIRU-VNTR is a PCR-based typing method that assigns the number of tandem repeats for each independent locus (MIRUs). In this context, all samples were analyzed with MIRU-VNTR typing based on 24 loci, whereby each locus was amplified by simple PCR. The PCR reaction was prepared for a final volume of 15 µl PCR mixture for each reaction, each consisting of the addition of 7.5 µl of GoTaq (DNA polymerase, green buffer,MgCl 2 , blue dye, yellow dye), 0.75 µl of primer forward (10 µM), 0.75 µl of primer reverse (10 µM), 5 µl of water, and 1 µl of DNA (Table 3). For thermal cycling, the setting was as follows: 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 61 °C for 1 min 30 s, and 20 s at 72 °C, then 72 °C for 10 min and finally 4 °C for 10 min.

Table 3. PCR primer sequences

Locus	Alias	Repeat unit length (tandem repeats length), bp	PCR primer pairs (5--->3)
580	MIRU 4	77	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC

2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG
802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
960	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
3192	MIRU 31	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT
424	MIRU 42	51	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC
577	MIRU 43	58	CGAGAGTGGCAGTGGCGGTTATCT AATGACTTGAACGCGCAAATTGTGA
2165	MIRU ETRA	75	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT
2401	MIRU 47	58	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCCATTAGTA
3690	MIRU 52	58	CGGTGGAGGCGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG
4156	MIRU 53	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT
2163b	MIRU QUB-11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT
1955	MIRU 1955	57	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA
4052	MIRU QUB-26	111	AACGCTCAGCTGTCGGAT CGGCCGTGCCGGCCAGGTCCTTCCCG AT
154	MIRU 2	53	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT
2531	MIRU 23	53	CTGTCGATGGCCGCAACAAAACG AGCTCAACGGGTTCGCCCTTTTGTC

4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT
2059	MIRU 20	77	TCGGAGAGATGCCCTTCGAGTTAG GGAGACCGCGACCAGGTACTTGTA
2687	MIRU 24	54	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA
3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA
2347	MIRU 46	57	GCCAGCCGCCGTGCATAAACCT AGCCACCCGGTGTGCCTTGTATGAC
2461	MIRU 48	57	ATGGCCACCCGATACCGCTTCAGT CGACGGGCCATCTTGGATCAGCTAC
3171	MIRU 49	54	GGTGCGCACCTGCTCCAGATAA GGCTCTCATTGCTGGAGGGTTGTAC

The primer sequences according to Supply. (2005)

The PCR products were analyzed by electrophoresis using a 2%-agarose gel. Electrophoresis was run under a constant 100 V current for 1 hour 15 min, and GeneRuler 50 bp DNA ladders were used as molecular size markers to determine bands sizes. The corresponding bands were obtained from the gel images and interpreted, based on a reference table (see the appendix 10.1), as copy numbers.

4.5.3 *Phylogenetic analysis*

Seventy-one MTB isolates were analyzed with the 24-loci MIRU-VNTR typing and lineages of the corresponding isolates were assigned by comparing the MIRU-VNTR patterns (user strains or user isolates) with those in the MIRU-VNTRplus platform (reference database). Also, a MIRU-based dendrogram and minimum spanning tree (MST) analysis using locus variant counts were performed to study the phylogenetic relationship.

For the transmissibility analysis, the MIRU-VNTR data of the strains of El Oro province were analyzed against the MIRU-VNTR data available for MTB strains of Peru and Ecuador with the software packages available at MIRU-VNTR plus platform using $(\delta\mu)^2$, and DSW (stepwise weighted distance) distance measures suggested for MIRU-VNTR data (pattern).

4.5.4 *Clonal complexes (CC) and Cluster*

Clonal complexes were determined due to one or two differences in MIRU-VNTR locus, and a cluster was assigned when there was an equality in the MIRU pattern of the strains studied.

5. RESULTS

5.1 *Bacterial strains and drug susceptibility testing*

Based on the drug susceptibility test conducted by INSPI, the available resistance tests were only 39 out of the 71 MTB isolates used in this study. 27/71 (38.03%) were phenotypically sensitive to isoniazid, 31/71 (43.66%) to streptomycin, 37/71 (52.11%) to ethambutol, 30/71 (42.25%) to rifampicin, and 37/71 (52.11%) to pyrazinamide, which are known as first-line antibiotics in the treatment of tuberculosis. The prevalence of phenotypically resistant isolates is described in Table 4, which shows that 9/71 (12.68%) isolates were classified as MDR (resistant to the two main anti-TB drugs known as isoniazid and rifampicin).

Table 4. Resistance to first line drug anti-TB and its percentage of prevalence in the study population

Drug resistance profile	***Number of isolates***	***Average (% of total)***
Isoniazid resistance	12	16.90
Streptomycin resistance	8	11.27
Ethambutol resistance	2	2.82
Rifampicin resistance	9	12.68
Pyrazinamide resistance	2	2.82
Isoniazid+rifampicin resistance (MDR)	9	12.68
Sensible to all drugs tested	25	35.21
No information available	32	45.07

5.2 *Identification of MIRU-VNTR genotype profiles of the MTB isolates from El Oro province*

The 24-loci MIRU-VNTR test provided information in each locus for the 71 samples analyzed, which generated MIRU patterns. These patterns were compared with the database available on the MIRU-VNTR plus platform; 52/71 (73.24%) strains were identified and 19/71 (26.76%) strains were unidentified which are defined as unknown (Table 5). The analysis showed that the principal lineage was lineage 4 (Euro-American), which represented 51/71 (71.83%) strains, and only 1/71 (1.41%) strain belonged respectively to the lineage 2 - family Beijing. The distribution of lineage 4 families included: LAM (20/71 or 28.17%), Haarlem (9/71 or 12.68%), S (1/71 or 1.41%), X (8/71 or 11.27%), and T family (13/71 or 18.31%).

Table 5. Genetic diversity. Quantity and percentages of the genotypes found in El Oro province

Lineage	*MTBC Families*	*Number of isolates*	*Average (% of total)*
Lineage 2 (East Asian)	**Beijing**	1	1.41
Lineage 4 (Euro-American)	**Haarlem**	9	12.68
	LAM	20	28.17
	S	1	1.41
	T	13	18.31
	X	8	11.27
	Unknown	19	26.76

5.3 *Population structure of the MTB strains from El Oro province*

The relation of MTB genotypes in the province of El Oro was analyzed with the help of the MIRU-VNTR plus platform. The Neighbor-Joining tree (dendrogram) based on the 24-loci

MIRU-VNTR for 71 strains (Figure 1), showed only two clusters with 2/71 (2.82%) strains each one (301 MACH 2012 and 377 MACH 2012; 545 MACH 2012 and 552 MACH 2012). Also, MST analysis presented three clonal complexes (CC1, CC2, CC3) that included 8/71 (11.27%) strains, and 59/71 (83.1%) remaining strains had MIRU patterns that vary by more than two loci concerning all strains (Figure 2).

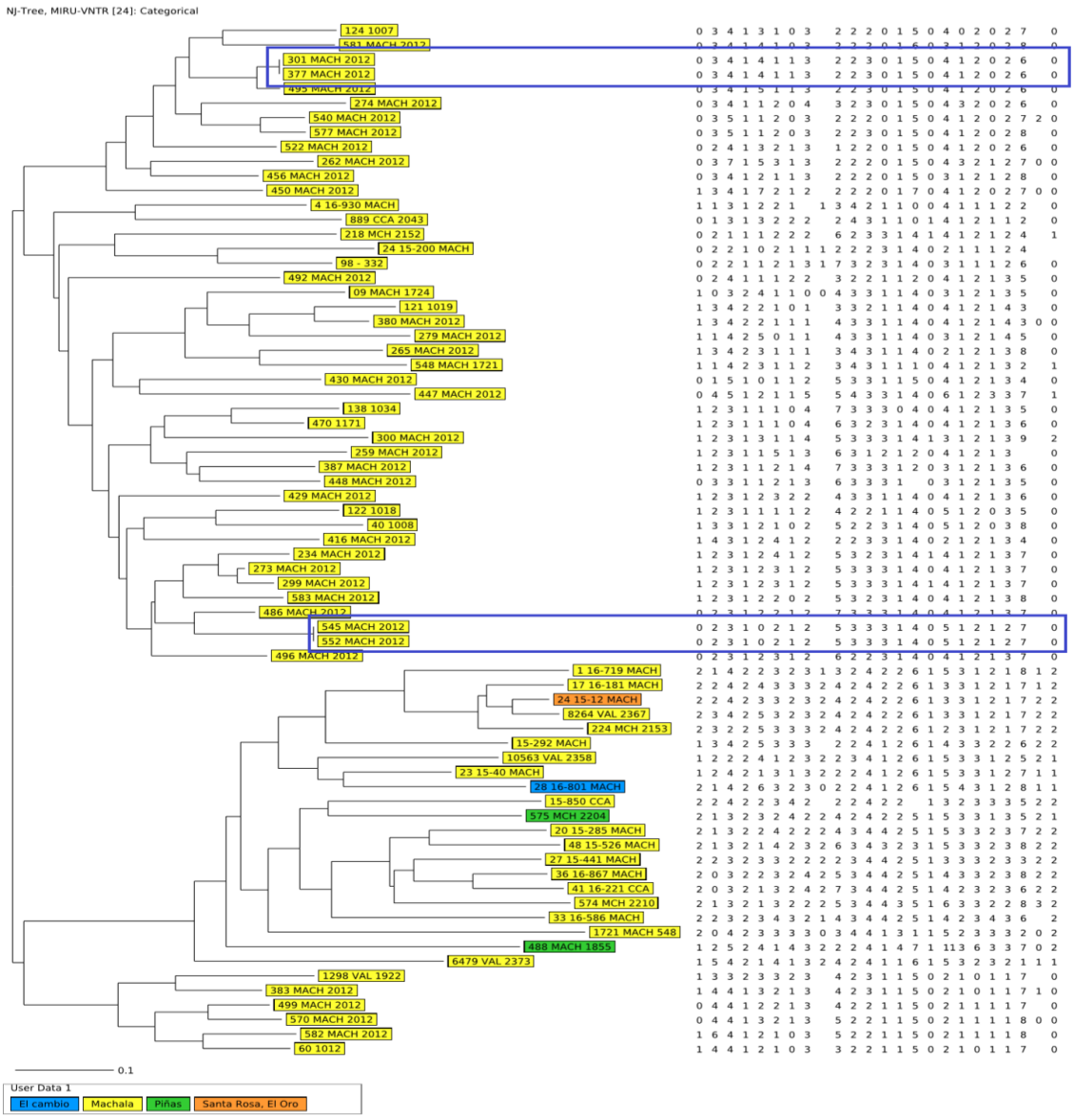


Figure 1. Neighbor joining tree and the 24-MIRU VNTR patterns of 71 strains from El Oro province. The 71 strains of El Oro province coming from Machala (yellow), El Cambio (blue), Piñas (green) y Santa Rosa (orange). On the left side of the figure shows, MIRU-based dendrogram and on the right side of the figure shows the MIRU patterns from each strain.

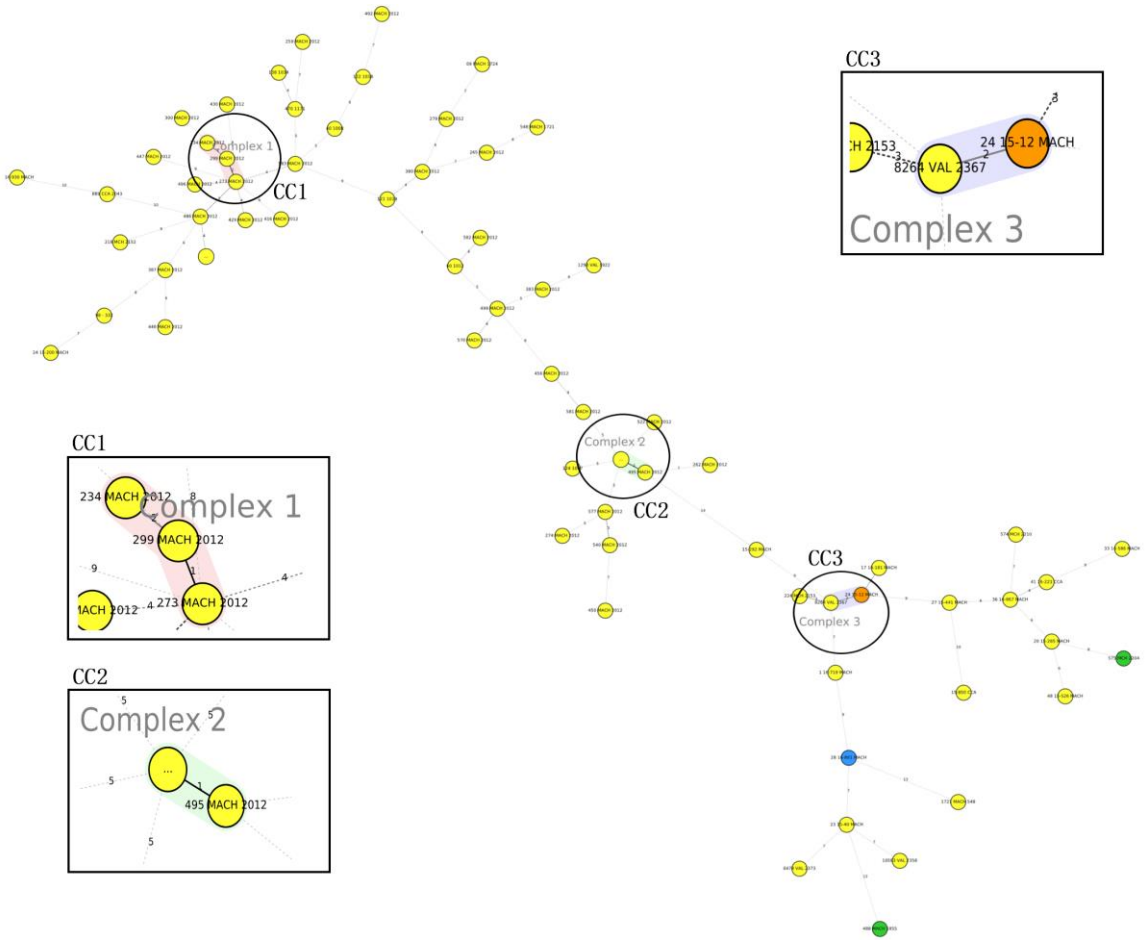


Figure 2. MST analysis from El Oro province. MST analysis of strains from Machala (yellow), El Cambio (blue), Piñas (green), and Santa Rosa (orange). The 24-loci MIRU-VNTR pattern for all strains included in this MST analysis is detailed in figure 1. CC1, CC2, CC3 show the clonal complexes formed between the strains. The loci difference between the genotypes is displayed by the style of the line (solid line = 1 or 2 loci difference, dashed line = more than 2 loci differences). Also, the distance is not proportional to line length.

5.4 *Comparison of MTB genotypes from El Oro province with database available from Peru and Ecuador*

To determine the relationship among the isolates of El Oro, Peru and Ecuador, a MIRU-based dendrogram and minimum spanning tree (MST) was drawn with the help of the MIRU-VNTR plus platform.

Concerning Peru, the MST analysis compared the 71 genotypes of El Oro against the reported genotypes in Peru. All the MIRU-VNTR patterns used for the MST are represented in Figure 3, which shows how the strains of Peru were grouped in specific clonal complexes for this

country. The seventy-one genotypes from El Oro are grouped into clusters, small and specific clonal complexes for this province. Also, it was observed that the strains from El Oro are genetically more diverse than the strains of Peru. Only 1/71 (1.41%) strains were linked to Peruvian genotypes. Despite this, we found no clustering with Peruvian clonal complexes.

As to Ecuador, seventy-one genotypes were compared against all available genotypes from Ecuador. The Neighbor-Joining tree (dendrogram) and MST analysis showed a clonal complex (CC4) comprising a cluster with 1/71 strains (1.41%), and another 2/71 strains (2.82%). Another three clonal complexes were found (CC13, CC15, and CC19), which had 1/71 strains (1.41%) each one. Also, there were 15/71 strains (21.13%) from El Oro linked with genotypes from other provinces that did not form clusters or clonal complexes, and 50/71 strains (70.42%) with different MIRU patterns concerning other provinces of Ecuador (Figures 4 and 5).

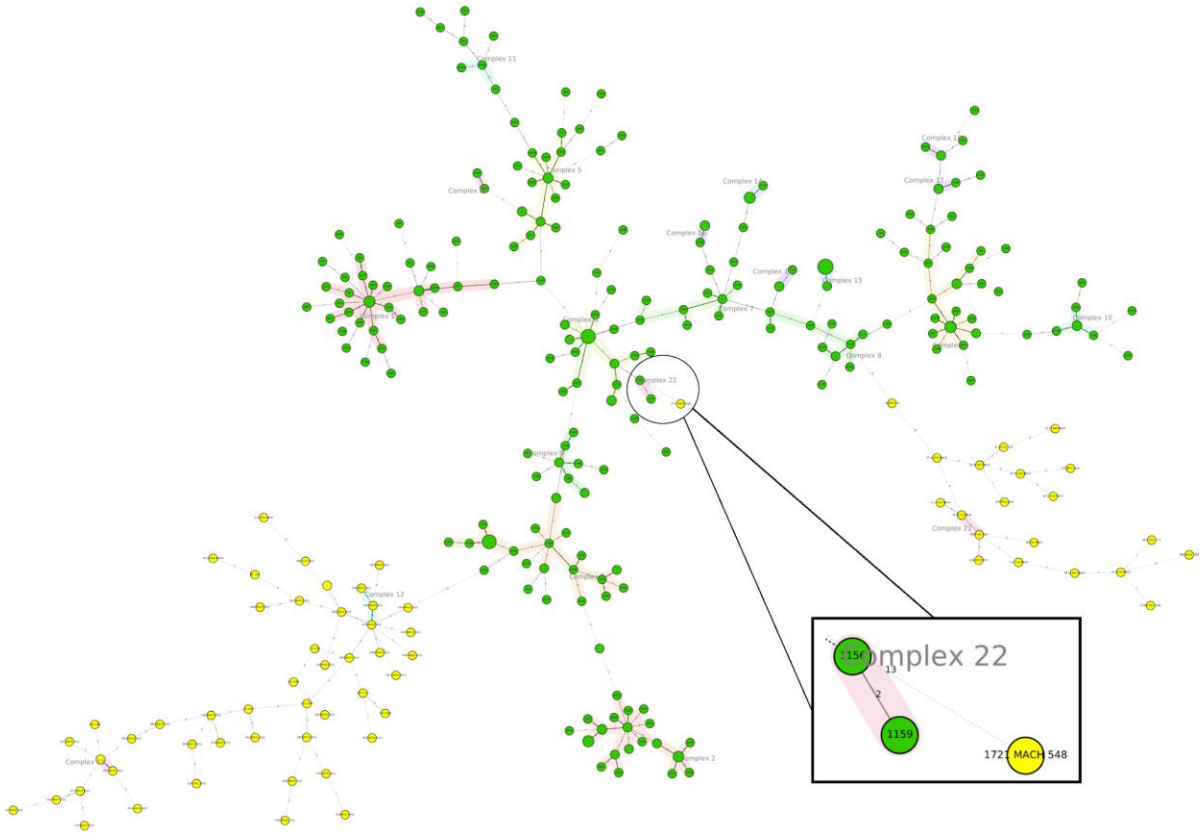


Figure 3. MST analysis between El Oro province and Perú. MST analysis of strains from El Oro (yellow) and Peru (green). The strains from El Oro are described in this study. The strains from Peru were described in Cáceres et al. (2014) and Barletta et al. (2015).

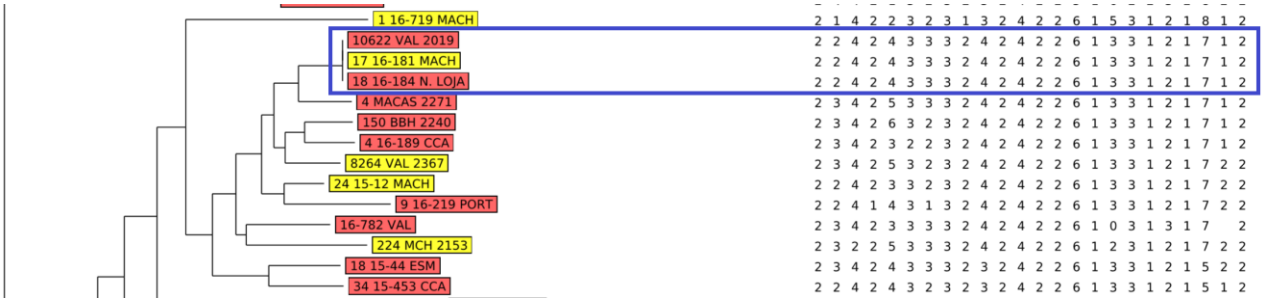


Figure 4. Neighbor joining tree and the 24-MIRU VNTR patterns of cluster between strains from El Oro province and Ecuador. The strains from El Oro province (yellow) and Ecuador (red) with 24-MIRU-VNTR patterns. The complete neighbor-joining tree can be found in the appendix 10.3

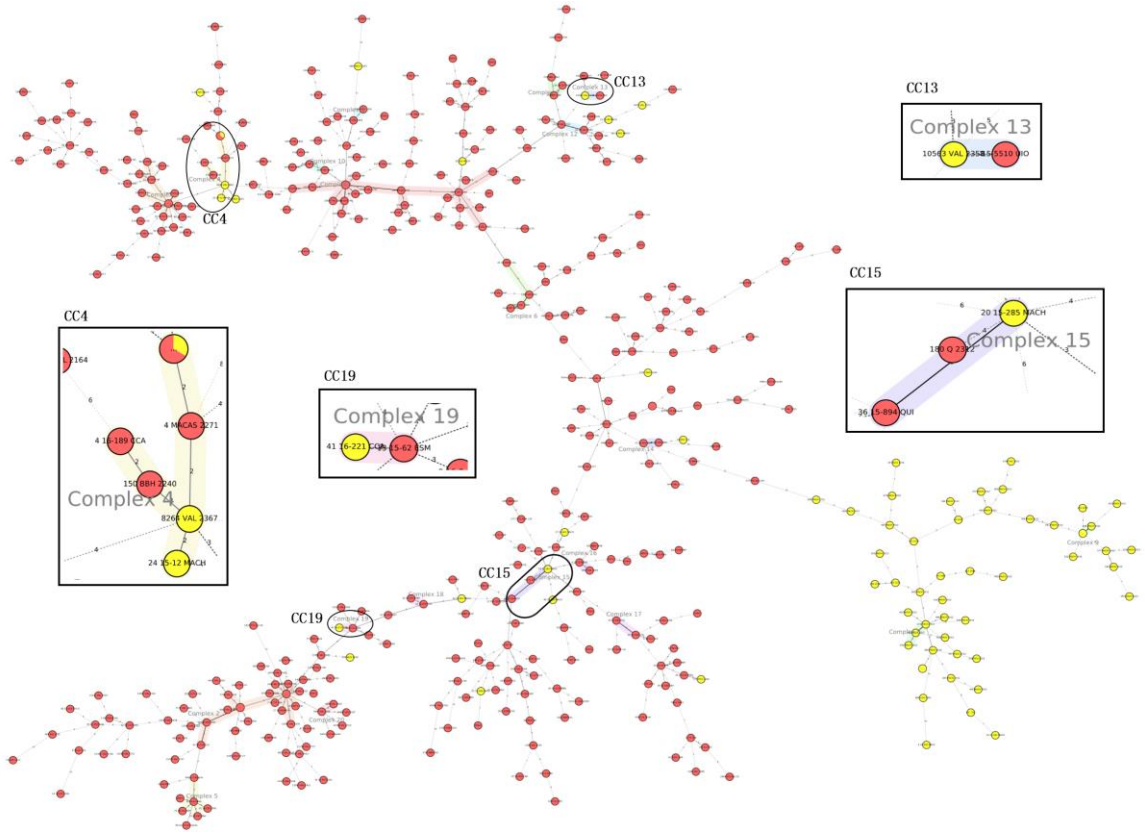


Figure 5. MST analysis between El Oro province and Ecuador. MST analysis of strains from El Oro (yellow) and Ecuador (red). The strains from El Oro are described in this study. The strains from Ecuador are described on Garzón-Chavez et al. (in press). CC4, CC13, CC15 and CC19 show clonal complexes formed between El Oro and Ecuador strains.

6. DISCUSSION

In this study, the genetic diversity of MTB isolates of El Oro province was characterized and compared with MTB isolates from Peru to gain insight into a possible transmission through the border between Ecuador and Peru. Also, we included a study of the prevalence of the resistance to anti-TB drugs in El Oro province. Some countries in Latin America like Paraguay, Colombia, Venezuela, Peru, Argentina, and Brazil have already reported their genetic diversity and established predominant strains as part of their epidemiological control policies (Cerezo et al., 2012) (Díaz Acosta et al., 2019) (Conceição et al., 2017) (Cerezo-Cortés et al., 2019) (Realpe et al., 2014). However, it should be considered that countries such as Peru and Brazil have focused their studies in densely populated urban areas, in a specific lineage or in strains associated with drug resistance, which is a limitation when conducting comparative studies within these countries. This is evident in Peru, where the molecular epidemiological studies available were focused in Lima and Callao (Cáceres et al., 2014) (Barletta et al., 2015). In the case of Ecuador, the information available about MTB isolates is scarce, since just four molecular epidemiology studies are available so far; two focused on the Beijing lineage, one on genetic diversity and drug resistance, and other in a nationwide analysis about the population structure of MTBC (Zurita et al., 2019) (Jiménez et al., 2017) (Garzón et al., 2019) (Garzón-Chavez et al., in press).

To investigate resistance, genetic diversity and transmission, a drug susceptibility test, a 24-loci MIRU-VNTR analysis, and MST analysis, respectively, were performed. The drug sensitivity study revealed a high resistance to isoniazid (12 or 16.9%) and rifampicin (9 or 12.68%), the two most effective first-line antituberculosis drugs available. These results are similar to the studies on resistance reported in Ecuador (Zurita et al., 2019) (Garzón-Chavez et al., in press). Also, a high prevalence of MDR-TB (9 or 12.68%) was found, which is very high compared to other regions inside and outside of Ecuador (Pan American Health Organization, 2017). Concerning the genetic diversity, El Oro showed more strains defined within lineage 4 (71.83%) and the LAM family (28.17%) associated with this lineage. These results were similar to studies reported on the phylogenetic distribution of MTBC in countries of South America, including Peru (Woodman et al., 2019) (Stucki et al., 2016) (Brynildsrud et al., 2018). In the case of lineage 2, family Beijing had a low prevalence (1.41%), which

are in agreement with the four studies carried out in Ecuador (Zurita et al., 2019) (Garzón et al., 2019) (Jiménez et al., 2017) (Garzón-Chavez et al., in press). Also, the presence of unknown genotypes must also be taken into account, which could be explained by the decrease in discriminatory power of the technique in each locus within each genetic family, which can generate a high frequency of unknown isolates. Due to this, the use of other complementary genotyping method like spoligotyping is recommended (Weniger et al., 2010) (Wirth et al., 2008) (Djelouadji et al., 2008) (Allix-Béguec et al., 2008).

The MST analysis from El Oro in the study of TB transmission showed the presence of two clusters and three clonal complexes (CCs) composed of 2-3 strains. In the case of CC3, a possible transmission between the cities of Machala and Santa Rosa was suggested, probably because between these cities, there is a constant commercial flow that generates the migration of population (Instituto Nacional de Estadística y Censo, 2010), thus playing an important role in the spread of the disease. Moreover, they remain groups of strains with similar MIRU patterns but without forming clonal complexes, indicating that they have a recent origin. This can be explained by changes in MIRU, which are caused by short insertions or deletions that can occur every 15 or 20 years, remembering that MIRU loci symbolize the most variable structures in the *M. tuberculosis* genome (Mizrahi & Andersen, 1998) (Sreevatsan et al., 1997). In the case of the route of transmission from Peru, the results showed that there was no clustering or CCs with the Peruvian genotypes. Similar findings were described in the research of Garzón-Chavez et al. (in press), where CCs were found with Colombia and not with Peru, although they only studied 21 strains of El Oro. Although this study used an increased number of strains, it was found no CCs. Neither the study did not find a correlation between the Beijing strain described above and the Peruvian strains. This result was similar to the research carried out by Garzón et al. (2019) based on the Beijing family in Ecuador, where it was shown that there was no transmission of the Beijing strains with Peru .

Interestingly, a set of El Oro strains were found to have a low relationship with Peruvian and Ecuadorian genotypes, so these strains were highly specific and isolated to this region. This finding may suggest that they were derived from a clone originally circulating in El Oro. However, being the first genotyping study focused in this province, we cannot assure this state. But this finding may be a baseline study for future research lines or the scope of another

study. Finally, in the case of transmission between provinces, it was possible to observe the formation of one cluster and four clonal complexes with whole Ecuador, this suggests the presence of possible TB transmission between provinces, which was reported in a previous study conducted by Garzón-Chavez et al. (in press).

7. CONCLUSIONS

- The study addressed the genetic diversity of *Mycobacterium tuberculosis* in El Oro province, prevalence of drug resistance and analyzed the possible transmission with the rest of Ecuador and Peru, using the 24-loci MIRU-VNTR technique.
- In El Oro province 28.17% of studied strains were identified in the LAM family, 12.68% in Haarlem family, 1.41% in S family, 11.27% in X family, 18.31% in T family, and 1.41% in Beijing family.
- Prevalence of drug resistance in El Oro shows that 16.90% of studied strains were resistant to isoniazid, 12.68% to pyrazinamide, and 12.68% to isoniazid-rifampicin (MDR-TB).
- Clonal complexes within El Oro province and a set of strains possibly derived from a clone originally of this region were found. Also, we found clonal complexes between El Oro and other regions of Ecuador but not with Peru, discarding active transnational transmission.

8. LIMITATIONS OF THE STUDY

Spoligotyping of MTB strains included in this study is ongoing for a better lineage identification, which is not possible only with MIRU-VNTR. MIRU pattern of MTB strains available on the database from Peru has a geographical bias, coming mostly from the Lima and Callao area. Besides, there are no epidemiological studies available from the northern region of Peru so a putative local transmission from northern Peru to El Oro cannot be utterly discarded.

9. REFERENCES

ALADI. (2013). *ALADI Asociación Latinoamericana de Integración*. ALADI. http://www.aladi.org/sitioaladi/

Allix-Béguec, C., Harmsen, D., Weniger, T., Supply, P., & Niemann, S. (2008). Evaluation and Strategy for Use of MIRU-VNTRplus, a Multifunctional Database for Online Analysis of Genotyping Data and Phylogenetic Identification of Mycobacterium tuberculosis Complex Isolates. *Journal of Clinical Microbiology*, *46*(8), 2692–2699. https://doi.org/10.1128/JCM.00540-08

Barletta, F., Otero, L., Collantes, J., Asto, B., de Jong, B. C., Seas, C., & Rigouts, L. (2013). Genetic variability of Mycobacterium tuberculosiscomplex in patients with no known risk factors for MDR-TB in the North-eastern part of Lima, Peru. *BMC Infectious Diseases*, *13*(1), 397. https://doi.org/10.1186/1471-2334-13-397

Barletta, F., Otero, L., de Jong, B. C., Iwamoto, T., Arikawa, K., Van der Stuyft, P., Niemann, S., Merker, M., Uwizeye, C., Seas, C., & Rigouts, L. (2015). Predominant Mycobacterium tuberculosis Families and High Rates of Recent Transmission among New Cases Are Not Associated with Primary Multidrug Resistance in Lima, Peru. *Journal of Clinical Microbiology*, *53*(6), 1854–1863. https://doi.org/10.1128/JCM.03585-14

Bermejo, M. C., Clavera, I., Michel de la Rosa, F. J., & Marín, B. (2007). Epidemiología de la tuberculosis. *Anales Del Sistema Sanitario de Navarra*, *30*, 07–19.

Bonilla Asalde, C. (2008). Situación de la tuberculosis en el Perú: Current status. *Acta Médica Peruana*, *25*(3), 163–170.

Brynildsrud, O. B., Pepperell, C. S., Suffys, P., Grandjean, L., Monteserin, J., Debech, N.,

Bohlin, J., Alfsnes, K., Pettersson, J. O.-H., Kirkeleite, I., Fandinho, F., Silva, M. A. da, Perdigao, J., Portugal, I., Viveiros, M., Clark, T., Caws, M., Dunstan, S., Thai, P. V. K., … Eldholm, V. (2018). Global expansion of Mycobacterium tuberculosis lineage 4 shaped by colonial migration and local adaptation. *Science Advances*, *4*(10), eaat5869. https://doi.org/10.1126/sciadv.aat5869

Burgos, M. V., Méndez, J. C., & Ribon, W. (2004). Molecular epidemiology of tuberculosis: Methodology and applications. *Biomédica*, *24*, 188–201. https://doi.org/10.7705/biomedica.v24iSupp1.1317

Cáceres, O., Rastogi, N., Bartra, C., Couvin, D., Galarza, M., Asencios, L., & Mendoza-Ticona, A. (2014). Characterization of the Genetic Diversity of Extensively-Drug Resistant Mycobacterium tuberculosis Clinical Isolates from Pulmonary Tuberculosis Patients in Peru. *PLOS ONE*, *9*(12), e112789. https://doi.org/10.1371/journal.pone.0112789

Cerezo, I., Jiménez, Y., Hernandez, J., Zozio, T., Murcia, M. I., & Rastogi, N. (2012). A first insight on the population structure of Mycobacterium tuberculosis complex as studied by spoligotyping and MIRU-VNTRs in Bogotá, Colombia. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *12*(4), 657–663. https://doi.org/10.1016/j.meegid.2011.07.006

Cerezo-Cortés, M. I., Rodríguez-Castillo, J. G., Hernández-Pando, R., & Murcia, M. I. (2019). Circulation of M. tuberculosis Beijing genotype in Latin America and the Caribbean. *Pathogens and Global Health*, *113*(8), 336–351. https://doi.org/10.1080/20477724.2019.1710066

Cohen, T., Murray, M., Abubakar, I., Zhang, Z., Sloutsky, A., Arteaga, F., Chalco, K., Franke, M. F., & Becerra, M. C. (2011). Multiple Introductions of Multidrug-Resistant Tuberculosis into Households, Lima, Peru. *Emerging Infectious Diseases*, *17*(6), 969–975. https://doi.org/10.3201/eid1706.101471

Coll, P., & García de Viedma, D. (2018). Epidemiología molecular de la tuberculosis. *Enfermedades Infecciosas y Microbiología Clínica*, *36*(4), 233–240. https://doi.org/10.1016/j.eimc.2018.01.001

Conceição, E. C., Rastogi, N., Couvin, D., Lopes, M. L., Furlaneto, I. P., Gomes, H. M., Vasconcellos, S. E. G., Suffys, P. N., Schneider, M. P. C., de Sousa, M. S., Sola, C., de Paula Souza E Guimarães, R. J., Duarte, R. S., & Batista Lima, K. V. (2017). Genetic diversity of Mycobacterium tuberculosis from Pará, Brazil, reveals a higher frequency of ancestral strains than previously reported in South America. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *56*, 62–72. https://doi.org/10.1016/j.meegid.2017.10.021

Del Castillo, H., Mendoza-Ticona, A., Saravia, J. C., & Somocurcio, J. G. (2009). Epidemia de tuberculosis multidrogo resistente y extensivamente resistente a drogas (TB MDR/XDR) en el Perú: Situación y propuestas para su control. *Revista Peruana de Medicina Experimental y Salud Publica*, *26*(3), 380–386.

Díaz Acosta, C. C., Russomando, G., Candia, N., Ritacco, V., Vasconcellos, S. E. G., de Berrêdo Pinho Moreira, M., de Romero, N. J., Morcillo, N., De Waard, J. H., Gomes, H. M., & Suffys, P. N. (2019). Exploring the "Latin American Mediterranean" family and the RDRio lineage in Mycobacterium tuberculosis

isolates from Paraguay, Argentina and Venezuela. *BMC Microbiology*, *19*(1), 131. https://doi.org/10.1186/s12866-019-1479-6

Djelouadji, Z., Arnold, C., Gharbia, S., Raoult, D., & Drancourt, M. (2008). Multispacer Sequence Typing for Mycobacterium tuberculosis Genotyping. *PLoS ONE*, *3*(6). https://doi.org/10.1371/journal.pone.0002433

Flores-López, C., Zenteno-Cuevas, R., Laniado-Laborin, R., Reynaud, Y., García-Ortiz, R., Gonzalez-Y-Merchand, J., Rivera, S., Vazquez-Chacon, C., Vaughan, G., Martinez-Guarneros, A., Victoria, N., Cruz-Rivera, M., Rastogi, N., & Muñiz-Salazar, R. (2017). Molecular epidemiology of Mycobacterium tuberculosis in Baja California, Mexico: A result of human migration? *Infection, Genetics and Evolution*, *55*, 378–383. https://doi.org/10.1016/j.meegid.2016.07.001

Frothingham, R., & Meeker-O'Connel, W. (1998). *Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats | Microbiology Society*. https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-144-5-1189

G. Canetti, N. Rist, J.M. Grosset. Mesure de la sensibilité du bacille tuberculeux aux drogues antibacillaires par la méthode des proportions. Méthodologie, critères de résistance, résultats, interprétation. Rev Tuberc Pneumol, 27 (1963), pp. 172-217

Garzón-Chavez, D., Garcia Bereguiain, M.A., Mora Pinargote, C., Granda-Pardo, J., Leon-Benitez, M., Franco-Sotomayor, G., Trueba, G., & de Waard, J. (in press). *Population structure and genetic diversity of Mycobacterium tuberculosis in Ecuador*. Sci Report.

García-Pachón, E., & Rodríguez, J. C. (2005). Epidemiología molecular de la tuberculosis: Principales hallazgos y su aplicación en España. *Archivos de Bronconeumología*,

41(11), 618–624. https://doi.org/10.1157/13081251

Garzón, D., Zurita, J., Mora Pinargote, C., Franco-Sotomayor, G., Leon-Benitez, M., Granda-Pardo, J., Trueba, G., Garcia Bereguiain, M. A., & de Waard, J. (2019). Prevalence, Drug Resistance, and Genotypic Diversity of the Mycobacterium tuberculosis Beijing Family in Ecuador. *Microbial Drug Resistance*, *25*. https://doi.org/10.1089/mdr.2018.0429

Gonzalo, X., Ambroggi, M., Córdova, E., Brown, T., Poggi, S., & Drobniewski, F. (2011). Molecular Epidemiology of Mycobacterium tuberculosis, Buenos Aires, Argentina. *Emerging Infectious Diseases*, *17*, 528–531. https://doi.org/10.3201/eid1703.100394

Instituto Nacional de Estadística y Censo. (2010). *Instituto Nacional de Estadística y Censo (INEC)*. https://www.ecuadorencifras.gob.ec/estadisticas/

Iwamoto, T., Grandjean, L., Arikawa, K., Nakanishi, N., Caviedes, L., Coronel, J., Sheen, P., Wada, T., Taype, C. A., Shaw, M.-A., Moore, D. A. J., & Gilman, R. H. (2012). Genetic Diversity and Transmission Characteristics of Beijing Family Strains of Mycobacterium tuberculosis in Peru. *PLOS ONE*, *7*(11), e49651. https://doi.org/10.1371/journal.pone.0049651

Jasmer, R. M., Nahid, P., & Hopewell, P. C. (2002). Latent Tuberculosis Infection. *New England Journal of Medicine*, *347*(23), 1860–1866. https://doi.org/10.1056/NEJMcp021045

Jiménez, P., Calvopiña, K., Herrera, D., Rojas, C., Pérez-Lago, L., Grijalva, M., Guna, R., & García-de Viedma, D. (2017). Identification of the Mycobacterium tuberculosis Beijing lineage in Ecuador. *Biomédica*, *37*(2), 233–237.

https://doi.org/10.7705/biomedica.v37i3.3450

Mathema, B., Kurepina, N. E., Bifani, P. J., & Kreiswirth, B. N. (2006). Molecular Epidemiology of Tuberculosis: Current Insights. *Clinical Microbiology Reviews*, *19*(4), 658–685. https://doi.org/10.1128/CMR.00061-05

Mazars, E., Lesjean, S., Banuls, A.-L., Gilbert, M., Vincent, V., Gicquel, B., Tibayrenc, M., Locht, C., & Supply, P. (2000). *High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology*. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC29354/

Ministerio de Salud Pública. (2017). *Ministerio de Salud Pública – El Ministerio de Salud Pública ejerce la rectoría del Sistema Nacional de Salud a fin de garantizar el derecho a la salud del pueblo ecuatoriano*. https://www.salud.gob.ec/

Ministerio de Salud-MINSA. (2017). *Ministerio de Salud—MINSA*. https://www.gob.pe/minsa

Mizrahi, V., & Andersen, S. J. (1998). DNA repair in Mycobacterium tuberculosis. What have we learnt from the genome sequence? *Molecular Microbiology*, *29*(6), 1331–1339. https://doi.org/10.1046/j.1365-2958.1998.01038.x

Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., Ginsberg, A., Swaminathan, S., Spigelman, M., Getahun, H., Menzies, D., & Raviglione, M. (2016). Tuberculosis. *Nature Reviews Disease Primers*, *2*, 16076. https://doi.org/10.1038/nrdp.2016.76

Pan American Health Organization. (2017). *PAHO | Tuberculosis*. https://www.paho.org/hq/index.php?option=com_topics&view=article&id=59&Itemid=40776&lang=es

Perdigão, J., Clemente, S., Ramos, J., Masakidi, P., Machado, D., Silva, C., Couto, I., Viveiros, M., Taveira, N., & Portugal, I. (2017). Genetic diversity, transmission dynamics and drug resistance of Mycobacterium tuberculosis in Angola. *Scientific Reports*, *7*, 42814. https://doi.org/10.1038/srep42814

Realpe, T., Correa, N., Rozo, J. C., Ferro, B. E., Ferro, B. E., Gomez, V., Zapata, E., Ribon, W., Puerto, G., Castro, C., Nieto, L. M., Diaz, M. L., Rivera, O., Couvin, D., Rastogi, N., Arbelaez, M. P., & Robledo, J. (2014). Population structure among mycobacterium tuberculosis isolates from pulmonary tuberculosis patients in Colombia. *PloS One*, *9*(4), e93848. https://doi.org/10.1371/journal.pone.0093848

Sheen, P., Couvin, D., Grandjean, L., Zimic, M., Dominguez, M., Luna, G., Gilman, R. H., Rastogi, N., & Moore, D. A. J. (2013). Genetic diversity of Mycobacterium tuberculosis in Peru and exploration of phylogenetic associations with drug resistance. *PloS One*, *8*(6), e65873. https://doi.org/10.1371/journal.pone.0065873

Sreevatsan, S., Pan, X., Stockbauer, K. E., Connell, N. D., Kreiswirth, B. N., Whittam, T. S., & Musser, J. M. (1997). Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. *Proceedings of the National Academy of Sciences*, *94*(18), 9869–9874. https://doi.org/10.1073/pnas.94.18.9869

Stucki, D., Brites, D., Jeljeli, L., Coscolla, M., Liu, Q., Trauner, A., Fenner, L., Rutaihwa, L., Borrell, S., Luo, T., Gao, Q., Kato-Maeda, M., Ballif, M., Egger, M., Macedo, R., Mardassi, H., Moreno, M., Tudo Vilanova, G., Fyfe, J., … Gagneux, S. (2016). Mycobacterium tuberculosis Lineage 4 comprises globally distributed and geographically restricted sublineages. *Nature Genetics*, *48*(12), 1535–1543.

https://doi.org/10.1038/ng.3704

Supply, P. (2005). *Multilocus Variable Number Tandem Repeat Genotyping of Mycobacterium tuberculosis Technical Guide*. https://www.researchgate.net/publication/265990159_Multilocus_Variable_Number_Tandem_Repeat_Genotyping_of_Mycobacterium_tuberculosis_Technical_Guide

Supply, P., Lesjean, S., Savine, E., Kremer, K., van Soolingen, D., & Locht, C. (2001). Automated High-Throughput Genotyping for Study of Global Epidemiology of Mycobacterium tuberculosis Based on Mycobacterial Interspersed Repetitive Units. *Journal of Clinical Microbiology*, *39*(10), 3563–3571. https://doi.org/10.1128/JCM.39.10.3563-3571.2001

Supply, P., Mazars, E., Lesjean, S., Vincent, V., Gicquel, B., & Locht, C. (2000). Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. *Molecular Microbiology*, *36*(3), 762–771. https://doi.org/10.1046/j.1365-2958.2000.01905.x

Weniger, T., Krawczyk, J., Supply, P., Niemann, S., & Harmsen, D. (2010). MIRU-VNTRplus: A web tool for polyphasic genotyping of Mycobacterium tuberculosis complex bacteria. *Nucleic Acids Research*, *38*(Web Server issue), W326–W331. https://doi.org/10.1093/nar/gkq351

Wirth, T., Hildebrand, F., Allix-Béguec, C., Wölbeling, F., Kubica, T., Kremer, K., van Soolingen, D., Rüsch-Gerdes, S., Locht, C., Brisse, S., Meyer, A., Supply, P., & Niemann, S. (2008). Origin, spread and demography of the Mycobacterium tuberculosis complex. *PLoS Pathogens*, *4*(9), e1000160. https://doi.org/10.1371/journal.ppat.1000160

Woodman, M., Haeusler, I. L., & Grandjean, L. (2019). Tuberculosis Genetic Epidemiology: A Latin American Perspective. *Genes*, *10*(1). https://doi.org/10.3390/genes10010053

World Health Organization. (2018). *Global tuberculosis report 2018*. World Health Organization.

Zurita, J., Espinel, N., Barba, P., Ortega-Paredes, D., Zurita-Salinas, C., Rojas, Y., & Alcocer, I. (2019). Genetic diversity and drug resistance of Mycobacterium tuberculosis in Ecuador. *The International Journal of Tuberculosis and Lung Disease: The Official Journal of the International Union Against Tuberculosis and Lung Disease*, *23*(2), 166–173. https://doi.org/10.5588/ijtld.18.0095

10. Appendix

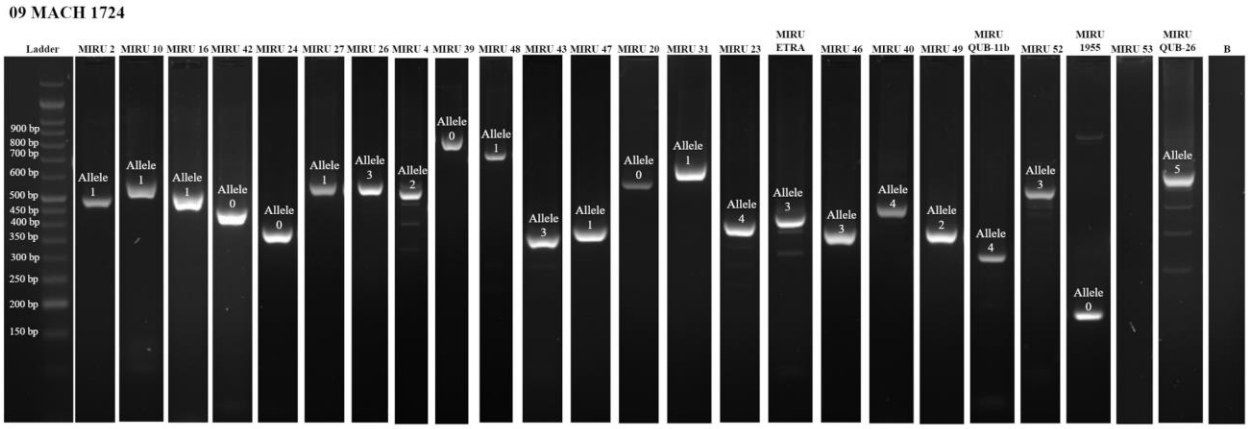
10.1 Reference table to 24-loci MIRU-VNTR

Locus	2	42	43	4	40	10	16	1955	20	QUB11	ETR-A	46	47	48	23	24	26	27	49	31	52	QUB26	53	39
Convention	154	424	577	580	802	960	1644	1955	2059	2163	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
Allele																								
0	402	537	171	175	354	482	565	116	437	67	195	335	252	347	150	395	285	498	326	492	272	153	563	540
1	455	588	208	252	408	537	618	149	514	136	270	392	305	404	200	447	336	551	380	545	330	264	622	593
2	508	639	266	329	462	590	671	206	591	205	345	449	363	461	253	501	387	604	434	598	388	375	681	646
3	561	690	324	406	516	643	724	263	668	274	420	506	421	518	306	555	438	657	488	651	446	486	740	699
4	614	741	382	483	570	696	777	320	745	343	495	563	479	575	359	609	489	710	542	704	504	597	799	752
5	667	792	440	560	624	749	830	377	822	412	570	620	537	632	412	663	540	763	596	757	562	708	858	805
6	720	843	498	637	678	802	883	434	899	481	645	677	595	689	465	717	591	816	650	810	620	819	917	858
7	773	894	556	714	732	855	936	491	976	550	720	734	653	746	518	771	642	869	704	863	678	930	976	911
8	826	945	614	791	786	908	989	548	1053	619	795	791	711	803	571	825	693	922	758	916	736	1041	1035	964
9	879	996	672	868	840	961	1042	605	1130	688	870	848	769	860	624	879	744	975	812	969	794	1152	1094	1017
10	932	1047	730	945	894	1014	1095	662	1207	757	945	905	827	917	677	933	795	1028	866	1022	852	1263	1153	1070
11	985	1098	788	1022	948	1067	1148	719	1284	826	1020	962	885	974	730	987	846	1081	920	1075	910	1374	1212	1123
12	1038	1149	846	1099	1002	1120	1201	776	1361	895	1095	1019	943	1031	783	1041	897	1134	974	1128	968	1485	1271	1176
13	1091	1200	904	1176	1056	1173	1254	833	1438	964	1170	1076	1001	1088	836	1095	948	1187	1028	1181	1026	1596	1330	1229
14	1144	1251	962	1253	1110	1226	1307	890	1515	1033	1245	1133	1059	1145	889	1149	999	1240	1082	1234	1084	1707	1389	1282
15	1197	1302	1020	1330	1164	1279	1360	947	1592	1102	1320	1190	1117	1202	942	1203	1050	1293	1136	1287	1142	1818	1448	1335

Reference table to assign the number of tandem repeats for each locus (see the allele column) according to Supply (2005)

10.2 Gel Electrophoresis

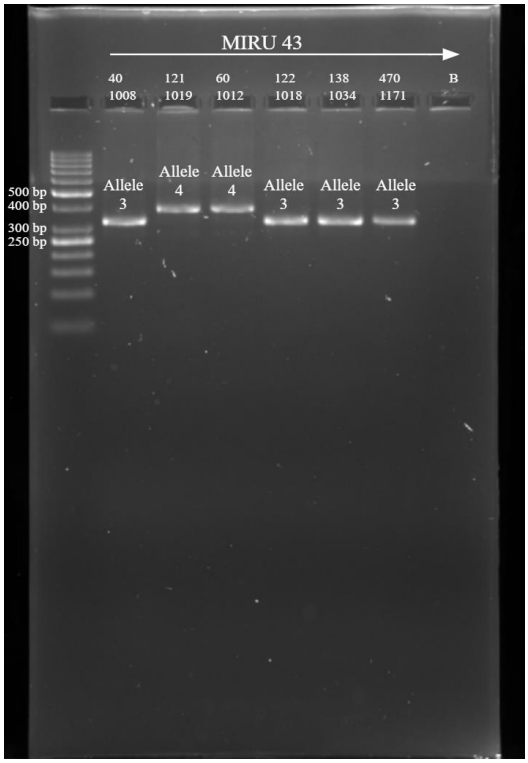
10.2.1 MIRU pattern assigned to the sample 09 MACH 1744



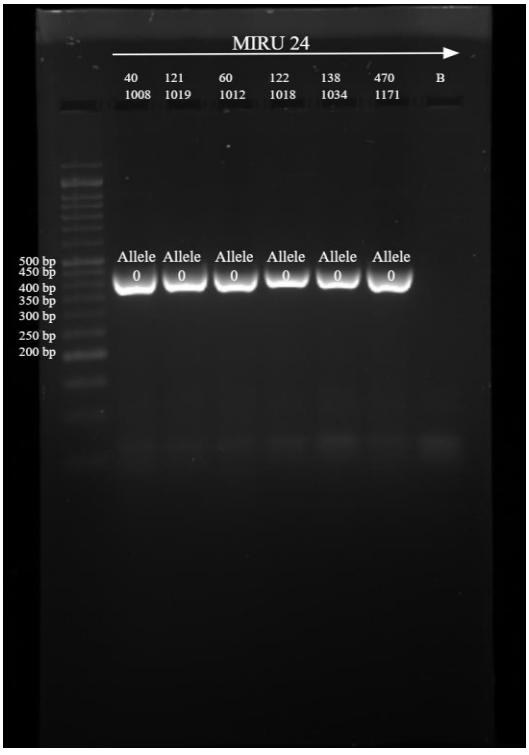
MIRU Pattern

1	1	1	0	0	1	3	2	0	1	3	1	0	1	4	3	3	4	2	4	3	0		5

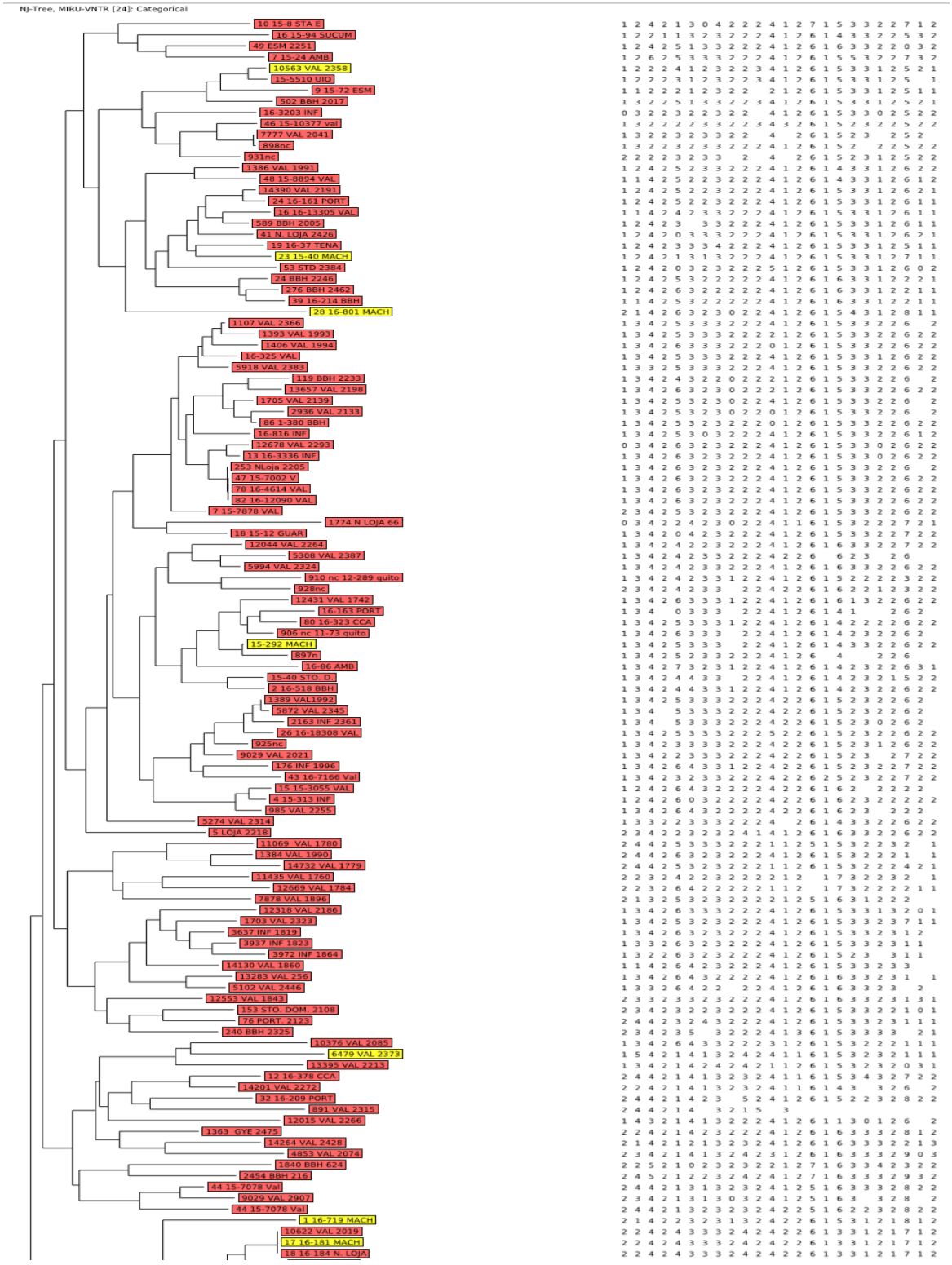
10.2.2 *M. tuberculosis* strains of El Oro. Reactions to MIRU 43 and identification of MIRU pattern



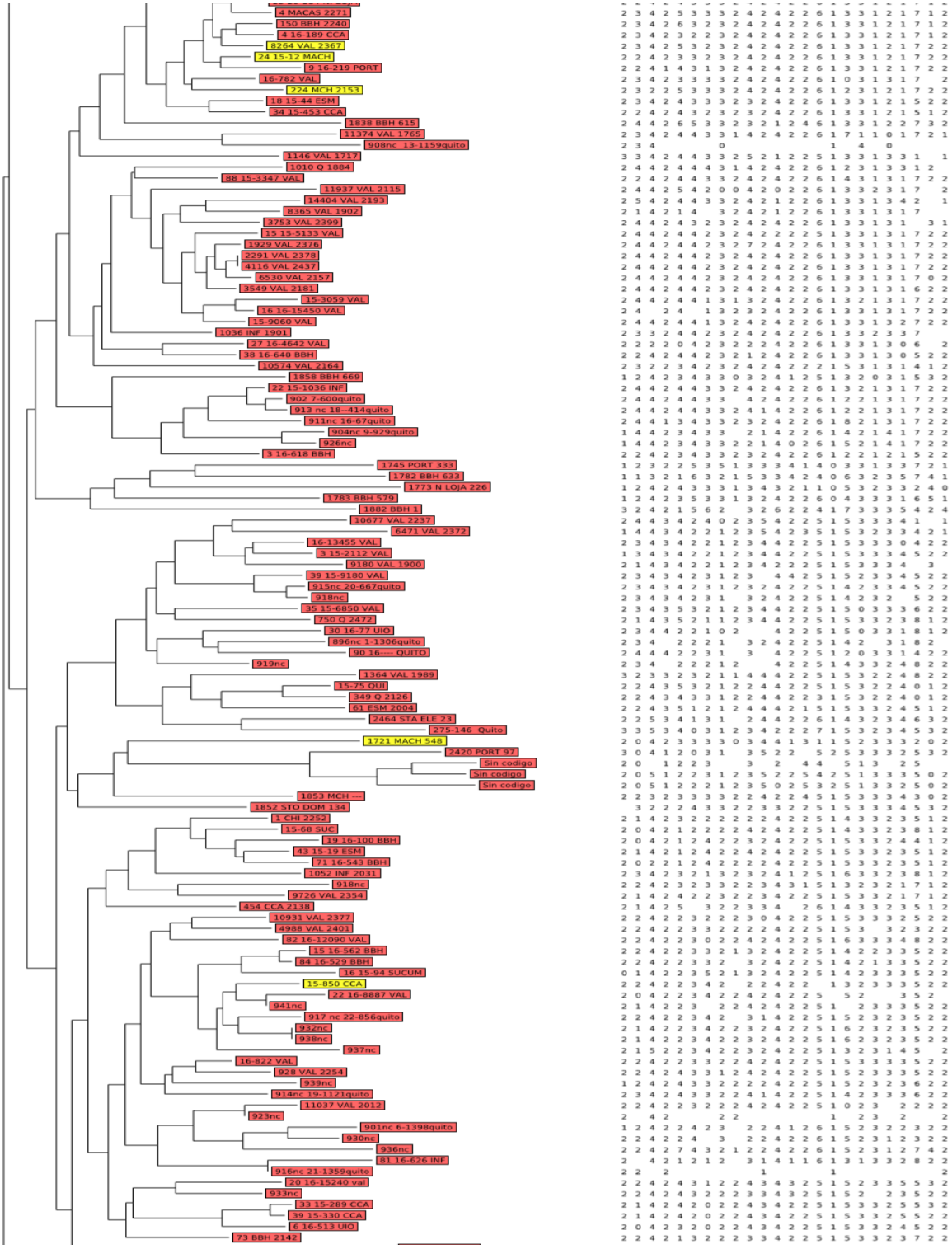
10.2.3 *M. tuberculosis* strains of El Oro. Reactions to MIRU 24 and identification of MIRU pattern



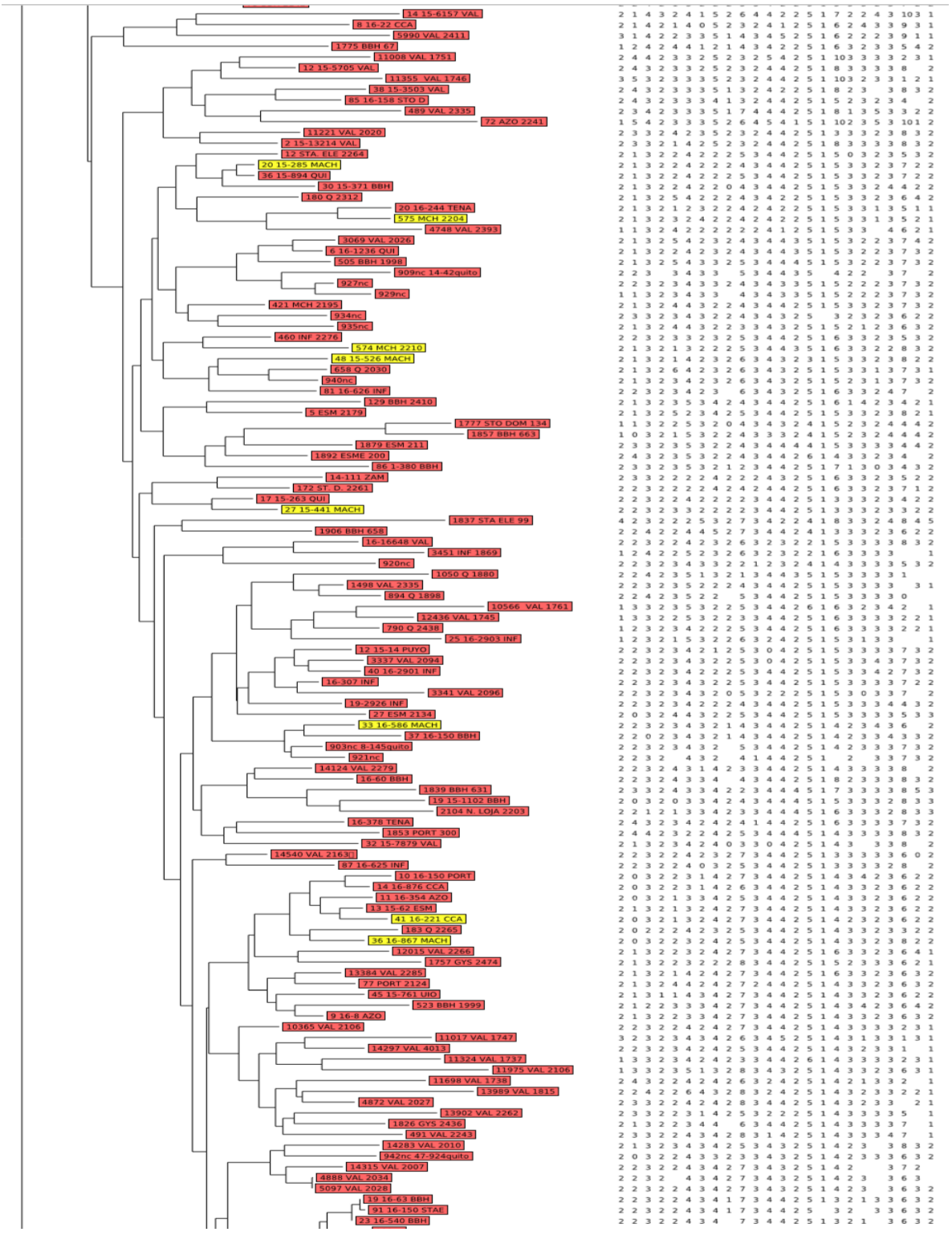
10.3 Neighbor joining tree and the MIRU pattern of MTB strains of El Oro province and Ecuador



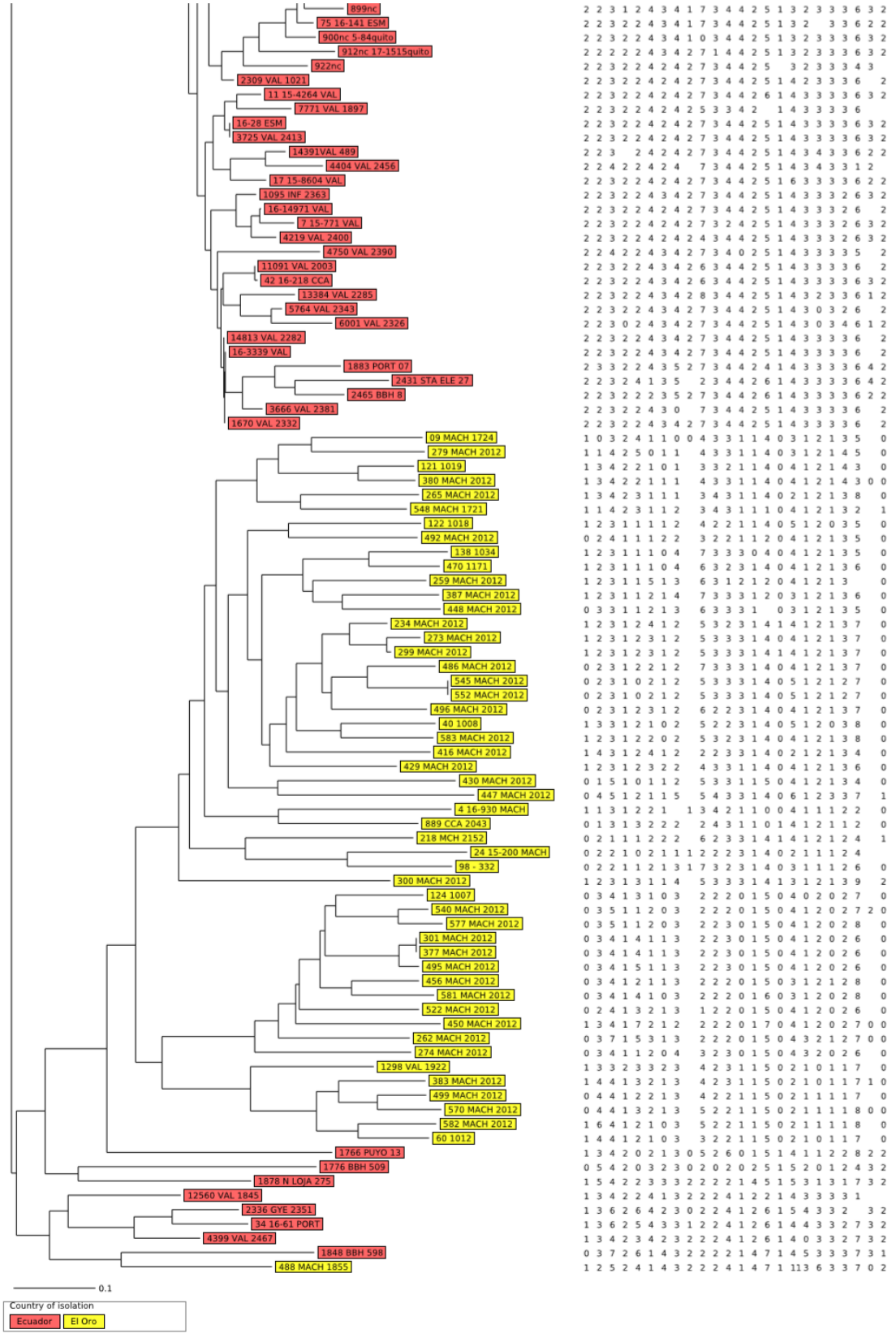
The strains are from El Oro province (yellow) and Ecuador (red). Part 1



The strains are from El Oro province (yellow) and Ecuador (red). Part 2

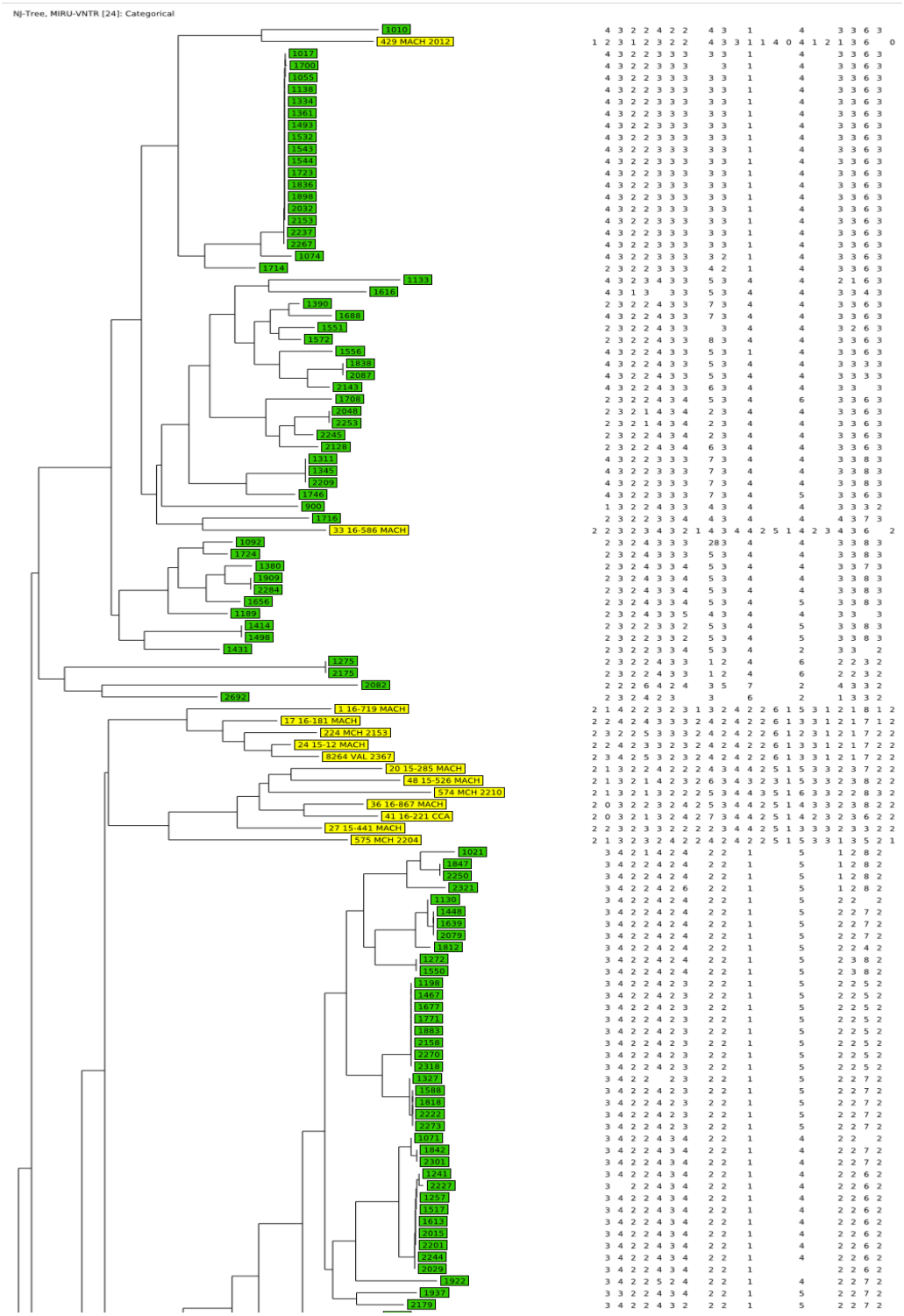


The strains are from El Oro province (yellow) and Ecuador (red). Part 3

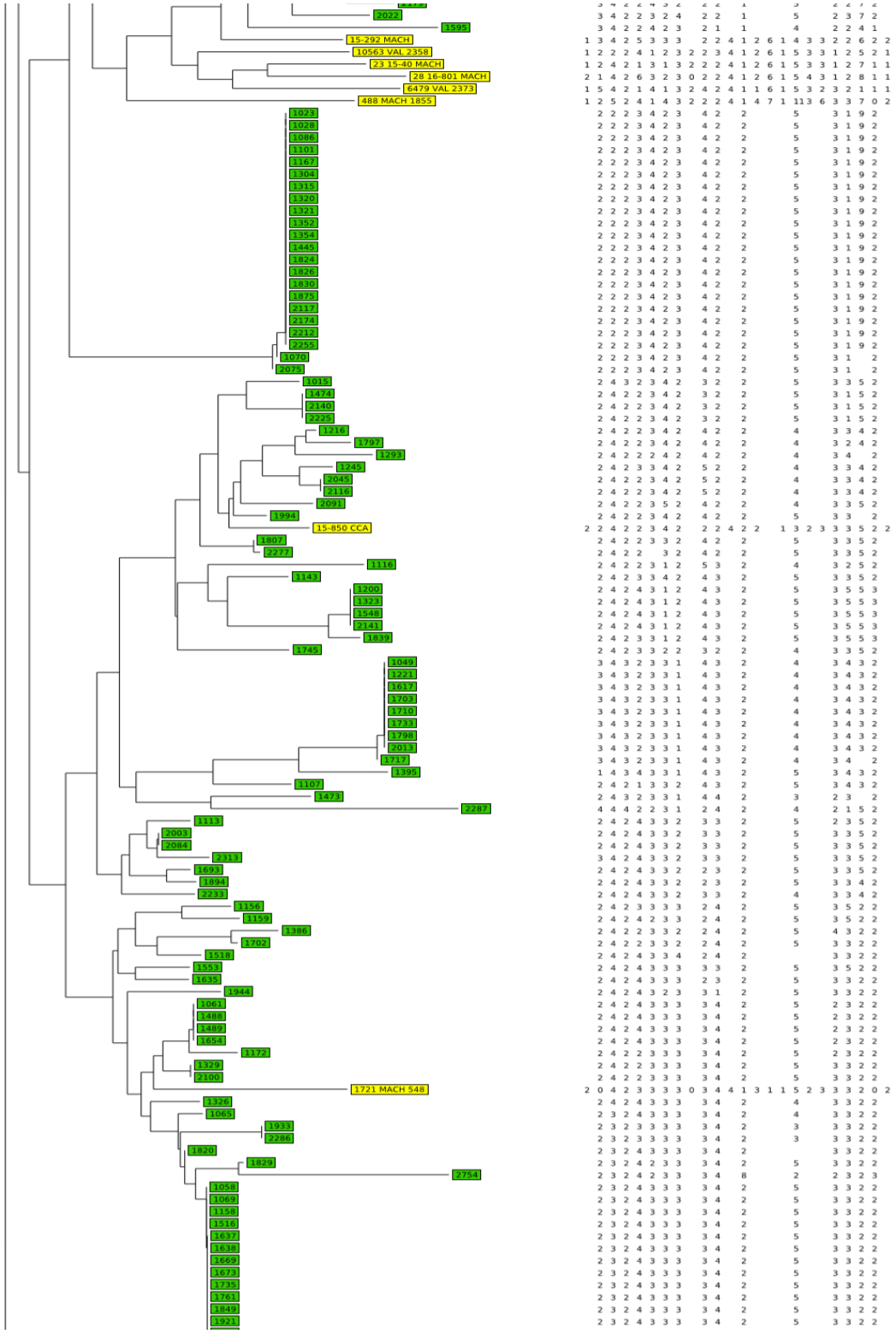


The strains are from El Oro province (yellow) and Ecuador (red). Part 4

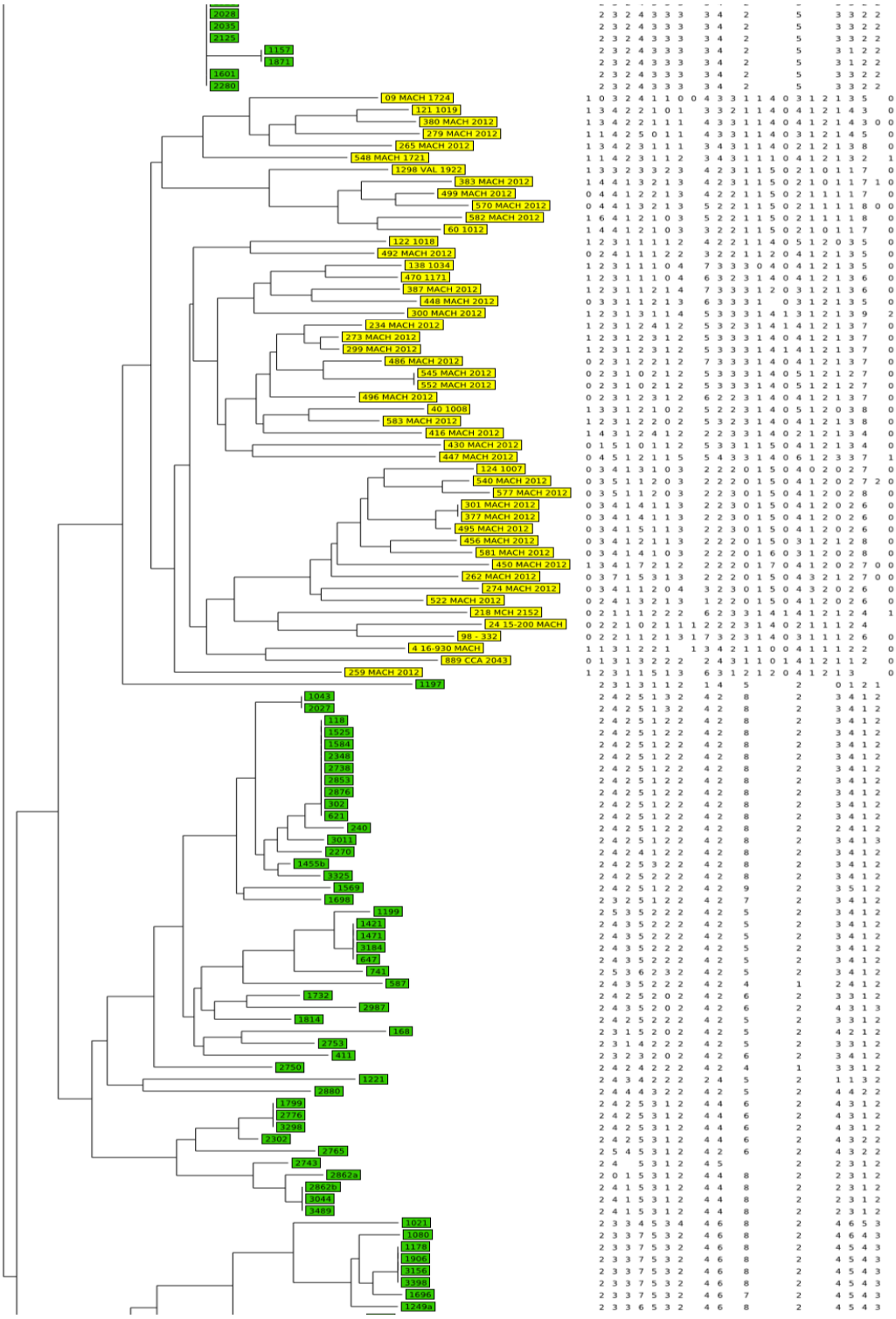
10.4 Neighbor joining tree and the MIRU pattern of MTB strains of El Oro province and Perú



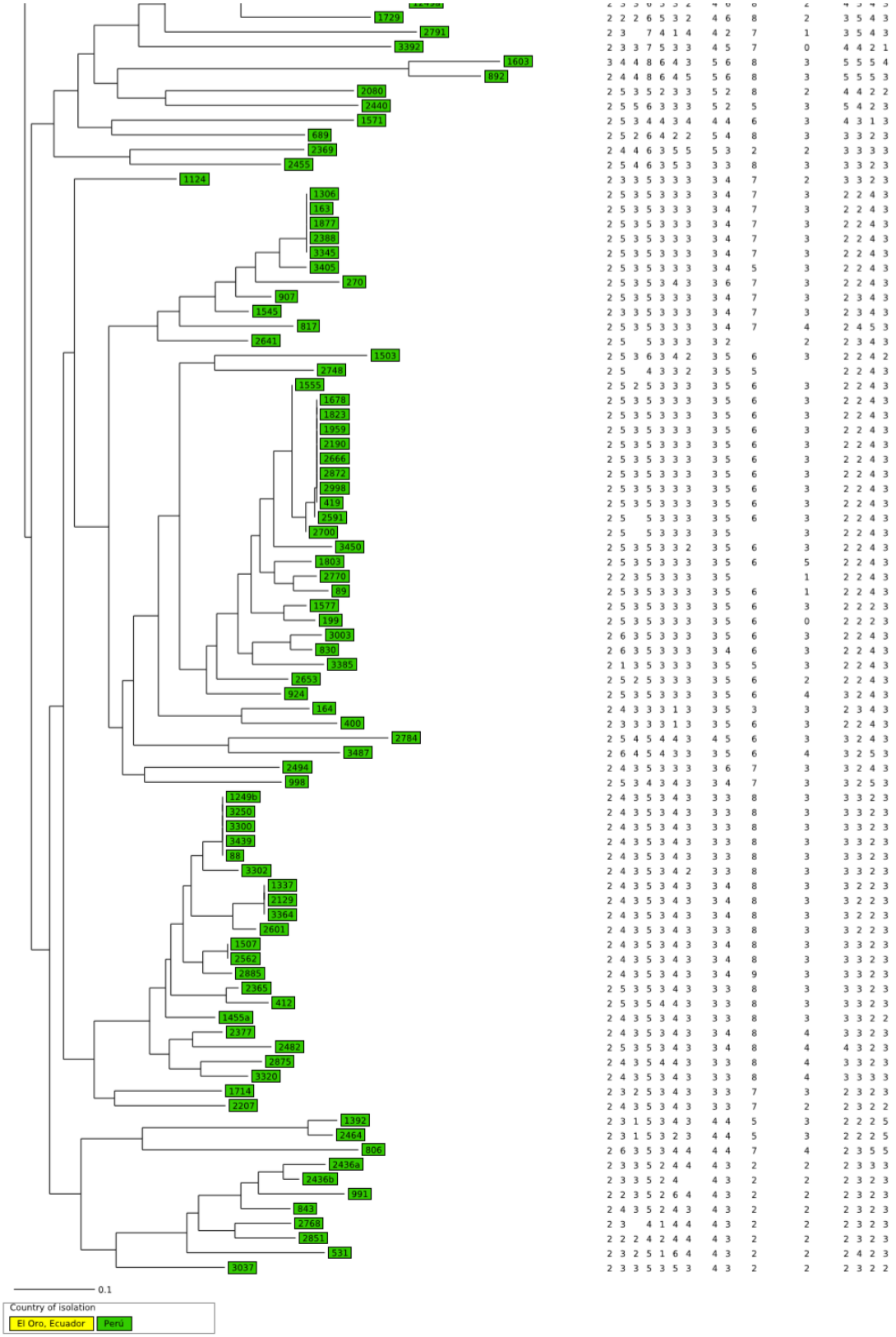
The strains are from El Oro province (yellow) and Perú (green). Part 1



The strains are from El Oro province (yellow) and Perú (green). Part 2



The strains are from El Oro province (yellow) and Perú (green). Part 3



The strains are from El Oro province (yellow) and Perú (green). Part 4