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# Intraspecific Typing and Phylogeny of the Causative Agent of the Plague—The Microbe *Yersinia pestis*: Problems and Perspectives

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**Abstract**—Two approaches to typing (analysis of intraspecific diversity) and reconstruction of the phylogeny (evolutionary history) of the causative agent of the plague (the microbe *Yersinia pestis*)—molecular genetic (MG) and ecological (adaptationist, on the basis of host adaptation)—are considered. It is shown that each of the approaches has its advantages and disadvantages. MG typing of pathogen strains in the studied foci of the world made it possible to characterize up to 30 subspecies/genovariants of the plague microbe, but the phylogeny of the microbe built on the basis of this diversity contradicts some obvious environmental facts. The ecological scenario of the origin and evolution of the causative agent of the plague has no obvious contradictions, and as an evolutionarily based hypothesis, it should be taken into account in MG reconstructions of the phylogeny of the plague microbe. The prospect of research in this direction is seen in integrating molecular genetic (statistical) and ecological (adaptationist) approaches.

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## INTRODUCTION

Knowledge of biodiversity, history, and evolution of microbial pathogens is highly in demand in the theory and practice of infectiology. Without this knowledge, it is impossible to build rational systems for diagnosing, treating, and preventing infectious diseases and predicting possible future epidemics and pandemics that cause colossal damage to human society. This also applies to the plague—an apocalyptic disease known since ancient times, which manifested itself particularly dramatically in three well-known pandemics. There is historical information about the "Plague of Justinian" in the 6th–8th centuries in the Mediterranean and North Africa. The "Black Death" engulfed Europe and North Africa for a long time, from the 14th to the 18th centuries. The last, third, pandemic began in the Chinese province of Yunnan in the middle of the 19th century; by 1894, it penetrated the seaport of Hong Kong, from where it spread with ship rats to all continents except Antarctica. In new previously infection-free territories of Asia (Indochina), the African continent, the New World and large oceanic islands (in Java, Madagascar, and Hawaii), the plague established itself for a long time in the form of synanthropic (rat) and secondary natural foci. The cause of the third pandemic has deep biological and historical roots in Hindustan, the homeland of the Black rat *Rattus rattus* and gerbil fleas *Xenopsylla astia*, and in Africa, the homeland of the "rat" flea *X. cheopis*. In fact, this flea is not a rat flea. The Black rat does not have its own specific fleas. In nature, this flea is a specific parasite of the Nile grass rat *Arvicanthis niloticus*, living in the Sahel and the Nile Valley. From the Nile Valley, it was spread throughout the world in the last two centuries by the synanthropic Black rat and became the main vector of plague in man-made rat outbreaks. In 1894, in Hong Kong, at the beginning of the third pandemic, a French doctor of Swiss origin, Alexandre Yersin, discovered the causative agent of the plague—a microbe that was later named in his honor *Yersinia pestis*.

The idea of the pathogen as an obligate parasite of burrowing rodents forms the basis of the theory of natural focality of plague (or sylvatic plague), which was fully formed by the 1960s–1970s. A great contribution to its creation was made by the work of Soviet and foreign scientists: D.K. Zabolotny, I.G. Ioff, Yu.M. Rall', N.P. Naumov, V.V. Kucheruk, I.I. Rogozin, I.S. Tinker, V.N. Fedorov, B.K. Fenyuk, Wu Lien-Te, H. Mollaret, R. Pollitzer, L. Cartman, M. Baltazard, and many others. Now the plague is considered one of the most studied especially dangerous infections. However, despite the enormous scientific and practical interest in this disease of a large number of scientists and practitioners who have been and are working on the problem of plague, definitive answers have not yet been obtained to the questions of where, when,

how, and under what circumstances its causative agent arose.

In the last 20–25 years, molecular genetic (MG) research methods have been introduced into the infectiology of plague and most other current infections and have become dominant. It was shown that the direct ancestor of the plague microbe is the causative agent of intestinal pseudotuberculosis infection, or more accurately Far East scarlet-like fever (FESLF)—*Y. pseudotuberculosis* 0:1b (Skurnik et al., 2000; Eppinger et al., 2007). Moreover, the causative agent of plague is the only pathogenic species of intestinal microbes of the family Enterobacteriaceae transmitted between hosts not by the typical alimentary route, but through flea bites. That is, the process of its speciation was unique and constitutes a gambling ecological and evolutionary intrigue. Its study requires an extraordinary approach. It is also shown that the separation of *Y. pestis* from the ancestral species occurred in the recent past, no earlier than 30000 years ago (Achtman et al., 1999, 2004; Morelli et al., 2010). More often, periods from 2000 to 8000 years ago are given (Cui et al., 2013; Demeure et al., 2019; Pisarenko et al., 2021). There are estimates of an earlier emergence of the plague microbe, up to 80000 years ago (Rasmussen et al., 2015). That is, the species *Y. pestis* in evolutionary terms is a very young infectious agent that arose in a biogeocenotic environment similar to the modern one. And yet the appearance of *Y. pestis* is more likely to be associated with changes in nature.

After the discovery by molecular geneticists of the ancestral form of the plague microbe and the establishment of its evolutionary youth, attempts were made to decipher the population genetic mechanisms of the evolutionary process. An important achievement that opened up prospects for further study of the history of the emergence of plague in the world was the identification of the probable original host of the plague causative agent—it turned out to be the Mongolian tarbagan marmot (*Marmota sibirica*) (Suntsov and Suntsova, 2000, 2006; Suntsov, 2022a). The focus of research shifted to Central Asia, and population ecology took up the relay race in solving the problem of the origin and evolution of the plague pathogen. Previously accumulated scientific data on the structure and historical dynamics of Central Asian biogeocenoses and the population genetic characteristics of the epizootic triad "Mongolian marmot–fleas–plague causative agent" have become invaluable. Unfortunately, the pathogen that circulates in Mongolian marmot populations remains poorly studied. It was suggested, which received well-reasoned support, that the aridization of Central Asian landscapes, which occurred from the mid-Cenozoic, led to the formation of a specific protective behavior in the Mongolian marmot—the use of its metabolic water (urine, excrement) to make a protective plug for the wintering burrow. In this case, excrement obligately enters the oral cavity of marmots preparing for winter. The consequence of this was the accumulation of the FESLF pathogen in the oral cavity of marmots sleeping in the cold season without the onset of an infectious process. Marmots do not feed during hibernation, so the FESLF pathogen does not enter the small intestine and does not interact with the M cells of Peyer's patches; i.e., there is no specific colonization and invasion by the pathogen of the mucous membranes of the host's gastrointestinal tract. But the infectious process of FESLF during the winter months in populations of hibernating Mongolian marmots was made possible under unique circumstances. Climate change in Central Asia at the turn of the Pleistocene and Holocene, or rather the maximum Sartan cold spell in North and Central Asia 22000–15000 years ago, caused a change in the behavior of a specific marmot flea *Oropsylla silantiewi*, which can still be observed today. In the cold period of the year, owing to deep freezing of the soil, the larvae of the marmot flea move to the warmer bodies of sleeping animals; with a stochastic pattern, they penetrate into the oral cavity and change their feeding method—they move from saprophagy in the lining of the nest to facultative hematophagy on the mucous membranes of the oral cavity (Suntsov, 2018b). Through scarifications created by flea larvae in the oral cavity of hibernating heterothermic (5–37°C) marmots, the FESLF pathogen directly, bypassing the M cells of the small intestine, penetrates into the "cold" blood of animals. This led to massive aberrant traumatic (not traditional alimentary!) "blood poisoning" of marmots by FESLF. Facultative hematophagy of flea larvae, apparently, marked the beginning of the population genetic transition of the FESLF pathogen to a new ecological niche—from the digestive tract to the lymphomyeloid complex, i.e., the process of becoming a new species *Y. pestis*.

According to ecological concepts, the process of speciation of the plague pathogen was launched by a change in the habitat of a certain population (clone) of its microbial ancestor at the turn of the Pleistocene and Holocene, when, against the background of global climate changes on Earth—the onset of the last maximum cooling–there was an intense extinction of old species and the formation of new species. The cooling significantly lowered the level of the world's oceans; the Beringian land bridge arose between the Asian and American continents, along which people from Asia settled in America. During this period of maximum cooling, the direct ancestor of the plague microbe, the psychrophilic pathogen FESLF, was perfectly preserved and, it is possible, significantly expanded its range (Suntsov and Suntsova, 2006). It is assumed that initially it infected only populations of Arctic coprophagous animals, such as lemmings, hares, which, owing to salt deficiency in Arctic plants, gnaw the bones and antlers of animal remains, eat the shells of bird eggs, lick salt licks and, most importantly, gnaw out snow and ice, soaked in urine and excrement, and

eat their excrement. This behavior of Arctic animals should have intensified the infectious process of FESLF. It can be assumed that, with the maximum cooling, the pathogen FESLF left the Arctic zone and expanded its host range and range, eventually penetrating the populations of the Mongolian marmot. Currently, the causative agent of FESLF is common in the cold regions of Northern and Central Asia and in the Far East, is not uncommon in Canada, and has penetrated into Japan. The habitats of simultaneously existing ancestral pseudotuberculosis and derived plague microbes can fully characterize the adaptive phenotypic (ecological, etiological, clinical, biochemical and other) properties and characteristics of these pathogens. And their comparative analysis makes it possible to reconstruct many aspects of the speciation process. In this case, it is clear that, when studying the origin and evolution of the plague pathogen, in addition to the MG methodology, the theoretical basis of which is the theory of neutral molecular evolution, ecological (in a broad sense) methods for studying the habitats and adaptations of plague pathogens and FESLF should be useful or even necessary.

Modern MG methodology, used to study the diversity of intraspecific forms of the plague microbe and its phylogeny, seems to be well developed, since it is fundamentally universal, was created on models of a wide variety of living organisms, and is technologically applicable in various ways to the study of any microorganisms, plants, and animals. At the same time, the molecular methodology of the historical reconstruction of the plague microbe in connection with its evolutionary youth, very rapid formation of species properties, coexistence with a direct ancestor, and unique position in the family of pathogens of intestinal diseases of the family Enterobacteriaceae to a certain extent should be ad hoc methodology adequate to the uniqueness of the research object (Achtman, 2008; Suntsov, 2021a). In other words, molecular and ecological methodologies for studying diversity *Y. pestis* and the histories of the formation of this diversity must be congruent, compatible, interpenetrating. The proposed article emphasizes that, when studying the intraspecific diversity of the plague microbe (typing) and the history of the formation of this diversity (phylogeny), an ecological (adaptationist) approach should be applied, revealing the evolutionarily important features of the relationships of ancestral and derivative microbes with each other and with their habitats, as well as a modified MG approach, the evolutionary model of which would take into account the systematic and evolutionary uniqueness of the plague pathogen.

## INTRASPECIFIC DIVERSITY OF *Y. PESTIS*

Owing to the recent origin and short period of evolution, the intraspecific diversity of the plague causative agent is small in comparison with many other pathogenic bacteria; therefore the species *Y. pestis* is generally considered monomorphic (Achtman, 2008, 2012). Nevertheless, its intraspecific radiation led to the modern polytypic structure. In a short evolutionary period of time, a new species *Y. pestis* emerged in the process of territorial expansion; it diverged into host forms characteristic of populations of one or several sympatric species of burrowing warm-blooded hosts. On the basis of these populations, mono-, di-, and polyhostal natural foci were formed. Various intraspecific forms have been characterized: systematically and taxonomically significant subspecies/genovariants and forms that do not represent taxa, but are operationally convenient for characterizing individual properties of the microbe: ecotypes, biovars, proteinovars, plasmidovars, ribotypes, etc. (Zhou et al., 2004a, 2004b; Vogler et al., 2016).

### *Molecular Typing*

For typing (description and diagnosis) and clarification of family relationships (phylogenetic analysis) of intraspecific forms of the plague microbe, biochemical, genetic, and molecular characteristics are used. At the same time, methods for analyzing nucleotide sequences—genetic markers that describe and characterize intraspecific diversity *Y. pestis* and diversity of species in the genus *Yersinia*—currently occupy a leading position in the study of phylogeny and system of the genus. However, despite the advanced methodology of genetic and molecular identification of the plague pathogen and undoubted achievements in its diagnosis, MG typing and MG phylogenetic constructions raise doubts owing to the fact that MG conclusions sometimes reveal obvious contradictions with the data of ecology, biogeography, and paleontology (Achtman, 2008, 2012; Suntsov, 2021a). Actually, this applies not only to the causative agent of the plague. Phylogenetic constructions based on MG analysis quite often raise doubts about the correctness of reflecting the history of the formation of taxa (Abramson, 2007, 2013).

Description of intraspecific diversity and modern systematization of the plague microbe are carried out using the gene composition and genetic markers IS, DFR, VNTR, SNP, CRISPR, and others, taking into account biochemical features (Platonov et al., 2013; Vogler et al., 2016; Vagaiskaya et al., 2019; Kislichkina et al., 2019). In modern publications, up to 30 subspecies/genovariants of *Y. pestis* identified (Achtman et al., 2004; Cui et al., 2013; Kutyrev et al., 2018). Subspecies may consist of several smaller subunits. In MG typing, alphanumeric names of the described forms are used (0.PE2, 0.ANT1, 1.ORI3, 1.IN2, 2.MED0, 3.ANT2, 4.ANT1, etc.) (Fig. 1a). For example, a subspecies that molecular geneticists classify as the most ancient of the modern ones, described from populations of the Siberian jerboa (*Allactaga sibirica*) in Tibet, is called 0.PE7. Another subspecies that claims

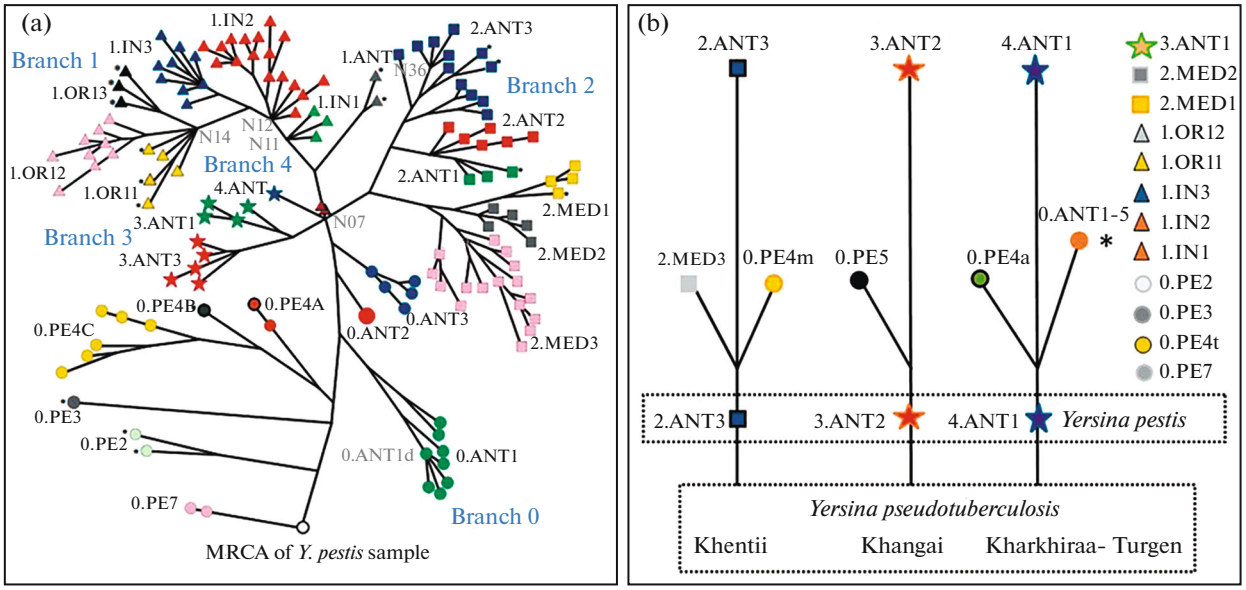


**Fig. 1.** Topology of phylogenetic trees of *Yersinia pestis*. (a) One of the most popular phylogenetic dendrograms of *Y. pestis* constructed on the basis of the analysis of SNP markers (Cui et al., 2013). A set of color marks shows the intraspecific genotypic diversity of the plague microbe. Letters and numbers indicate subspecies/genovariants/geographical populations. Basal branch 0 unites subspecies circulating in populations of voles and pikas (cluster 0.PE) and subspecies characteristic of populations of Altai and red marmots (cluster 0.ANT); "marmot" subspecies 0.ANT according to some characteristics can be considered the most ancient (Anisimov et al., 2016). (b) Three-rooted "ecological" phylogenetic tree of *Y. pestis* (Suntsov, 2022b). Three original subspecies/genovariants 2.ANT3, 3.ANT2, and 4.ANT1 diverged from three independent populations (clones) of the FESLF pathogen (almost) simultaneously in three geographical populations of the Mongolian marmot: Khentei, Khangai, and Kharkhira-Turgen. * Diversification and Asian expansion of subspecies/genovariants.

to be the most ancient (Pisarenko et al., 2021), circulating in populations of the Common vole in the Caucasus, is known by its biochemical and genetic properties as *Y. pestis caucasica*, in the MG classification is called 0.PE2. The first numbers (0–4) indicate the main phylogenetic branches emanating from the abstract statistically based ancestral form of the plague microbe MRCA (most recent common ancestor) (Achtman et al., 1999, 2004). Branch 0 is basal, comes directly from the MRCA, and is considered the most ancient; its representatives, according to selected molecular markers, are most similar to the ancestral pseudotuberculosis microbe (Achtman et al., 2004). Letter abbreviations denote the biovars Antiqua (ANT), Mediaevalis (MED), Orientalis (ORI), Pestoides (PE) and Intermedia (IN), which have specific biochemical properties. Abbreviations identify first- and second-order subbranches. At the same time, biochemical properties are not always stable and can also be the result of various mutations. In such cases, the described forms may be polyphyletic and should belong to different phylogenetic lineages (Achtman et al., 2004). The names of biovars were proposed by Devignat (1951) back in the middle of the last century, supposedly in accordance with the pandemics they caused—ancient (Antiqua), medieval (Mediaevalis), and modern, which began in the east (Orientalis). It is now clear that these names, on one hand, do not correspond to the historical events described; on the other hand, they can only partially characterize the related relationships of genovariants/subspecies. Their use is largely associated with established tradition. The last numbers, in some cases with an additional letter, characterize subspecies (=geographic populations) or populations of the microbe of varying scale and hierarchical position in specific geographically defined foci. Thus, populations of different ranks that fall under MG typing have a ternary digital and letter designation: number—abbreviation–number (+letter). This is the designation that the original population of the plague microbe should have—the abstract so-called most modern common ancestor MRCA (the ecological approach described below justifies the simultaneous peripatric formation of three equivalent initial populations of the plague microbe—2.ANT3, 3.ANT2, and 4.ANT1, which can be seen in Fig. 1b). In general, the name of subspecies/populations proposed by molecular geneticists seems operationally convenient, but owing to the genetic uncertainty of biovars and in some cases the lack of directly related relationships between representatives of the same biovar, the abbreviation of biovars in the designation of subspecies should still be abandoned.

In MG nomenclature and, accordingly, in molecular typing of intraspecific forms of *Y. pestis*, there are uncertainties, inconsistencies, ecological incidents,

and, in some cases, a noticeable lack of evolutionary logic. Let us give two examples.

Root subspecies 0.PE7 and 0.PE3 are represented by only one or several strains, which, according to statistical indicators, cannot in any way represent populations (subspecies). Moreover, the natural host of subspecies 0.PE3 (*Y. pestis angolica*) remains unknown, and the subspecies 0.PE7 (*Y. pestis tibetica*) was isolated from two infected people and two Siberian jerboas in Eastern Tibet (Cui et al., 2013), but there are no plague foci with this rodent species as the main host of the pathogen in nature (places of isolation of cultures 0.PE7, according to the publication of Cui et al. (2013), are located within the boundaries of a natural focus with the main host of the microbe—the Himalayan marmot *Marmota himalayana*). The natural source of infection in humans is not clear (it is logical to believe that this is a Himalayan marmot, and not a Siberian jerboa, for which, owing to the biological characteristics of the species, contact with humans is extremely unlikely). In this case, molecular typing refers only to individual genotypes (strains), possibly aberrant and not related to the ancestral taxa in whose populations genetic modifications that led to the formation of the plague causative agent occurred.

Another example. In the intraspecific structure of the plague microbe, a subspecies *Y. pestis central-asiatica* (0.PE4) was recently described (Eroshenko et al., 2015; Kutyrev et al., 2018). This subspecies includes four local populations that have a high degree of similarity in molecular markers (SNPs): *Y. pestis hissarica*, *Y. pestis talassica*, *Y. pestis altaica*, and *Y. pestis microtus* (=*Y. pestis xilingolensis*). But it is quite obvious that the similarity of MG markers in this case cannot characterize the unity of the subspecies. By definition, a subspecies is a holophyletic group of directly related forms that have a single habitat and have the same ecological properties. MG typing, contrary to the generally accepted idea of a subspecies, combines into one subspecies *Y. pestis central-asiatica* four different intraspecific forms forming a radically fragmented range, including Hissar, Talas, Altai Mountains, and Northeast China (Xilin Gol Grassland), and circulating in populations of far from related hosts: the Juniper vole (*Microtus carruthersi*), Silver mountain vole (*Alticola argentatus*), Narrow-headed vole (*Lasiopodomys gregalis*), Mongolian pika (*Ochotona pallasi pricei*), and Brandt's voles (*L. brandti*). The assertion of the direct relationship of the Hissar, Talas, Altai, and Chinese strains of the plague microbe and their inclusion in one subspecies taxon only on the basis of the similarity of the nucleotide structure of the selected molecular markers does not find ecological and biogeographical support.

On the other hand, molecular typing made it possible to specify the structure of the so-called "main" subspecies *Y. pestis pestis*, established in the 1980s on the basis of a phenotypic characteristic, according to medical indicators—according to the degree of virulence in relation to humans. The concept of main/non-main subspecies continues to be widely used in modern scientific literature. On the basis of biochemical properties and medical indicators, all highly virulent forms circulating in nature in populations of marmots, gophers, gerbils, and rats in many natural and anthropogenic centers of the world are combined into one main subspecies. This multitude of forms is divided into the above-mentioned biovars, which by and large have independent habitats and completely different properties. For example, pathogens classified as biovars Antiqua and Orientalis have stable biochemical differences in their ability to ferment glycerol, have independent habitats, form foci with different biocenotic structures, and circulate in populations of different primary hosts. The Antiqua biovar is characteristic of Asian natural foci of the marmot type; i.e., the main host in them are marmots (*Marmota*). During the first and/or second pandemics, marmot plague with synanthropic rats was brought by humans to the African continent, where it still circulates in populations of local wild burrowing rodents in the Congo, Uganda, Kenya, Zambia, and Tanzania and represents a newly emerged independent subspecies/genovariant 1.ANT1.

Primary natural foci with the biovar Orientalis and the "rat" subspecies/genovariant 1.ORI1 are located in Hindustan. The main host in them is the Indian gerbil (*Tatera indica*). These outbreaks became the source of the third pandemic through synanthropic rat outbreaks. In the process of economic development of Hindustan, populations of synanthropic Black rats were formed, which, through gerbil fleas *Xenopsylla astia* came into parasitic contact with populations of the Indian gerbil, the main host of infection in natural foci with the 1.ORI1 genovariant. In this way, synanthropic foci arose, initially supported by the parasitic system *R. rattus*–*X. astia*, to which was later added *R. rattus*–*X. cheopis* (Suntsov and Suntsova, 2006). Subsequently, with the Black rat, the plague penetrated into the Chinese province of Yunnan and further to Hong Kong, from where at the end of the 19th century it spread throughout the world. At the same time, to date, the pathogen has not changed its properties in the United States (1.ORI1), but has changed somewhat in Southeast China (Yunnan) and Myanmar (1.ORI2), Madagascar (1.ORI3), Peru, Bolivia, and Zimbabwe (1.ORI4) (Pisarenko et al., 2021). Thus, the biovars Antiqua and Orientalis, in addition to biochemical differences, have many differences in ecological features, habitat structure, and distribution history, and they should hardly be classified as one collective so-called main subspecies *Y. pestis pestis*. The same should be said about the biovars Mediaevalis and Intermedia. In addition, the names of the subspecies—main and non-main—should be considered inappropriate; they do not reflect the essence of the taxa but represent the classification of the

plague microbe in an extremely asymmetrical form—the "motley" and "bloated" main subspecies dominates all others combined. Molecular typing logically rejects the existence of a single, in fact quasi-basic, subspecies and subdivides it into many independent subspecies/genovariants characteristic of host populations of a particular species or a set of populations of two or more species in di- and polyhostal foci, representing various phylogenetic branches, subbranches, and lines and having unique "life" stories: 0.ANT1–5, 1.ORI1–4, 1.IN1–4, 2.ANT1–3, 3.ANT1–2, 4.ANT1–2 (Morelli et al., 2010; Cui et al., 2013; Kutyrev et al., 2018).

In general, molecular typing, despite the existing limitations of the approach, when supported by environmental facts, can provide a completely plausible explanation for the currently known intraspecific diversity of the plague microbe.

### *Ecological Typing. The Host Principle*

Molecular typing technologies of *Y. pestis* are based on the analysis of predominantly neutral nucleotide sequences using genetic and partly biochemical characteristics, and this methodology has generally yielded positive results. But there are also negative sides, similar to those mentioned above. These negatives can be overcome by using an ecological approach to typing according to the host trait, i.e., on the basis of adaptation to a specific host or to two or more hosts.

Hostal specialization is an integral property of the plague pathogen. The microbe circulates in the "rodent/pika-flea" parasitic system; i.e., it is adapted to live in two environments—the host and the vector. In the body of the flea vector, the microbe resides only in the contents of the digestive tract, i.e., in a substrate derived from the host, and does not interact with flea tissues and does not have specific adhesins and invasins. Fleas largely play the role of a "living syringe." Adaptation of the microbe to the flea body was aimed at a long stay in the anterior sections of its digestive tract and intensive reproduction with the formation of a mechanical "block" of the forestomach ("bone in the throat") as a specific mechanism of transmission. From this, it is clear that the adaptation of the microbe to the flea body is not deep and was aimed only at optimizing the process of transfer to a new host, most clearly expressed in the development of a key innovation—synthesis of gene *ymt* on the virulence plasmid pFra (Sun et al., 2014; Hinnebusch et al., 2016). This gene encodes the ability of the microbe to form a biofilm and a mechanical "block" of the flea forestomach and thereby intensifies the process of transmission into the body of a new host, which was initially ensured by contamination of the flea's oral apparatus with the pathogen during the host's bite (Hinnebusch et al., 2017). Although flea species are conventionally divided into highly effective, effective, weakly effective, and ineffective vectors, the specialization of the microbe to certain species of fleas cannot be traced at the biochemical, genetic, and molecular levels. For this reason, typing based on transmission ability and species of vector is not carried out. At the same time, there are significant reasons to believe that the formation of "block" transmission took place in a cold environment in populations of the cold-loving flea *O. silantiewi* (Suntsov, 2018a, 2018b). Fleas of genus *Oropsylla* reproduce year-round; in any season of the year, a large number of imagoes of these fleas can be found on their hosts and in their nests; they actively form a "block" at low environmental temperatures and are highly effective vectors of plague (Williams et al., 2013; Lemon et al., 2020).

As for warm-blooded hosts, they have active immunity against infectious agents. The pathogen adapts to the host body, developing specific protective mechanisms. In the plague causative agent, these mechanisms have been studied quite well (*Yersinia pestis*: *Retrospective*..., 2016; *Yersinia pestis Protocols*, 2018).

Tumansky (1957) was the first to propose dividing intraspecific "varieties" of the plague microbe according to host characteristics. He saw the reason for the emergence of varieties in the physiological and immunological characteristics of the body of rodents—the main hosts of the plague microbe in nature. He identified three varieties according to the genera of rodents from which they were regularly isolated: *Y. pestis ratti* was identified for the first time by Yersin and Kitazato in 1894 in Hong Kong from rats and people; *Y. pestis marmotae* was identified from the Mongolian marmot in 1911 by D.K. Zabolotny in Transbaikalia; *Y. pestis citelli* was identified from a Little Ground squirrel in 1912 in the Volga region by I.A. Deminsky. Later, a gerbil variety *Y. pestis gerbilli* and vole subspecies *Y. pestis microti* were proposed. In view of the fact that different species of rats, marmots, ground squirrels, gerbils, and voles form autonomous foci, often with specific properties of the circulating pathogen, this rather crude typology has not taken root in the scientific literature. Of the three subspecies proposed by V.M. Tumansky, one, according to modern ideas, turned out to actually exist—the rat; this is a subspecies/genovariant 1.ORI1, in nature characteristic of populations of the Indian gerbil and transferred in Hindustan to sympatric populations of the synanthropic Black rat (Suntsov, 2020a).

We made an attempt to typify the plague causative agent according to the main host in known natural and anthropogenic foci (Suntsov and Suntsova, 2006, 2008). It was assumed that the adaptation of the pathogen to the host species in monohostal foci should be evident, and "good" subspecies of the pathogen can be named according to the species of the main host in each specific focus. For example, it was proposed to call the subspecies circulating in populations of the Mongolian tarbagan marmot *Y. pestis tar-*

*bagani*, the subspecies in the outbreaks of the Great gerbil *Y. pestis rhombomys-opimus*, the subspecies of Indian gerbil populations *Y. pestis tatera-indica*, and so on. This somewhat cumbersome nomenclature of the plague microbe, despite its positive aspects, did not take root, as it seems to us, for three reasons. Firstly, it is not provided for by the rules of binomial/trinomial nomenclature of bacterial species, since it includes a fourth nomen. Secondly, the name of the subspecies *Y. pestis tarbagani* as the original one turned out to be not entirely correct; in fact, this "subspecies" includes three real original subspecies 2.ANT3, 3.ANT2, and 4.ANT1, which formed autonomously in three geographical populations of the Mongolian marmot (Suntsov, 2020a); a similar situation may occur with other subspecies. Thirdly, there are numerous examples where the same subspecies/genovariant circulates stably in populations (foci) with different main hosts of the microbe. A few of the most striking examples.

• Subspecies/genovariant 1.ORI1 is characteristic of natural foci of Hindustan, where it circulates in Indian gerbil populations. After the emergence of synanthropic populations of the Black rat in the process of human economic activity between the populations of the Indian gerbil and the Black rat through local gerbil fleas *X. astia* and African "rat" fleas *X. cheopis*, close parasitic contact arose, thanks to which the microbe passed from the Indian gerbil to the Black rat. In Black rat populations, anthropogenic synanthropic plague foci with the same 1.ORI1 genovariant of the plague microbe formed as in Indian gerbil populations. Further expansion from Hindustan led to the formation of close subspecies 1.ORI2, 1.ORI3, and 1.ORI4 in different parts of the world, but in North America, in populations of local burrowing wild rodents, the subspecies 1.ORI1, introduced more than 100 years ago, did not change its molecular properties. So, at present, a single subspecies/genovariant of the pathogen, 1.ORI1, circulates in Hindustan in populations of the Indian gerbil and Black rat and in North America in populations of wild rodents of various species and synanthropic rats (Cui et al., 2013).

• As indicated above, in accordance with the ecological scenario of the origin of the plague causative agent, the original subspecies/genovariants are 2.ANT3, 3.ANT2, and 4.ANT1, circulating in three geographical populations of the Mongolian marmot: Khentei, Khangai, and Kharkhira-Turgen, respectively. In Northeast China (Song Liao Plain), Eastern Mongolia, and Transbaikalia, in foci of plague in populations of the Daurian ground squirrel (*Spermophilus dauricus*), the same marmot subspecies/genovariant 2.ANT3 circulates as in the marmot foci of Khentei, Transbaikalia, and Inner Mongolia (Hulun Buir Plateau). This subspecies, having arisen in populations of the Mongolian marmot, upon introduction to the sympatric population of the Daurian ground squirrel has not changed its genetic and biochemical characteristics, presumably owing to the systematic proximity and physiological and biochemical similarity of the hosts—marmots and ground squirrels, which are systematically classified as one tribe—Marmotini. After the extermination of marmots in Transbaikalia, the plague epizootic remained in the populations of the Daurian ground squirrel. The same can be said about marmot foci on the Khangai and Kharkhira-Turgen-Mongun-Taiga mountain range, where subspecies/genovariants 3.ANT2 and 4.ANT1 from the Mongolian marmot populations passed without changes into sympatric populations of the Long-Tailed ground squirrel (*S. undulatus*). At the same time, the transition of the pathogen from these foci to sympatric populations of Brandt's vole and Mongolian pika led to a change in the subspecies circulating in them; 0.PE4a, 0.PE4m, and 0.PE5 arose. It is clear that the physiological and immunological characteristics of non-hibernating voles and pikas are not similar to those of hibernating members of the tribe Marmotini.

• Many synanthropic foci have several primary hosts or a primary and additional hosts and are called di- or polyhostal. For example, the synanthropic focus of plague in Vietnam, until its elimination in 2002, supported populations of Small (*R. exulans*), Black (*R. rattus*), Buff-Breasted (*R. flavipectus*), Himalayan (*R. nitidus*), and Gray (*R. norvegicus*) rats. At the same time, the Small and Buff-Breasted – rats were of greatest epidemiological importance. One subspecies/genovariant 1.ORI2, imported from Hong Kong at the end of the 19th century, circulated in populations of all rat species.

Intraspecific genetic diversity *Y. pestis* may be determined not only by the adaptation of subspecies developed in the process of evolution to specific species and populations of the main host(s) but also by the processes of transition to new hosts under various environmental circumstances. Therefore, as in many other species, the real polytypic intraspecific structure of the young plague microbe consists not only of more or less clearly defined discrete, so-called "good" subspecies but also of transitional polymorphic, "blurred" forms. On the basis of MG traits, gerbil-gopher (Central Caucasus) and vole-marmot (Hissar, Talas) forms (variants) of the plague microbe have been identified (Gorshkov et al., 2000; Savostina et al., 2009), but such forms have not yet been obtained a trinomial name or a molecular name, which, for example, in the second case must somehow combine the abbreviations PE ("vole" characteristics) and ANT ("marmot" characteristics). These "transitional" forms arise when the epizootic systems "rodent–flea–pathogen" and the parasitic systems "rodent–flea" are combined for natural or anthropogenic reasons, the exchange of components between them increases, and, as a consequence, a transition of the pathogen in the system "rodent–flea" to additional hosts occurs, which may eventually become the main ones. In such cases, on the basis of the two-membered parasitic "rodent–

flea" system, a new epizootic triad with a monohostly specialized subspecies of the pathogen can be formed (Zhou et al., 2004b; Suntsov and Suntsova, 2006, 2008).

The above shows that consideration of the issue of typing *Y. pestis* from the standpoint of two approaches—molecular and ecological—can clarify many unclear and controversial issues. Therefore, at the present stage, when creating consistent phylogenetic reconstructions, the results of both approaches should be taken into account.

## PHYLOGENESIS OF *Y. PESTIS*

The MG approach reconstructs first the emergence of an abstract common ancestor from the FESLF clone of *Y. pestis*—MRCA, but its host habitat and population characteristics are not discussed. The faceless MRCA is characterized only statistically by marker traits that are not functionally determined (ecological functions). It remains unclear whether MRCA can be classified as an already established species *Y. pestis* or is this still a transitional form *Y. pseudotuberculosis/pestis*. Thus, the MG approach on a phylogenetic tree of *Y. pestis* clearly records the ancestral species (unfortunately, not its specific population/subspecies), but does not reveal the unique features of the population-genetic speciation process and does not name the original host of the plague microbe. This gives rise to different interpretations of molecular data: the overwhelming number of researchers, without any ecological validity, consider the original host to be any species of small burrowing mammals (voles, jerboas, African grass mice) (Achtman et al., 1999; Cui et al., 2013; Pisarenko et al., 2021); others, also without referring to ecology, suggest the origin of plague in marmot populations (Tong et al., 2005; Wang et al., 2006; Anisimov et al., 2016). Such discrepancies among molecular geneticists are understandable: molecular methods are not sufficient to reveal the true history of taxa; here, accumulated knowledge from the field of ecology, paleontology, biogeography, and other classical scientific fields comes to the rescue.

The most important drawback of the MG approach at the present stage is the limited ability to identify the original host of the plague pathogen using purely statistical methods. It remains unclear in what rodent species the transformation of the population (clone) of the FESLS pathogen into the population of the plague pathogen occurred. However, knowing the characteristics of the population of the original host, in which the transformation of the intestinal pathogen FESLF into a "blood" parasite could have occurred, and taking into account the facts of the evolutionary youth of the plague microbe and its coexistence with the immediate ancestor (FESLF), the evolutionary history of the plague pathogen can be fully reconstructed by ecological (in a broad sense) methods (Suntsov, 2020a, 2021a). According to the above environmental scenario, the formation of the plague microbe took place in parallel in three geographical populations of the Mongolian marmot—on Khentei and Khangai and on the Kharkhira-Turgen-Mongun-Taiga mountain complex (Fig. 1b). The three original geographic populations (=three original subspecies) are designated 2.ANT3 (Khentei), 3.ANT2 (Khangai), and 4.ANT1 (Kharkhira-Turgen-Mongun-Taiga). Further host specialization was associated with the territorial expansion of three initial populations/genovariants of the plague microbe along independent routes and the formation of three independent (almost) nonoverlapping zones of primary natural foci in Asia and partly in southeastern Europe (Eastern Ciscaucasia) (Suntsov, 2020a).

From an ecological point of view, there are considerable objections to existing MG phylogenetic constructions. The proposed ecological scenario for the origin and expansion of the plague microbe requires significant amendments in the reconstruction of the phylogeny of *Y. pestis* MG methods, primarily correction of the basic evolutionary model, and indicates the need to adjust the topology of the phylogenetic tree constructed on the basis of molecular traits.

• The MG approach connects the speciation of the plague microbe with the recent horizontal transfer of genes from the external environment or from other microorganisms into the cells of the pathogen FESLF. In this case, the plasmids pFra and pPst borrowed from outside are called species-specific for the plague microbe. But gene structures species-specific to *Y. pestis* must be synthesized during the formation of the species *Y. pestis*. Plasmids pFra and pPst are complex multifunctional genetic structures carrying genes encoding various virulence, transmission, and communication functions specific to the plague pathogen. The formation of these plasmids in the genome of the plague microbe took place, presumably, gradually, and speciation of *Y. pestis* proceeded according to the principle of mosaic evolution (Suntsov, 2020b, 2021b), which should be taken into account when constructing or choosing a phylogenetic evolutionary model.

• The MG approach proclaims the greatest antiquity of the "vole" subspecies that make up the 0.PE branch/cluster. Of these, subspecies 0.PE5 (*Y. pestis ulegeica*), circulating in populations of the Mongolian pika in Mongolia, which, according to MG characteristics, is closest to highly virulent subspecies (Riehm et al., 2012; Demeure et al., 2019), is believed to have entered the populations of the Altai marmot in the Tien Shan on the eve of the first pandemic, where it transformed into the highly virulent subspecies 0.ANT1. The likelihood of such an extended geographical transit of the Mongolian subspecies 0.PE5 in the recent past is beyond ecological, biogeographical, and historical comprehension.

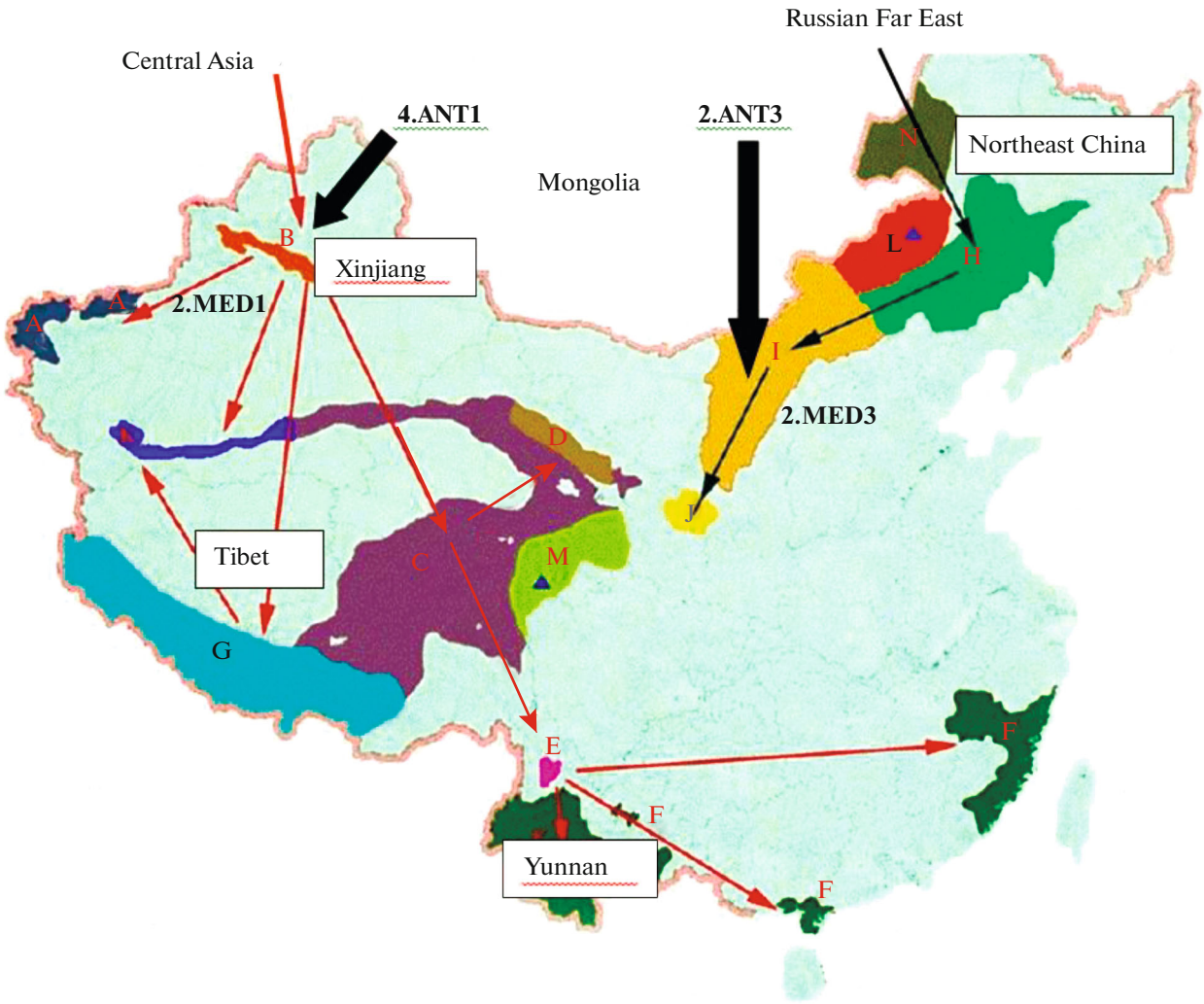


**Fig. 2.** Expansion of *Yersinia pestis* in China. The work of Chinese authors (Zhou et al., 2004a, 2004b) suggests that the Antiqua biovar entered China from various northern regions (thin arrows). According to the ecological scenario (Suntsov, 2020a), the expansion routes of the Antiqua biovar to China (thick black arrows) began in western (4.ANT1) and eastern (2.ANT3) Mongolia. In any case, the derived genovariants 2.MED1 and 2.MED3 arose in different phylogenetic lineages. A–N mark natural plague foci.

• Subspecies/genovariants 2.MED1 and 2.MED3, which have a common biochemical property of nitrification/denitrification, are assigned to a single phylogenetic branch 2.MED according to the similarity of the selected MG markers. However, this property is caused by different mutations in subspecies, and these subspecies/genovariants cannot be considered directly related (Zhou et al., 2004a, 2004b; Pavlova et al., 2012). Ecological analysis suggests that these subspecies diverged from different original subspecies of the plague microbe—4.ANT1 and 2.ANT3, respectively (Fig. 2). Therefore, in principle, they should be placed in different phylogenetic branches (Suntsov, 2020a); i.e., there is no single phylogenetic branch of 2.MED.

• The question of MG dating of the emergence of subspecies/genovariants 2.MED1 and 2.MED3 arises. The most ancient genovariant of the 2.MED branch according to the MG version is considered to be 2.MED0, which circulates in Mountain Ground squirrel populations in the Caucasus (Nosov et al., 2016; Kutyrev et al., 2018; Pisarenko et al., 2021). On the basis of the selected molecular markers, it is closest to the subspecies corresponding to the Antiqua biovar. In Central Asia, two other genovariants were noted—2.MED1 and 2.MED3; as is commonly believed in the MG approach, these are younger subspecies/genovariants. Therefore, it is believed that the territorial expansion of branch 2.MED0 took place in the direction from the Caucasus to the east, to Central Asia. But the logic of the spread of the plague in this direction contradicts the widely accepted idea of the origin of the plague in Central Asia and its further Asian and world expansion from the center of microbial speciation of *Y. pestis* with the formation of the modern zone

of natural focality of the plague. Thus, in nature, in populations of burrowing rodents, plague most likely spread from Central Asia to the Caucasus, and not vice versa, and the Caucasian subspecies 2.MED0 should be younger.

• As a rule, work on the phylogenetics of the plague microbe ends with the demonstration of phylogenetic dendrograms showing the molecular statistical relationships of genovariants (subspecies, geographic populations), and these relationships are not tied to natural and/or historical events. Thus, all molecular phylogenetic dendrograms record the presence of polytomy ("Big Bang," node N07 in Fig. 1a), which took place in the Tien Shan in accordance with the "molecular clock" dating on the eve of the second pandemic ("Black Death," 1346). Over the next few centuries, from the Tien Shan region, the virulent pathogen allegedly spread naturally through populations of marmots, gophers, gerbils, and rats across vast areas of Asia. But the reasons for the occurrence of this completely nontrivial natural event—recent on an evolutionary time scale, the almost simultaneous, explosive emergence of the main genealogical branches of *Y. pestis* in the Tien Shan on the eve of the "Black Death"—the MG approach does not name or even assume. At the same time, ecological data confidently indicate the occurrence of this polytomy during the process of speciation of *Y. pestis*, initiated by the last maximum (Sartan) climate cooling in North Asia (Suntsov, 2020a, 2021a).

• Molecular phylogenies proclaim a two-stage formation of natural foci in Eurasia (Li et al., 2009; Cui et al., 2013; Demeure et al., 2019). At the first stage, a habitat was allegedly formed by the "vole" subspecies of cluster 0.PE, including vast spaces from Northeast China and Eastern Tibet in the east to the Caucasus and the Middle East in the west and from Transbaikalia, Northern Kazakhstan, and the Northern Caspian region in the north to the south of Hindustan. The main hosts in the primary most ancient foci are believed to be jerboas (Eastern Tibet), voles (Hissar, Talas, Northeast China), and the Mongolian pika (Altai Mountains and Western Mongolia). The pathogens in these foci have weak virulence against marmots, ground squirrels, and gerbils and, according to the selected molecular markers, are close to the FESLF pathogen. At the second stage, from the "pika" subspecies 0.PE5u, which penetrated from Mongolia to the Tien Shan under unknown circumstances, the highly virulent subspecies 0.ANT1 was formed in the Tien Shan populations of the Altai marmot, which subsequently diverged into many of the same virulent subspecies that spread within the borders of "vole" foci, and also penetrated into Hindustan. The proposed MG phylogenetic schemes for the two-stage formation of primary natural foci of plague in Eurasia do not agree with the data of classical scientific directions: in nature, plague spreads in populations of burrowing rodents according to the principle of an oil stain spreading across paper, and the two-stage formation of the vast Euro-Asian area of natural foci from an epidemiological point of view seems to be logical nonsense (Suntsov, 2021a). The ecological approach calls into question the greatest antiquity of the "vole" subspecies of the plague microbe, representing the 0.PE cluster, and, accordingly, all positions within the framework of MG methodology that lead to such conclusions.

• In modern MG phylogenies, to assess the degree of relatedness of the studied strains and groups, CO92 is used as a reference (standard) strain, isolated in the United States from a sick person infected from a domestic cat, which in turn was infected from wild rodents in nature. Foci of plague in populations of wild rodents in North America are of secondary origin—they arose after the introduction of plague with ship rats to San Francisco during the third pandemic. The ship rats acquired the infection from synanthropic rats from Yunnan, China (or possibly India). In Yunnan, the plague spread from synanthropic populations of the Black rat, which at one time formed synanthropic foci in Hindustan. Synanthropic foci in Hindustan owe their origin to natural foci in Indian gerbil populations. So the American strain CO92 has a long anthropogenic history, was transferred by humans from the Eastern Hemisphere to the Western Hemisphere, and cannot be a standard for natural strains. The method of constructing phylogenetic lines by comparing the nucleotide sequences of selected markers in the genotypes of the plague microbe under study with the genotype CO92, which has been under the long-term influence of anthropogenic factors throughout its history, is questionable. The widespread use of this strain as a reference strain is associated with the priority of decoding its complete genome in the United States and inclusion in the NCBI GenBank database, accessible to researchers. As a reference, it would be much more reliable to choose the genome of a strain circulating in natural populations of the Mongolian marmot in the uninhabited expanses of Central Asia, belonging to subspecies 2.ANT3, 3.ANT2, or 4.ANT1.

• In the MG approach, the strain *Y. pseudotuberculosis* 0:1b IP32953 isolated from a sick person in France is often used as an outgroup, one of the first sequenced and deposited in GenBank. Owing to the highly probable origin of the plague in Central Asia in geographic populations of the Mongolian marmot and wide geographic variability of *Y. pseudotuberculosis*, the selected strain cannot reliably characterize the root of the phylogenetic tree of the plague microbe. It is more reliable to choose a strain of pseudotuberculosis microbe, also isolated from the Mongolian marmot in Central Asia, as a representative of the external group.

• The cladistic methodology for constructing phylogenetic dendrograms in the MG approach uses the concept of sister groups, the use of which is inevitable

in all cases where the direct ancestor remains unknown, and such cases are the absolute majority. By virtue of this circumstance, cladistics can only provide a reduced view of the history of the taxon being studied (Pavlinov, 2005). In the unique case of the plague microbe, its direct ancestor is known for certain. Therefore, in phylogenetic reconstructions of *Y. pestis*, there is no need to limit oneself to cladistic methodology, and the use of a "non-cladistic" ancestor–descendant concept becomes fully justified. In this case, an environmental (adaptive) approach acquires special value, which allows us to create a more complete narrative of the history of the plague microbe, rich in biological information.

## CONCLUSIONS

As we can see, typologies and phylogenies of *Y. pestis* constructed using the methods of two different approaches—MG and ecological—have their advantages and disadvantages. None of them can be considered perfect. So far, few strains of plague pathogens and FESLF have been sequenced and analyzed from Mongolia and its adjacent regions, which does not allow us to judge the true diversity and degree of relatedness of intra- and interspecific forms circulating in the most likely homeland of the plague—in Central Asia. The relationship between the Mongolian marmot, the marmot flea, and the causative agents of plague and FESLF has not been properly studied. There is little information about the functioning of plague and pseudotuberculosis microbes in a heterothermic and heteroimmune environment—marmot populations during hibernation. Nevertheless, both approaches are not only useful, but necessary for reconstructing the most plausible picture of the origin and evolution of the plague pathogen today. Both of these approaches should be seen as complementary to each other. At the same time, the ecological scenario of the origin and evolution of the plague pathogen has no obvious contradictions and it is important to take it into account as an evolutionarily based hypothesis in MG reconstructions of the phylogeny of the plague microbe.

Natural plague foci of the Russian Federation, located in Southern Siberia, belong to the group of Central Asian foci in which the secrets of the origin of the plague are hidden. These foci are being intensively studied by employees of the Irkutsk Anti-Plague Institute of Siberia and the Far East and specialists from the Gorno-Altai, Tuva, and Chita Anti-Plague Stations, and one can hope that priority in solving the problem of the origin and territorial expansion of the plague pathogen through the efforts of specialists from these organizations will be assigned to Russian researchers.

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## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

## CONFLICT OF INTEREST

The author of this work declares that he has no conflicts of interest.

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