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Authors

Tibayrenc, Michel
Ayala, Francisco J

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New Insights into Clonality and Panmixia in *Plasmodium* and *Toxoplasma*

Michel Tibayrenc^{*,1}, Francisco J. Ayala[†]

^{*}Maladies Infectieuses et Vecteurs Ecologie, Génétique, Evolution et Contrôle, MIVEGEC (IRD 224-CNRS 5290-UM1-UM2), IRD Center, Montpellier, France

[†]Department of Ecology and Evolutionary Biology, University of California, Irvine, California, USA

¹Corresponding author: e-mail address: michel.tibayrenc@ird.fr

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Abstract

Until the 1990s, *Plasmodium* and *Toxoplasma* were widely considered to be potentially panmictic species, because they both undergo a meiotic sexual cycle in their definitive hosts. We have proposed that both parasites are able of clonal (nonrecombining) propagation, at least in some cycles. *Toxoplasma* was soon shown to be a paradigmatic case of clonal population structure in North American and in European cycles. But the proposal provoked an outcry in the case of *Plasmodium* and still appears as doubtful to many scientists. However, the existence of *Plasmodium* nonrecombining lines has been fully confirmed, although the origin of these lines is debatable. We discuss the current state of knowledge concerning the population structure of both parasites in the light of the recent developments of pathogen clonal evolution proposed by us and of new hypotheses presented here.

1. INTRODUCTION

The population structure of pathogens is a fundamental parameter, not only for understanding the biology of these organisms but also for applied studies. The population structure of pathogens needs to be ascertained for molecular epidemiology (strain typing), vaccine and drug design, follow-up of genes of interest, and control measures. Discussions of pathogen population structure have focused on the “clonality/sexuality debate” (Tibayrenc et al., 1990). Clonality is here understood as scarcity or absence of genetic recombination, a definition widely accepted for all kinds of pathogens (Tibayrenc and Ayala, 2012), including *Plasmodium* (Anderson et al., 2000; Annan et al., 2007; Beck et al., 2009; Conway, 2007; Griffing et al., 2011; Heitman, 2006; Mu et al., 2005; Nkhoma et al., 2013; Razakandrainibe et al., 2005; Volkman et al., 2012a) and *Toxoplasma* (Beck et al., 2009; Heitman, 2006; Khan et al., 2011; Rajendran et al., 2012; Sibley and Ajioka, 2008; Smith, 2009; Su et al., 2003, 2010; Wendte et al., 2011). Clonality does not amount to genetic monomorphism, an erroneous definition still used by some researchers (Tibayrenc and Ayala, 2012). A clonal species can have considerable genetic diversity, such as *Trypanosoma cruzi*, while some sexual organisms can be extremely monomorphic, after a drastic bottleneck effect, for example.

Clonality strongly influences the population structure of pathogens. If a species is potentially panmictic (freely recombining), its multilocus genotypes (MLGs) are transient individual variants that vanish in the common gene pool in each generation. A panmictic pathogen behaves like sexual metazoa (*Drosophila* and humans), with the peculiarity that pathogens have very short generation times. If clonal evolution predominates in a given species, its MLGs are stable in space and time and can persist over years and over wide geographical ranges. Even when clonal evolution has a limited impact, it introduces a stratification component and discontinuities in the pathogen’s population structure, which must be taken into account in association studies and in surveys dealing with the dynamics of genes of interest. Importantly, all systems that restrain genetic recombination amplify the impact of natural selection, by generating coadapted multigenic complexes (Avise, 2004).

Plasmodium and *Toxoplasma* were widely considered panmictic until the early 1990s (Grigg and Sundar, 2009; Walliker, 1991), an apparently logical inference from the well-known feature that they undergo sexual recombination and meiosis in their definitive hosts (anopheline mosquitoes for the

Plasmodium species that infect humans and felids for *Toxoplasma*). However, *a priori* inferences may not be correct. The only adequate means to settle the issue is population genetic analysis of natural populations.

2. INITIAL PROPOSALS

Early proposals of clonal population structure emerged from linkage disequilibrium (LD) analyses of natural populations of *Plasmodium falciparum* (Tibayrenc et al., 1990, 1991) and *Toxoplasma* (Tibayrenc et al., 1991). LD analysis seeks nonrandom association of genotypes at different loci. In a pan-mictic species, genotypes from different loci recombine at random (except in the case of physical obstacles to genetic exchange: isolation by space and time, the so-called Wahlund effect). Knowing the genotype at a given locus provides no information about which genotypes will occur at other loci. Such information may become apparent if recombination is rare or absent (clonality). LD is therefore taken as a strong circumstantial evidence of clonality, when a sufficient number of loci are surveyed and the significance of statistical tests is high (Tibayrenc and Ayala, 2012; Tibayrenc et al., 1990). In an initial survey (Tibayrenc et al., 1990), *P. falciparum* was chosen as a counterexample. Yet, it was surprising to observe significant LD in the populations surveyed. The inference followed that “uniparental and biparental lineages may coexist within this species, for which a sexual cycle has been a classical notion” (Tibayrenc et al., 1990). Although this inference was corroborated by further studies (Ben Abderrazak et al., 1999; Urdaneta et al., 2001), it resulted in a vehement outcry (Dye et al., 1990; Walliker, 1991; Walliker et al., 1990). Our proposal was widely rejected. The “pan-mictic prejudice” about *Plasmodium* was kept for long and still has not been rejected by all. The sample we analysed in *Toxoplasma* was less limited, which allowed us to suggest more firmly that “not only that a uniparental cycle exists in *T. gondii* (which was already known), but also that it is common and perhaps predominant” (Tibayrenc et al., 1991).

3. INDISPENSABLE RECALLS

The model of preponderant clonal evolution (PCE) proposed by us includes, as again recently stated (Tibayrenc and Ayala, 2012, 2013), the following: (i) It refers to a pathogen’s population structure, not to a particular cytological mechanism of propagation; (ii) its definition is based on restrained recombination (see Section 1); (iii) it affirms that recombination

is severely restricted, not that it is completely absent; and (iv) it definitely includes selfing and strong inbreeding and considers them as particular cases of clonality. That selfing and inbreeding amount to clonality is a view shared by most authors working on *Plasmodium* (Anderson et al., 2000; Annan et al., 2007; Beck et al., 2009; Conway, 2007; Griffing et al., 2011; Mu et al., 2005; Mzilahowa et al., 2007; Nkhoma et al., 2013; Razakandrainibe et al., 2005; Volkman et al., 2012a) and *Toxoplasma* (Beck et al., 2009; Grigg and Sundar, 2009; Lehmann et al., 2004; Sibley and Ajioka, 2008; Su et al., 2012; Wendte et al., 2010). However, clonality does not limit itself to selfing and inbreeding and can have other origins (mitotic propagation and several cases of parthenogenesis, gynogenesis, and hybridogenesis).

4. RECENT DEVELOPMENTS

We have proposed (Tibayrenc and Ayala, 2012) that the two main features of PCE are (i) strong LD and (ii) near-clading.

LD analysis has been criticised as a measure of genetic exchange (De Meeûs et al., 2007). However, (i) it is the very and only statistic appropriate for testing rarity or absence of recombination, on which the definition of PCE is based; (ii) when enough loci are surveyed, its power of resolution is high (Tibayrenc and Ayala, 2012; Tibayrenc et al., 1990); and (iii) it is widely used as a circumstantial evidence for clonality by many authors working on *Plasmodium* (Anderson et al., 2000; Arnott et al., 2012; Branch et al., 2011; Chenet et al., 2012; Conway, 2007; Ferreira et al., 2007; Imwong et al., 2007; Iwagami et al., 2009, 2012; Karunaweera et al., 2008; Mu et al., 2005; Nkhoma et al., 2013; Orjuela-Sánchez et al., 2010; Rezende et al., 2010; Volkman et al., 2007, 2012a,b) and *Toxoplasma* (Khan et al., 2011; Lehman et al., 2004; Rajendran et al., 2012; Su et al., 2006), as well as on other pathogens (Tibayrenc and Ayala, 2012). As asserted here, only the LD approach has been able to refute the panmictic prejudice in *Plasmodium* and *Toxoplasma* (Tibayrenc et al., 1990, 1991).

Near-clading refers to the tendency of pathogen natural populations to differentiate into discrete genetic subdivisions that are stable in space and time. This is evidenced by a clear phylogenetic signal. The term “near-clade” has been coined (Tibayrenc and Ayala, 2012) because some recombination nearly always interferes with PCE in pathogens, which renders the term “clade” improper (there is no recombination between true clades). We have proposed to ascertain whether PCE is apparent within near-clades, which leads to a “Russian doll pattern” (Tibayrenc and Ayala, 2013): one

observes a miniature picture of the whole species within each near-clade, which includes LD and lesser near-clades (Fig. 5.1).

The expectations of a strict cladistic approach are not fulfilled in pathogens due to occasional recombination. Near-clading and a Russian doll pattern should be therefore explored by means of a flexible phylogenetic approach relying on the congruence principle (Avice, 2004), which states that evidence increases when additional suitable data, coming from various sources, are considered. For example, in the case of multilocus sequence typing, evidence accumulates when additional loci are considered. Also, the evidence becomes stronger when microsatellite and multilocus enzyme electrophoresis data are jointly considered. The phylogenetic signal should be looked for by genes that do not undergo strong selective pressure (neutral genes or nearly so). Antigen genes, which are strongly selected, are not an appropriate population genetic tool.

Figure 5.1 “Russian doll” model (Tibayrenc and Ayala, 2013). When population genetic tests are practised with appropriate markers (of sufficient resolution) within each of the near-clades that subdivide the species under study (large tree, left part of the figure), they evidence within these near-clades a miniature picture of the whole species, with the two main PCE features, namely, linkage disequilibrium and lesser near-clades (two small trees, right part of the figure). This is an evidence that the near-clades do not correspond to cryptic, biological species that are potentially panmictic, and that they undergo predominant clonal evolution too.

5. POPULATION STRUCTURE OF *PLASMODIUM* AND *TOXOPLASMA* IN THE LIGHT OF THE PCE MODEL

5.1. *Plasmodium*

A drastic reduction in the panmictic prejudice was caused by a seminal paper (Anderson et al., 2000) dealing with microsatellite variability in a large number of *P. falciparum* worldwide strains. LD analysis led to the inference that *P. falciparum* exhibits a broad spectrum of population structures, ranging from panmictic to highly inbreeding (clonal) populations. Stable, widespread MLGs were observed. For example, the same MLG was sampled in Bolivia in 1994 and in Brazil in 1997–1998. Identical genotypes were obtained from different individuals in Bolivia, Brazil, Colombia, Thailand, Papua New Guinea, and Zimbabwe. The hypothesis advanced to account for restrained recombination was that, in low transmission areas, multiclonal infections are rare, so that identical MLGs frequently mate together (selfing and inbreeding). In other words, recombination is restrained by the absence of different MLGs. We will call it here the “starving sex hypothesis”. It has been accepted by many authors in order to account for the observed population structure of *Plasmodium* (Arnott et al., 2012; Branch et al., 2011; Chenet et al., 2012; Conway, 2007; Ferreira et al., 2007; Gupta et al., 2012; Imwong et al., 2006, 2007; Iwagami et al., 2009; Mobegi et al., 2012; Mu et al., 2005; Mzilahowa et al., 2007; Neafsey, 2013; Neafsey et al., 2008; Nkhoma et al., 2013; Schultz et al., 2010; Volkman et al., 2012a,b). It can be challenged, as we will further see, since many observations are at odds with it, both in *P. falciparum* and in *Plasmodium vivax*.

Many studies have ascertained evidence for clonality and restrained recombination, based on the analysis of LD in many populations of *P. falciparum* (Annan et al., 2007; Branch et al., 2011; Chenet et al., 2012; Griffing et al., 2011; Iwagami et al., 2009; Manske et al., 2012; Nkhoma et al., 2013; Razakandrainibe et al., 2005; Volkman et al., 2007, 2012a) and *P. vivax* (Chenet et al., 2012; Imwong et al., 2007; Iwagami et al., 2012; Karunaweera et al., 2008; Rezende et al., 2010). Consequently, the panmictic model was considered to be “oversimplified” in *Plasmodium* (Heitman, 2006). In confirmation of Anderson et al.’s (2000) observations, persistent MLGs were observed in *P. falciparum* for as long as 8 years (Nkhoma et al., 2013). Many authors have come to use the very terms “clones” and “clonal population structure” for describing the population structure of *Plasmodium* (Annan et al., 2007; Chenet et al., 2012; Ferreira

et al., 2007; Griffing et al., 2011; Heitman, 2006; Karunaweera et al., 2008; Nkhoma et al., 2013; Razakandrainibe et al., 2005), which would have been unthinkable 20 years ago. In some populations, restrained recombination manifests itself, not only by LD and ubiquitous MLGs but also by a tendency of natural populations to be structured into lasting, stable clusters, which cannot be accounted for by isolation by distance and time. This has been observed in *P. falciparum* (Branch et al., 2011; Griffing et al., 2011) and *P. vivax* (Gupta et al., 2012; Iwagami et al., 2009; Orjuela-Sánchez et al., 2010; Rezende et al., 2010). However, *Plasmodium* clusters cannot be equated to near-clades similar to the ones observed in *T. cruzi* and *Leishmania* (Tibayrenc and Ayala, 2013), because their stability is limited in time and is blurred by genetic recombination from 1 year to another. Still, the fact remains that they evince a major factor of population structuration and heterogeneity. This is all the more the case, because LD can be observed, not only in whole populations but also within the clusters that subdivide them (Griffing et al., 2011), like a nascent and labile “Russian doll” pattern (Tibayrenc and Ayala, 2013) (Fig. 5.1). In several cases, the strength of LD and structuration is not linked to transmission intensity (see further).

5.2. *Toxoplasma*

The proposal that *Toxoplasma* could undergo clonal evolution in certain cycles (Tibayrenc et al., 1991) did not face strong opposition such as it did in *Plasmodium*, although “a clonal population structure was entirely unexpected, especially since cats are both highly prevalent and widely distributed” (Grigg and Sundar, 2009). Clonality in *Toxoplasma* has been confirmed by numerous papers (Boothroyd, 2009; Dubey et al., 2011; Khan et al., 2009, 2011; Sibley and Ajioka, 2008; Sibley and Boothroyd, 1992; Smith, 2009; Su et al., 2003; Wendte et al., 2010). It is preponderant in Europe, North America, and Africa, while recombination has a greater impact in South America (Lehman et al., 2004, 2006; Mercier et al., 2011; Su et al., 2006, 2010). *Toxoplasma* clonality is common also in North American wildlife (Dubey et al., 2011; Wendte et al., 2011). Authors use the terms “clonal” and “clonal population structure” in the case of *Toxoplasma* more so than for *Plasmodium* (Boothroyd, 2009; Grigg and Sundar, 2009; Khan et al., 2011; Sibley and Ajioka, 2008; Smith, 2009; Su et al., 2003, 2010; Volkman and Hartl, 2003; Wendte et al., 2011). Even in South America, *Toxoplasma*’s population structure is far from “panmictic” (Grigg and Sundar, 2009). The signal of clonal propagation is not “largely absent” (Minot et al., 2012); rather, it is obvious. One MLG isolated from sheep

in Brazil is identical to a MLG isolated years apart in France from a human congenital case (Da Silva et al., 2011). Identical MLGs have been isolated in French Guiana from humans, cats, dogs, and chickens 50 km apart (Mercier et al., 2011). The “clonal types” I, II, and III, preponderant in Europe and North America, are present in Africa (Mercier et al., 2010) and have been also recorded in Central and South America (Da Silva et al., 2011; Mercier et al., 2011; Rajendra et al., 2012; Su et al., 2012).

Near-clading in *Toxoplasma*

Toxoplasma natural populations show, in contrast to *Plasmodium*, a strong tendency for persistent and widespread clustering patterns that meet our definition of near-clades (Tibayrenc and Ayala, 2012). The three major “clonal lineages” (Boothroyd, 2009; Sibley and Boothroyd, 1992) can definitely be equated to near-clades. This near-clading pattern in *Toxoplasma* is not limited to the three major clonal genotypes. When a sufficiently broad sampling of genetic markers and stocks is used (Su et al., 2012), the whole species appears to be subdivided into six major “clades” (near-clades). Three of them (clades A, B, and F) exhibit a distinctive Russian doll pattern (Tibayrenc and Ayala, 2013): LD and lesser near-clades are observed within them. Within-near-clade clonality (Russian doll pattern) has been also observed within “group 12” (Khan et al., 2011). The term clade (Su et al., 2012) is improper, since hybridisation is ubiquitous in *Toxoplasma* evolution, hence the relevance of the new term “near-clade” (Tibayrenc and Ayala, 2012). The three main *Toxoplasma* clonal lineages, although they are stable in space and time and propagate clonally, are thought to have a hybrid origin (Minot et al., 2012; Sibley and Ajioka, 2008; Su et al., 2010, 2012; Volkman and Hartl, 2003). They show striking similarities to some *T. cruzi* near-clades that also have a hybrid origin (Zingales et al., 2012). Clonal propagation of successful hybrid genotypes may represent a specific adaptation to human environments. *T. cruzi* hybrid near-clades are indeed widespread in human cycles of the southern range of Chagas disease (Zingales et al., 2012). Similarly, *Toxoplasma* clonality and major clonal genotypes are obviously associated (although not exclusively) with human environments (Boothroyd, 2009; Mercier et al., 2011).

6. PASSIVE CLONALITY (STARVING SEX) VERSUS IN-BUILT CLONALITY IN *PLASMODIUM*

We have seen that the existence of clonal propagation and a clonal population structure in some *Plasmodium* populations (Tibayrenc et al.,

1990) is now widely accepted, although many researchers still consider that clonality is doubtful in *Plasmodium* and clashes with the notion of an obligatory sexual cycle in these parasites. We propose to move the debate from panmixia versus clonality, to passive clonality versus active (in-built) clonality. As we have noted, passive clonality (starving sex hypothesis) is the widely accepted working hypothesis to account for restrained recombination in *Plasmodium*. It is epidemiologically highly relevant. Indeed, if clonality is strictly correlated to low transmission, LD could be used as a measure of transmission intensity (Volkman et al., 2012a).

Starving sex can be challenged by the hypothesis that clonality in *Plasmodium* cannot be purely passive, “mechanical”, but could be due in some cases to in-built biological properties of the parasite. This would allow it to actively restrain recombination and escape the recombinational load (Agrawal, 2006), an evolutionary strategy that could be common in many pathogens (Tibayrenc and Ayala, 2012). The presence of putative meiotic genes, evidenced in many eukaryotic pathogens (Heitman, 2006), could be attributed to a “clonality/sexuality genetic machinery” (Tibayrenc and Ayala, 2012). The hypothesis of active clonality in *Plasmodium* is grounded on the fact that many observations are at odds with starving sex in *P. falciparum* and even more so in *P. vivax*. In Anderson et al.’s article (2000), African populations show no LD, which fits the starving sex hypothesis. However, a strong LD is observed in *P. falciparum* populations of Papua New Guinea and Zimbabwe, even though transmission is high in New Guinea and multiclonal infections are frequent in Zimbabwe (Anderson et al., 2000). LD is stronger in Zimbabwe than in Brazil, although transmission rate is lower in Brazil (Anderson et al., 2000). Weak sample size in Brazil could bias the power of LD analysis in this country. However, this does not lower the strength of the many other observations that challenge the starving sex hypothesis. Strong inbreeding (clonality) with high transmission has been confirmed in Papua New Guinea (Manske et al., 2012). Strong indications for clonality in spite of high transmission rates have been found in Kenya and Cameroon too (Annan et al., 2007; Razakandrainibe et al., 2005). In Brazil, high inbreeding has been evidenced, although multiclonal infections seem to be “highly prevalent”, a result considered as “puzzling” by Ferreira et al. (2007). Departures from starving sex expectations are even more obvious in *P. vivax*. In this species, low transmission is frequently associated, not only with a strong LD but also with high genetic diversity (Gupta et al., 2012), which does not favour the starving sex hypothesis. In Brazil, strong LD and high levels of inbreeding in *P. vivax* are “at odds” with high

genetic diversity and prevalent multiclonal infections (Ferreira et al., 2007; Rezende et al., 2010). The same pattern has been observed in Sri Lanka (Karunaweera et al., 2008).

We do not claim that the starving sex hypothesis should be rejected. However, considering the many cases that are at odds with it, the alternative hypothesis (in-built, active clonality) deserves to be considered and is highly falsifiable. The two hypotheses are, in fact, not mutually exclusive.

7. ARE CLONALITY AND NEAR-CLADING IN *PLASMODIUM* AND *TOXOPLASMA* MAINLY DUE TO NATURAL SELECTION?

It has been proposed that natural selection, strongly favouring some variants to the detriment of others, plays a major role in the stability of the main clonal lineages in *Toxoplasma* (Su et al., 2003). It is most probable that natural selection, both diversifying and purifying, plays a significant role in the population structure of pathogens, as it does in other organisms. The PCE model specifically proposes that LD, clonality, and near-clading are mainly due to in-built genetic properties of the pathogens, rather than to the drastic elimination by selection of most possible variants in an otherwise panmictic species (Tibayrenc and Ayala, 2012). The proposal of a preponderant role of natural selection would indeed imply a heavy burden of elimination of most genotypes at each generation (Lehman et al., 2004).

8. ARE THE NEW *PLASMODIUM* “SPECIES” NOT MERE NEAR-CLADES?

In recent years, impressive sets of data have been gathered on *Plasmodium* parasites closely related to *P. falciparum* isolated from apes (Duval et al., 2010; Liu et al., 2010; Prugnolle et al., 2010, 2011a,b). Due to severe technical constraints, the description of these new taxonomical entities has relied on limited sets of genes, except for Prugnolle et al. (2011b). Proposals to describe several new “species” based on limited phylogenetic evidence (Liu et al., 2010; Rayner et al., 2011) are questionable (Tibayrenc and Ayala, 2012; Valkiunas et al., 2011). If the same level of evidence were used for *T. cruzi*, there would be even stronger arguments to describe six or seven “species” within the agent of Chagas disease! We have warned (Tibayrenc and Ayala, 2012, 2013) against the inflation of various different terms used for many pathogens (“clades”, “clonal lineages”, “clusters”, and “haplogroups”,

among many others) to designate what is probably the same evolutionary entity, namely, the near-clade. The new *Plasmodium* “species” described using limited phylogenetic evidence could be possibly equated to near-clades, evolutionarily equivalent to the near-clades described in *T. cruzi* and other pathogens (Tibayrenc and Ayala, 2012, 2013). Near-clading seems to be omnipresent in most pathogen species (Tibayrenc and Ayala, 2012). Describing new “species” based only on phylogenetic data would lead to a misleading inflation of new species.

The question whether or not the new *Plasmodium* entities deserve to be described as new species will only be settled by additional phylogenetic, population genetic, and phenotypic analyses of these “species”.

9. CONCLUDING REMARKS

The panmictic prejudice in *P. falciparum* and *Toxoplasma* teaches us that “logical” *a priori* inferences (here based on a known sexual cycle in both species) have severe limitations. Suitable evidence should rather be gathered with adequate tools—in this case, population genetic analysis of natural populations (Tibayrenc et al., 1990). Population genetic analyses refute the panmictic prejudice in these parasites and, at the same time, evince clear differences between *Plasmodium* and *Toxoplasma*. Although *P. falciparum* has a spectrum of population structures (Anderson et al., 2000; Tibayrenc et al., 1990) and shows a clonal population structure in many populations, its clonal genotypes and near-clades are made unstable by frequent recombination, even in the populations where clonality is apparent. The same obtains for *P. vivax*. The fact remains that clonality introduces a major stratification factor in *P. falciparum* and *P. vivax* populations, a parameter that must be taken into account in all studies dealing with epidemiological surveillance, vaccine and drug design, and pathogenicity. Additional analyses should aim at falsifying the starving sex versus active clonality hypotheses, since their epidemiological and biological implications are dramatically different.

The present study focused on *P. falciparum* and *P. vivax* for two reasons: (i) They are medically the most relevant ones and (ii) more population genetic data are available for these two species than for others. Further studies should test the hypotheses proposed here on *Plasmodium malariae* and *Plasmodium ovale*, as well as in the many *Plasmodium* species that do not affect humans.

Toxoplasma exhibits a more typical PCE pattern, with stable clonal MLGs and near-clades, not only preponderant in the Northern Hemisphere and Africa but also present in South America.

In *Plasmodium* and in *Toxoplasma*, prior to all applied studies, a reliable population genetic framework should be firmly ascertained, by means of phylogenetic character mapping (Avisé, 2004; Tibayrenc and Ayala, 2012). New powerful tools based on whole genome sequencing and the use of large sets of SNPs will help to determine such a robust population genetic framework.

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